Spatial and Temporal Dissection of Immediate and Early Events following Cadherin-mediated Epithelial Cell Adhesion

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Abstract. Cell-cell adhesion is at the top of a molecular cascade of protein interactions that leads to the remodeling of epithelial cell structure and function. The earliest events that initiate this cascade are poorly understood. Using high resolution differential interference contrast microscopy and retrospective immunohistochemistry, we observed that cell-cell contact in MDCK epithelial cells consists of distinct stages that correlate with specific changes in the interaction of E-cadherin with the cytoskeleton. We show that formation of a stable contact is preceded by numerous, transient contacts. During this time and immediately following formation of a stable contact, there are no detectable changes in the distribution, relative amount, or Triton X-100 insolubility of E-cadherin at the contact. After a lag period of ~10 min, there is a rapid acquisition of Triton X-100 insolubility of E-cadherin localized to the stable contact. Significantly, the total amount of E-cadherin at the contact remains unchanged during this time. The increase in the Triton X-100 insoluble pool of E-cadherin does not correlate with changes in the distribution of actin or fodrin, suggesting that the acquisition of the Triton X-100 insolubility is due to changes in E-cadherin itself, or closely associated proteins such as the catenins. The 10 minute lag period, and subsequent prompt and localized nature of E-cadherin reorganization indicate a form of signaling is occurring.

SPECIFIC cell-cell adhesion is essential for the proper functioning and development of all cells in the body. For example, guidance of neurons to target tissues (Hortsch and Goodman, 1991; Jessell, 1988), activation or silencing of cells in the immune system (Kosco and Gray, 1992; Vitetta et al., 1991) and determination of cell fate and function in epithelial cells (Fleming and Johnson, 1988; Rodriguez-Boulan and Nelson, 1989), are all dependent on the temporal and spatial regulation of cell adhesion (see also, Singer, 1992). While much is known about the immediate effects of cell adhesion in lymphocytes (Dustin and Springer, 1991), little is known in adherent and morphologically highly differentiated cells such as epithelial cells.

Epithelial cell adhesion is mediated by E-cadherin, a member of a family of Ca^{2+}-dependent cell adhesion proteins termed cadherins (see review by Takeichi, 1991). Homotypic recognition and binding between cadherins on adjacent cells results in the formation of an intercellular junctional complex (Gumbiner et al., 1988) and the establishment of strong cell-cell adhesion that results in cell aggregation (Nagafuchi et al., 1987; Mege et al., 1988) and sorting of mixed cell populations (Friedlander et al., 1989; Jaffe et al., 1990; Nose et al., 1988). The formation of this junction is preceded by and requires homotypic binding between cadherin molecules (Behrens et al., 1985; Gumbiner et al., 1988; Wheelock and Jensen, 1992). On a time course of hours and days after cell adhesion, there is a reorganization of the cytoskeleton and of the biosynthetic machinery of the cell, which leads to the remodeling of the plasma membrane into structurally and functionally distinct domains and results in the development of cellular polarity (see review by Rodriguez-Boulan and Nelson, 1989). These results establish that interactions between cell adhesion molecules are at the top of a molecular cascade that lead to the remodeling of epithelial cell structure and function. However, the mechanisms involved in the transduction of extracellular contact between cells into intracellular reorganization and differentiation are unknown.

One pathway of reorganization involves physical interactions between cadherins and components of the cortical cytoskeleton. Cadherins co-localize at cell-cell contacts with actin (Hirano et al., 1987; Matsuzaki et al., 1990; Takahashi et al., 1990) and fodrin (Nelson et al., 1990), and complexes of cadherin and several cytoskeletal proteins, including ankyrin, fodrin, and actin have been isolated (Balsamo et al., 1991; Nelson et al., 1990; Itoh et al., 1991). In addition, co-localization of cadherins and the cortical cytoskeleton correlates with a dramatic increase in resistance of cadherin to extraction with nonionic detergents (Shore and Nelson, 1991). Cadherin interactions with the cytoskeleton may be mediated by cytosolic proteins, termed catenins (Ozawa et al., 1989; Nagafuchi and Takeichi, 1989; McCrea et al., 1991; Herrenknecht et al., 1991). Disruption of cadherin/catenin binding, by deletion of the cytoplasmic domain

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of cadherins, results in loss of both catenin binding and association of cadherins with the cytoskeleton (Ozawa et al., 1989, 1990). Significantly, this disruption also results in loss of cell–cell contacts even though the extracellular binding domain of cadherin is intact (Nagafuchi and Takeichi, 1988; Jaffe et al., 1990; Ozawa et al., 1989, 1990). Together, these results have lead to a model in which cell recognition and adhesion requires interactions between cell adhesion proteins and the cortical cytoskeleton, which may mediate clustering of cadherins and the formation of an adhesive contact (reviewed in Takeichi, 1990; McNeill and Nelson, 1992).

To examine early events involved in the initiation and subsequent remodeling of cell–cell contacts we have developed a novel technique that allows us to determine distributions of cadherins and the cytoskeleton and correlate them with the exact duration and distribution of cell–cell contacts at the single cell level. Our results show that there is no significant change in the amount or distribution of E-cadherin during formation of initial, transient contacts, or for ∼10 min following the establishment of a stable contact. After this lag period, E-cadherin at the stable contact rapidly becomes resistant to extraction by detergents. Significantly, the total amount of E-cadherin at the contact remains unchanged during this time. In addition, there is no detectable change in the organization of cortical cytoskeletal proteins actin and fodrin during this period. The fine spatial and temporal resolution of this technique has allowed us to dissect steps in cell–cell contact that have important implications for models of cell adhesion.

Materials and Methods

Growth and Maintenance of Cells

MDCK cells were maintained in DME/FBS as described previously (Nelson and Veshnock, 1987*a*). Before all experiments, cells were grown for 2 d at low cell density, to generate a population of contact-naive cells (for details see Nelson and Veshnock, 1987). Cells were briefly trypsinized and plated at low cell density (∼10^5 cells/22 mm^2) on collagen-coated coverslips and incubated for 5–7 h to allow recovery of cell surface E-cadherin expression. Immediately before observation, cells were transferred to a viewing chamber and placed into DME/FBS lacking phenol red and with the addition of 10 mM Hepes. Cells were maintained at 35°C during experiments by means of a heated viewing stage.

Immunofluorescence

Cells were either fixed with 100% methanol at −20°C for 10 min, or extracted with CSK buffer (50 mM NaCl, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, 300 mM sucrose, 1.2 mM PMSF, 100 μg/ml RNAse, 100 μg/ml DNAse) (Boehringer Mannheim Biochemicals, Indianápolis, IN) for 5 min, and then fixed with 2% formaldehyde in D-PBS for 10 min at 25°C. Fixation of cells in methanol results in little or no extraction of E-cadherin (Shore and Nelson, 1991), and E-cadherin distribution in cells fixed in this manner is indistinguishable from that of cells fixed with formaldehyde. Fixed cells were incubated overnight in 10% normal goat serum in D-PBS at 4°C and then incubated with a 1:100 dilution of the primary antibodies (see below) in D-PBS for 1 h. Coverslips were washed extensively in D-PBS, and then incubated with a 1:200 dilution of FITC-conjugated goat anti-rabbit or goat anti-mouse from Boehringer Mannheim Biochemicals. Coverslips were washed extensively in D-PBS before viewing. mAb 3G8 against MDCK E-cadherin was obtained from Dr. Warren Galin (Department of Zoology, University of Alberta, Canada) and the hybridoma supernatant was used at 1:100 dilution. The 3G8 mAb recognizes E-cadherin in cells that have been fixed in either methanol or formaldehyde. Western analysis demonstrated that this mAb was specific for E-cadherin (data not shown). Polyclonal antibodies against fodrin were generated as described previously (Nelson and Veshnock, 1987*a*). Bodipy-conjugated phallacidin was obtained from Molecular Probes Inc. (Junction City, OR) and used according to the manufacturer's instructions.

Microscopy

Time lapse measurements were performed using a customized DIC system which was adapted to laser scanning confocal microscopy on a Zeiss IM35 microscope (Carl Zeiss, Oberkochen, Germany) (see also Cooper and Smith, 1992). Most time lapse sequences were obtained using a Nikon 20× (0.75 NA; Nikon Inc., Melville, NY), or an Olympus 40× (1.3 NA; Olympus Corp., Lake Success, NY) objective, Zeiss Wollaston prisms, and a Zeiss DIC condenser (0.63 NA). The computerized robotic stage was custom built and interfaced with Bio-Rad software (Bio-Rad Laboratories, Cambridge, MA) used in conjunction with a modified Bio-Rad MRC 500 laser scanning unit. Confocal fluorescence measurements were performed with the pinhole in the full open configuration, using a FITC filter set. Images were recorded onto an optical memory disk (Panasonic 2028F), digitized, and analyzed using a Data Translation Quick Capture board and Adobe Photoshop on a Macintosh IIfx. Retrospective analysis of cells was achieved by recording the coordinates of the cells being measured in timelapse with respect to a fudicial mark on the coverslip.

Fluorescence Quantitation

Quantitative fluorescence intensity measurements were performed in order to estimate the relative concentration of protein at the site of cell contact relative to that of noncontacting regions. Intensity measurements were made of the average pixel values in the region of the contact typically over an area of ∼50 μm^2 and compared to the average intensity over noncontacting cell borders. The intensity values were corrected for background by subtracting the intensity value measured over a small region adjacent to the cell in question. The ratio of these two quantities was then calculated to determine the relative protein concentrations (fluorescence intensity ratio; FIR)[1]. All measurements were performed over the linear range of the data acquisition hardware. Since the axial resolution in confocal microscopy is still much greater than the thickness of the cell, these measurements may also reflect the cross-sectional thickness of the specimen. However since the measurements performed on the total E-cadherin pool appear constant over the range of contact times (Fig. 3), we do not feel that the enrichment in Triton X-100 E-cadherin results from a changing morphology (e.g., thickening along the axial direction of the contact site), but rather is due to a local concentration increase of this species.

Results

Initial analysis of individual MDCK cells revealed that motility of these cells is relatively slow, on the order of microns per minute, and that it was difficult to predict if a contact was going to be made between two cells, even when cells were plated at high densities. To increase the probability of observing initial contacts between cells, we used a motorized, computer-controlled stage attached to a microscope adapted for laser-scanning confocal and timelapse microscopy. This allowed us to sequentially record images from many different sites on a single coverslip (for details see Cooper and Smith, 1992; and Materials and Methods).

Typically, five to eight imaging sites, with an average of 12 cells per site, were selected on each coverslip and then observed over time using high resolution, differential interference contrast (DIC) optics; images were recorded on an optical memory disk. Of ∼5,000 cells examined by this method, 67 made stable contacts within a recording period of 30–120 min. After a period of recording, the coverslip was fixed and processed for immunostaining with specific antibodies, and then the same cells were analyzed by laser-scanning confocal microscopy (retrospective histochemis-

1. *Abbreviations used in this paper*: DIC, differential interference contrast; E-$cadherin^{TX1}$, Triton X-100 insoluble pool of E-cadherin; FIR, fluorescence intensity ratio.

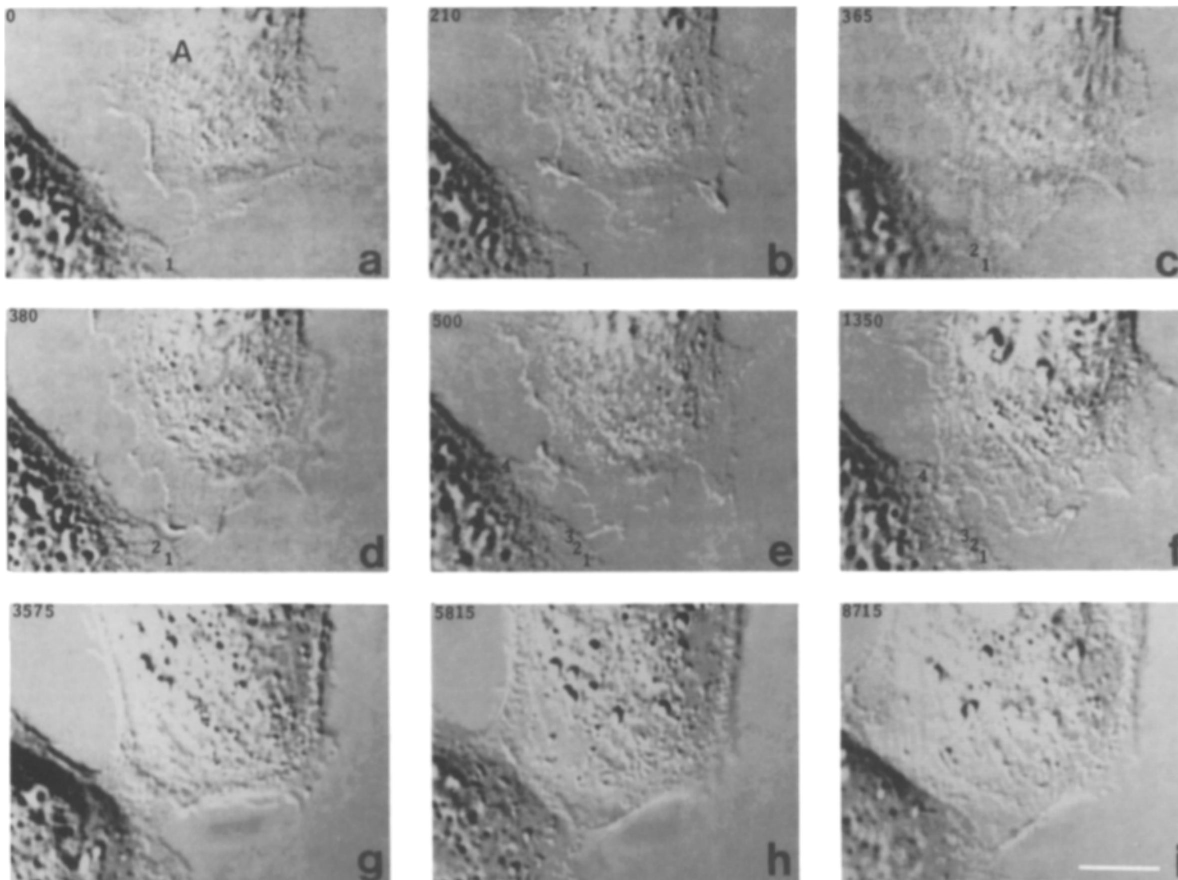

Figure 1. Morphological analysis of contact formation in MDCK epithelial cells. Time lapse, high resolution, differential interference contrast microscopy was used to observe contact formation between cells. Cells make transient contacts (*a*, *c*, and *e*; contacts #1, 2, and 3), which are subsequently released (*b* and *d*), before formation of a stable contact (contact #4; *f*–*i*). Upon formation of a stable contact, the contact extends in a zippering fashion (*f*–*h*). Time of viewing from first tentative contact is recorded in seconds in upper left corner. Bar, 10 μm.

try). After acquisition, individual time-lapse sequences of a single contact site were collated onto a separate optical disk, and inspected to determine the history of the formation of each cell–cell contact. The distribution of specific proteins in these cells that had been determined by retrospective histochemical analysis could then be correlated directly with temporal and spatial events involved in the formation of the contact site between cells.

Multiple Transient Contacts Precede Formation of Stable Contacts

High-resolution observations of motile MDCK cells revealed that establishment of a stable cell–cell contact is preceded by the formation of multiple, independent, and unstable contacts. These unstable contacts are characterized by the formation of a membrane extension from one cell that touches the apposing cell and is then rapidly retracted. This exploratory stage is shown in Fig. 1. Initially, a fine filopod is extended from cell A towards the other cell and a contact is made (Fig. 1 *a*). Two hundred and ten seconds later (Fig. 1 *b*), this contact has been retracted, and the cells are separate. After a further 155 s (Fig. 1 *c*), a second contact is made at approximately the same membrane region as the first contact, although the area of the membrane involved is broader. This contact dissociated 15 s later (Fig. 1 *d*). After a further 120 s (Fig. 1 *e*), a third and fourth contact have formed, of which one is novel and the other is close to the site of second unstable contact, respectively. The novel contact is dissociated, while the fourth contact remains, and eventually extends to form the stable contact. This exploratory stage of formation and dissociation of cell contacts can occur for a variable length of time. On a number of occasions, cells were observed making multiple, transient contacts, but did not form a stable cell–cell contact.

Formation of the stable contact is defined by morphological stability over time (i.e., it is not retracted), and by the absence of a dark line at the contact in Nomarski DIC optics which, when present, is indicative of a gap between the membranes of adjacent cells. Time-lapse analysis of the formation of a stable contact shows that it extends along the membrane from the point of initial stable contact in a "zippering" manner, rather than through the coalescence of multiple individual contacts along the interacting membranes (Fig. 1, *g*–*i*). The speed of the zippering varies between cells (~0.17–2.5 μm/min), and appears to be dependent on the speed at which the cells approached each other, and the amount of membrane apposed at the contact site. The direction of zippering does not appear to be biased towards previous sites of unstable contacts.

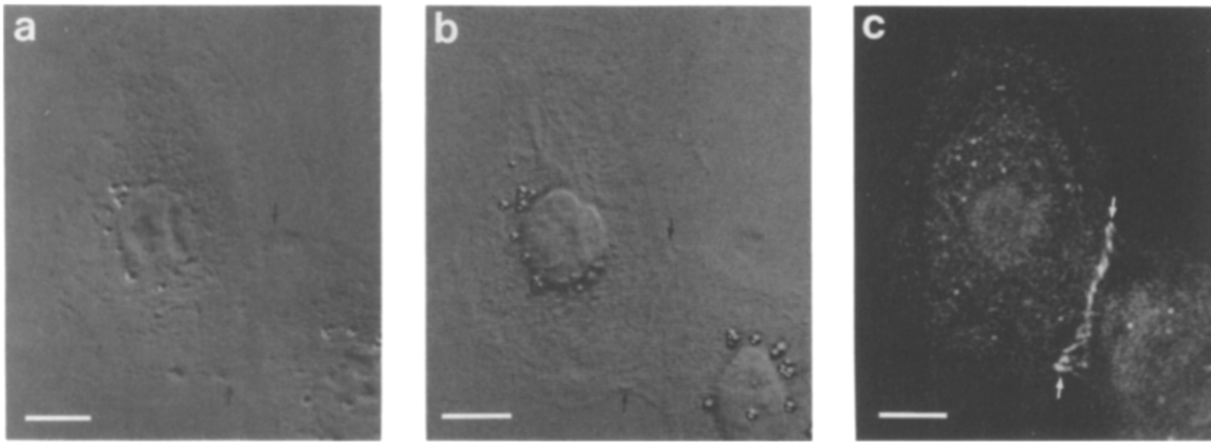

Figure 2. Correlation of formation of a stable contact and distribution of E-cadherin. The morphology of stable contacts between cells is preserved during fixation and extraction. (*a*) Morphology of a stable cell–cell contact that had formed 22.4 min before extraction and fixation. (*b*) Morphology of the same contact after extraction in 0.5% Triton X-100 and fixation with formaldehyde. (*c*) Cells were processed for indirect immunofluorescence with mAb directed against E-cadherin, and observed using laser scanning confocal fluorescence microscopy. Note that the E-cadherin^{TXI} specifically enriched within the boundary of the contact region. Bar, 10 μm.

A Triton X-100 Insoluble Pool of E-Cadherin Is Enriched at Stable Contacts

We chose to analyze the distribution of E-cadherin during the formation of stable cell contacts. Although different elements of the intercellular junctional complex of MDCK cells contain cell adhesion proteins, previous studies have demonstrated that initial cell–cell adhesion mediated by E-cadherin is required for, and precedes formation of the junctional complex (Gumbiner et al., 1988; Jenson and Wheelock, 1992).

It was important for this analysis that the morphology of the contact site was maintained during the manipulations required to process the cells for retrospective immunohistochemistry. Preservation of this morphology permits precise correlation of the distributions of specific proteins with stable contacts. Fig. 2 shows the formation of a stable contact between two cells, and the subsequent morphology of the stable contact before, and then after extraction with Triton X-100 and immunostaining for E-cadherin. Fig. 2 shows two cells that have formed a stable contact of 22.4-min duration, as defined above. A DIC image of this contact is shown before extraction and fixation (Fig. 2 *a*). Subsequently, the cells were extracted at 4°C in an isotonic buffer containing 0.5% Triton X-100, fixed with formaldehyde at room temperature, and processed for immunostaining with E-cadherin antibodies (for details see Materials and Methods). Retrospective analysis of these cells shows that the morphology of the stable contact appears identical after extraction and fixation (Fig. 2 *b*). Laser-scanning confocal microscopy of E-cadherin staining shows that there is a restricted enrichment of E-cadherin within the precise boundary of the cell–cell contact (Fig. 2 *c*). Some punctate staining is observed by confocal microscopy in the cytoplasm of the cells, but this staining pattern is spatially separate from the plasma membrane (see Discussion). Regions of the membrane outside the boundary of the contact do not stain for E-cadherin under these extraction conditions (Fig. 2 *c*). Some punctate stain is observed in the cytoplasm of the cell distal to the plasma membrane (see Discussion). Staining with a nonimmune antibody, or with only the fluorescein-conjugated secondary antibody yielded little detectable fluorescence and no enrichment at regions of cell contact (data not shown), demonstrating that the staining was specific for E-cadherin.

Recruitment of E-Cadherin to a Triton X-100 Insoluble Pool ~10 min after Formation of a Stable Contact

To determine the time course of enrichment of the Triton X-100 pool of E-cadherin at the contact site, we analyzed cells which had formed stable contacts for 0.5-120 min. Fig. 3 shows examples of cells that were extracted, fixed, and processed for immunostaining at different times following formation of stable contacts. At times of 0.5 (Fig. 3 *a*), 1.1 (Fig. 3 *b*), 1.5 (Fig. 3 *c*), and 8.3 min (Fig. 3 *d*) after formation of a stable contact, we detected little or no difference in E-cadherin staining at the contact site compared to regions of the cell surface outside the boundary of the contact. In contrast, we detected a dramatic increase in staining intensity of the E-cadherin^{TXI} in cells which had maintained a stable cell contact >10 min: 10.8 (Fig. 3 *e*), 11.7 (Fig. 3 *f*), 13.3 (Fig. 3 *g*), and 13.8 min (Fig. 3 *h*). In all cases, the E-cadherin^{TXI} was restricted to the site of stable contact, and was absent from areas of the cell surface outside the contact. The E-cadherin staining at these relatively early times has a fibrous and punctate appearance (Fig. 3 *e–h*), which represents interdigitation at the contacting membranes of closely apposed cells; this contrasts with a more homogeneous appearance at contacts formed after hours and days (data not shown). It is noteworthy that we also detected by confocal microscopy a punctate staining pattern of E-cadherin in the cytoplasm of cells; the small spherical structures may represent individual vesicles. At present, we do not know the relationship between the cytoplasmic and plasma membrane pools of E-cadherin.

We compared the fluorescence intensity of the E-cadherin^{TXI} at the membrane with the duration of stable cell contacts. The intensity of E-cadherin immunofluorescence at the contact point was normalized to the fluorescence inten-

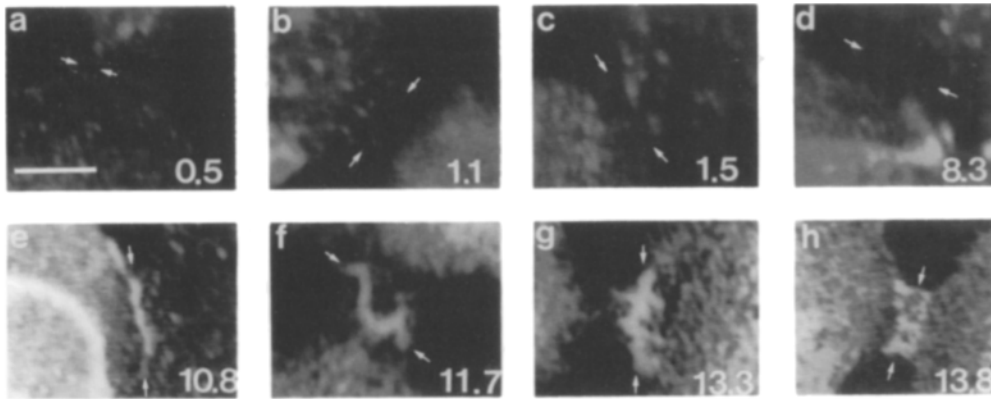

Figure 3. Time course of acquisition of the E-cadherin^{TXI} at stable cell contacts. The time course of acquisition of the E-cadherin^{TXI} at newly formed stable cell contact was determined in cells processed for immunofluorescence at various times after contact formation; the region of contact was determined by DIC microscopy, and is designated by arrows. Note the lack of E-cadherin staining at the membranes of cells that had formed stable contacts for a short duration (up to 8.3 min: *a–d*), and the marked accumulation of E-cadherin^{TXI} specifically at stable contacts of longer duration (>10.3 min: *e–h*) compared to noncontacting regions of the membrane.

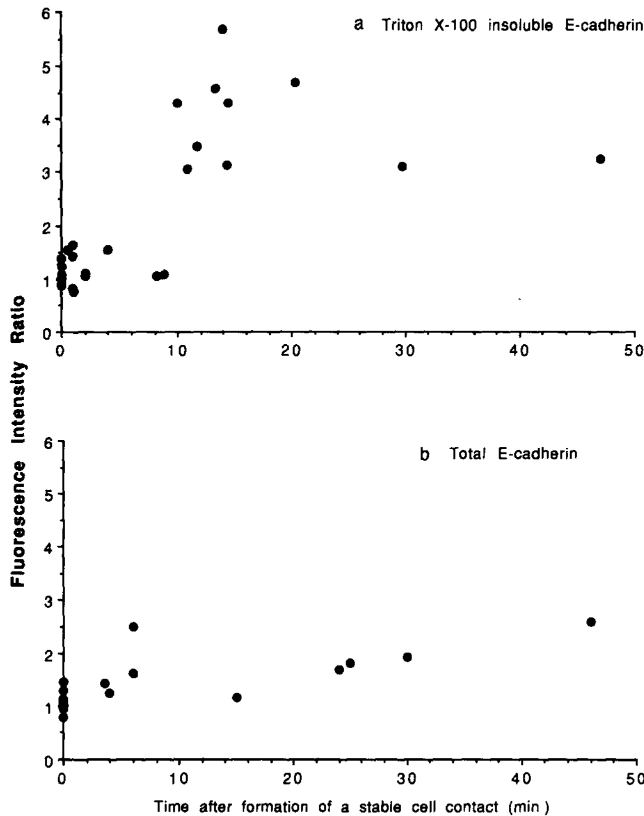

Figure 4. Quantitation of fluorescence intensity ratios of E-cadherin at stable cell contacts. Estimates of the relative concentration of E-cadherin at the membrane were obtained for both the total E-cadherin pool (methanol fixation) and the E-cadherin^{TXI} pool (Triton X-100 extraction) by quantitating the fluorescence intensity at cell–cell contacts and normalizing the amount to that in a non-contacting region of the membrane in the same cell pair (see Materials and Methods). Representative images of cells that were analyzed are shown in Fig. 2; note that Figs. 3 and 5 show only the areas of cell–cell contact from images similar to those in Fig. 2. The derived (FIR) are plotted as a function at the duration of each stable contact. (*a*) E-cadherin^{TXI}; note the dramatic enrichment of E-cadherin^{TXI} at stable contacts of duration longer than 10 min. (*b*) Total E-cadherin in methanol-fixed cells; note that there is no significant change in the amount of E-cadherin at the contact up to 50 min after formation of a stable cell contact.

sity of noncontacting regions of the membrane at the edge of the same cells (FIR); mean ratios are given with the standard deviation (see Materials and Methods).

Analysis of E-cadherin fluorescence at times up to ~10 min after formation of a stable contact shows no significant difference between the intensity at contacting and noncontacting regions of the membrane (Fig. 4 *a*; mean FIR of 1.2 +/− 0.3 in cells in stable contact for 0.5–10 min). However at times after ~10 min, we detected a dramatic increase in the fluorescence intensity of the E-cadherin^{TXI} at the contact site (Fig. 4 *a*). Specifically, the fluorescence intensity at the cell contact was three to six times greater than that present at a noncontact region of the membrane (mean FIR of 4.0 +/− 0.9 at times between 10 and 30 min).

The Increase in the Triton X-100 Insoluble Pool of E-Cadherin Is Not Caused by an Increase in the Total Amount of E-Cadherin at the Contact Site

The increase in the E-cadherin^{TXI} at the stable contact could reflect an increase in the total amount of E-cadherin, or the titration of E-cadherin from a preexisting Triton X-100 soluble pool into an insoluble pool. Discrimination between these possibilities has ramifications for models of cell adhesion (see Introduction). To approach this problem, we examined the distribution of total E-cadherin at different times after formation of a stable cell contact (Fig. 5). Cells were fixed and permeabilized in 100% methanol at −20°C; under these conditions little or no protein is extracted from the cell (Pasdar and Nelson, 1988; Shore and Nelson, 1991); similar results are obtained when cells were fixed in formaldehyde and then extracted in buffers containing Triton X-100 (data not shown). Cells were processed for immunostaining and retrospective analysis as described in detail above. Comparison of the morphology of the contact before and after fixation revealed little or no detectable changes (data not shown).

Cells fixed/permeabilized in 100% methanol at −20°C exhibit a higher level of E-cadherin staining intensity than cells extracted with buffers containing Triton X-100. This staining is specific, as indicated by virtually no detectable fluorescence when cells are incubated with only secondary antibody. Analysis of E-cadherin staining at cell contacts that had formed for 4 (Fig. 5 *a*), 6 (Fig. 5 *b*), or 15 min (Fig.

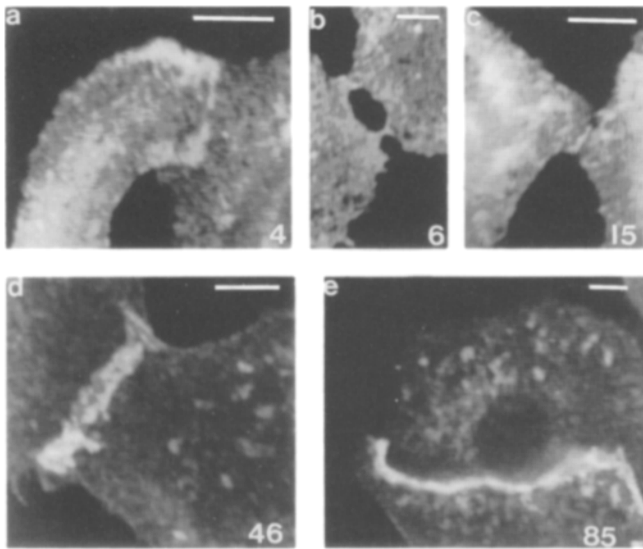

Figure 5. Distribution of total pool of E-cadherin as a function of duration of stable cell–cell contacts. MDCK cells were analyzed for formation and duration of cell–cell contacts using time-lapse, DIC microscopy, fixed in −20°C methanol and processed for E-cadherin immunostaining as described in the legends to Figs. 1 and 2. (*a*) 4-min stable contact; (*b*) 6-min contact; (*c*) 15-min contact; (*d*) 46-min contact; and (*e*) 85-min contact. Note E-cadherin staining in very early contacts (*a* and *b*), and the increase in staining after 85 min of contact (*e*). Bars, 10 μm.

5 *c*) revealed little enrichment of E-cadherin staining at the contact beyond that which can be accounted for by summing the staining of two closely apposed membranes (see below). E-cadherin staining at contacts that had formed for >45 min (e.g., 46 and 85 min; Fig. 5, *d* and *e*) revealed increased fluorescence intensity compared to a noncontact region of the membrane. In some cases the staining pattern appears fibrous due to the interdigitation of membranes at the contact site (see also Fig. 3). However, interpretation of this increased fluorescence intensity is complicated by the fact that at these later times the height of membrane at the contact increases, as determined by analysis with the laser-scanning confocal microscope.

To quantitate the changes in E-cadherin staining at different times after formation of a stable contact, we measured the fluorescence intensity ratio, as described in detail above. There was little variation in the fluorescence intensity ratio at the edge of the noncontact regions in different cells (mean FIR 1.0 +/− 0.1). We detected no significant increase in the fluorescence intensity ratio of E-cadherin at the contact for 30 min (Fig. 4 *b*). The FIR is 1.7 (+/− 1.31) in cells which have been in contact for 0.1–10 min, and 1.6 (+/− 0.34) in cells in contact for 10–30 min. At later times (60–120 min), the FIR increases (~3.0 +/− 0.98), however, we cannot attribute this change solely to increases in protein concentration since the vertical extension of the contact becomes significant at these times (see Materials and Methods).

These results demonstrate a significant difference in the kinetics of accumulation of the Triton X-100 soluble and insoluble pools of E-cadherin at stable contacts. The size of the Triton X-100 insoluble pool increases at the stable contact following a lag period of ~10 min. In contrast, we detected little or no increase in the total amount of E-cadherin at stable contacts for up to 30 min. These results indicate that the increase in size of the E-cadherin^{TXI} does not reflect recruitment of protein from outside the contact, but the conversion of a pre-existing pool of Triton X-100 soluble E-cadherin into a Triton X-100 insoluble form specifically at the contact site.

The Increase in the Pool of Triton X-100 Insoluble E-Cadherin Does Not Correspond to Changes in the Distributions of Actin or Fodrin

We sought to determine whether the increase in the E-cadherin^{TXI} could be accounted for by a change in the distributions of components of the cortical cytoskeleton. We analyzed the distributions of actin and fodrin in relation to the duration of a stable contact, and compared the results with the time course of the increase in E-cadherin^{TXI}. Actin and fodrin are major components of the cortical cytoskeleton that co-localize with E-cadherin in cells that have been in contact for periods of hours and days (Hirano et al., 1987; Ozawa et al., 1990; Matsuzaki et al., 1990; Balsamo et al., 1991; Takahashi et al., 1992; Nelson et al., 1990; Itoh et al., 1991).

Actin filament distribution was determined using bodipy-conjugated phallacidin (Fig. 6). Actin filaments were detectable at the area of cell contact, however there was no enrichment of actin at the contacting membrane compared to noncontacting membranes over the first 30 min of stable cell contact. For example, in Fig. 6 *a*, contacts that had been stable for 16 min (contact A and B), or for 28 min (contact B and C) exhibit no enrichment of actin at the contacting membranes compared to noncontacting membranes. Similarly, in Fig. 6 *b*, neither the 9 min (contact A and B), nor 29-min stable contact (contact B and C), show enrichment of actin compared to the noncontacting membranes. Other contacts that had formed for 29 min (Fig. 6 *c*) or 30 min (Fig. 6 *d*) showed a similar organization of actin. Actin filaments do appear to be oriented towards the contact site(s), however a similar orientation of actin filaments is observed at noncontacting membrane extensions (see Fig. 6 *b*). At later time points (hours and days after cell contact), there is an apparent increase in the level of actin at cell contacts, however the interpretation of this increase is complicated by the increase in height of the cell contact at these times (data not shown).

The distribution of the Triton X-100–insoluble pool of fodrin was also analyzed in noncontacting single cells, and in cells in which contacts had formed for a few minutes, or for up to 30 min (Fig. 7). We detected little or no increase in the amount of the Triton-X-100–insoluble pool of fodrin at contacts compared to noncontacting regions of the membrane in the same cells. Furthermore, we did not detect any significant enrichment of fodrin at the contact over this time period (FIR 1.1 +/− 0.1 in cells in contact for 10–30 min). For example in Fig. 7, a cell which has no contact (cell *a*) has the same pattern of membrane-associated fodrin staining as cells which have been in contact for 12 min (contact C and D), or for >28 min (contact B and C). Thus, there appears to be little or no correlation between the increase in the E-cadherin^{TXI} and a spatial reorganization of these components of the cortical cytoskeleton at immediate and early stages in formation of a stable cell–cell contact.

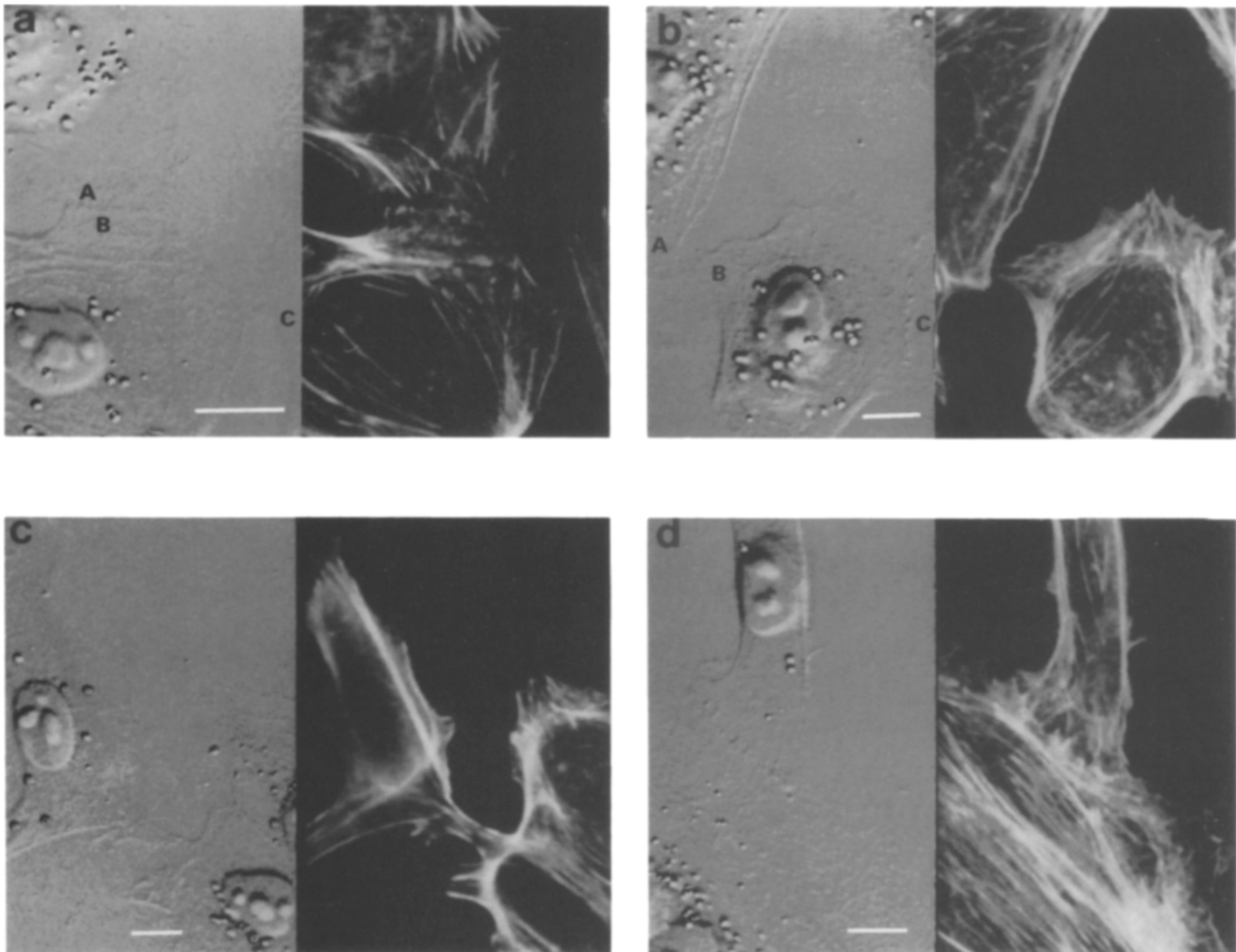

Figure 6. Actin does not become enriched at stable cell contacts of short duration. Times of stable contact formation were determined as described in the legend to Fig. 2. Cells were fixed and stained with Bodipy-phallacidin. Simultaneously recorded DIC and fluorescent images are presented in each panel. (*a*) *A–B*, 16-min stable contact; *B–C*, 28-min stable contact. (*b*) *A–B*, 9-min stable contact; *B–C*, 29-min stable contact. (*c*) 30-min stable contact. (*d*) 31-min stable contact. Note the lack of enrichment of actin staining at stable cell contacts of up to 30-min duration compared to noncontacting membranes. Bars, 10 μm.

Discussion

Cell–cell adhesion is an essential component in the development of cell diversity in complex multicellular organisms (Takeichi, 1991). Much is known about the proteins involved in cell recognition and adhesion, and of the mechanisms involved in cellular remodeling which occurs on the order of hours and days after cell adhesion. However, little is known about the very early steps in the molecular cascade leading to cellular differentiation. To temporally and spatially dissect these early, critical steps in cell–cell adhesion, we sought to develop a system in which the duration and distribution of cell–cell contacts can be correlated in the same cell with the spatial organization of proteins thought to be important in cell–cell recognition and adhesion. This precise analysis allows us to discriminate between different models of cell recognition and adhesion. This study is unique in that it provides first insights into immediate and early events of cell contact between epithelial cells, and consequential changes in the distributions of a specific adhesion protein and components of the cortical cytoskeleton.

Three Morphologically Distinct Steps in Epithelial Cell Recognition and Adhesion

High spatial resolution analysis of many independent cell–cell contacts between MDCK cells demonstrated that epithelial cell recognition and adhesion are characterized morphologically by at least three sequential steps: (*a*) an exploratory step involving numerous, transient contacts; (*b*) formation of a stable contact; and (*c*) extension of the stable contact by zippering of closely apposed membranes.

The first step in cell recognition and adhesion is the formation and release of numerous, transient contacts. Although this exploratory stage was not necessarily followed by a stable contact, formation of a stable contact was always preceded by these transient contacts. The time of transition between these steps may be a stochastic process, and formation of transient contacts may simply reflect the fact that the concentration of adhesion proteins in that area of the membrane is below a critical level. Experiments using cells expressing different amounts of transfected adhesion proteins could distinguish between these possibilities. Alternatively,

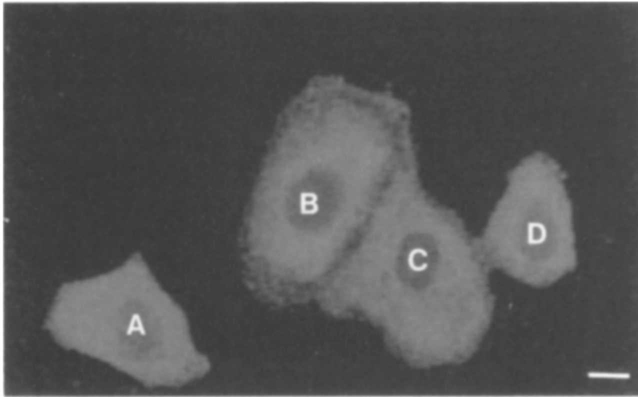

Figure 7. Fodrin does not accumulate at stable cell contacts of short duration. Formation of stable contacts within a group of four cells was recorded by time-lapse DIC microscopy in the same microscopic field. The cells were extracted with Triton X-100 buffer, fixed, and then processed for immunostaining with antibodies directed against fodrin. Cell A lacks any cell contacts. The stable contact between cell C and cell D was of 12-min duration, and the contact between cell B and C was of > 28-min duration. Note the low level of Triton X-100 insoluble staining of fodrin at the membranes of stable contacts of greater than 10-min duration. Bar, 10 μm.

it is possible that constant probing by transient contacts induces an increase in the adhesivity of that area of the membrane, which aids the transition of a transient contact into a stable contact. An increase in adhesivity may reflect localized recruitment of cell adhesion proteins to that membrane, or transduction of a signal that modulates the activity of a pre-existing pool of protein. By examining the amount of E-cadherin present at noncontacting cells and in cells in various stages of cell contact we could discriminate between these mechanisms. Note that although elements of the intercellular junctional complex of MDCK cells contain different cell adhesion proteins, previous studies have demonstrated that initial cell-cell adhesion mediated by E-cadherin is required for, and precedes formation of the functional complex (Gumbiner et al., 1988; Jenson and Wheelock, 1992).

Formation of a stable cell contact is defined by the lack of release of the contact and the loss of a dark line separating the cells that can be observed in the microscope with Nomarski DIC optics, and is characterized by the cessation of the probing activity of transient contacts. After a variable period of time, the stable contact begins to spread along the apposed membranes by a zippering activity, rather than through the coalescence of multiple individual contacts along the apposed membranes. This suggests that a nucleating event may be needed in order to form a stable contact. The direction of zippering does not appear to be biased towards previous sites of transient contacts. Interestingly, a similar progressive lengthening of cell-cell contacts, also termed "zippering", was inferred in a study of neuromuscular junction formation (Buchanan et al., 1989).

Transitional Stages in E-Cadherin and Cytoskeleton Organization during Formation of Stable Contacts

We have sought to investigate E-cadherin organization by extracting cells under different conditions to visualize either the total (methanol fixation; see Shore and Nelson, 1991), or E-cadherin^{TXI}. It is possible that there are subtle changes that might occur in the antigenicity of E-cadherin under these different fixation conditions; note, however that the mAb recognizes epitopes on E-cadherin following either formaldehyde or methanol fixation. Therefore, we do not directly compare the fluorescence intensities of E-cadherin at cell contacts, but rather the accumulation of E-cadherin at cell contacts relative to noncontacting membranes in the same cell (FIR; see Fig. 4).

Analysis of total E-cadherin distribution (methanol fixation), at short times after formation of a stable contact revealed that there is no significant difference in the amount of E-cadherin present at the contact in cells which are either noncontacted, have just initiated a stable contact, or have been in stable contact for up to 30 min. Analysis of E-cadherin^{TXI} revealed only a small amount of E-cadherin^{TXI} at the contact at the time of initiation of a stable cell contact or after 10 min. However after ~10 min of stable cell contact there is a rapid increase in Triton X-100 insolubility specifically at the contact site. We interpret the acquisition of Triton X-100 insolubility as an association of E-cadherin with the cytoskeleton after 10 min of stable cell-cell contact. Triton X-100 insolubility is commonly used as an indicator of association of proteins with the cytoskeleton (Ben-Ze'ev et al., 1979; Brown et al., 1976; Fey et al., 1984; Fulton et al., 1980; Kaufman et al., 1985; Prives et al., 1982), and specifically has been used to indicate that E-cadherin is interacting with the cytoskeleton (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989; Hirano et al., 1987; Shore and Nelson, 1991). However it is also possible that this is a reflection of another form of molecular reorganization of E-cadherin (see below).

Our results shed new light on the role of the interaction of E-cadherin with the cortical cytoskeleton in the formation of cell-cell contacts. Previous studies have shown that loss of binding of E-cadherin to the cytoskeleton inhibits cell aggregation in suspension cultures (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989). In contrast, our studies indicate that stable contacts precede the interaction with the cortical cytoskeleton and the formation of E-cadherin^{TXI} by ~10 min. Differences between these studies may reflect the fact that analysis of cell adhesion in suspension culture necessitates constant stirring, which results in shearing forces on the cell; such shearing forces are not present in our assay. We suggest that interactions between E-cadherin and the cytoskeleton are not required for initial cell recognition and adhesion. In support of this is the observation that the isolated extracellular domain of E-cadherin binds to E-cadherin present on cells, suggesting that cadherin-cadherin binding can occur independent of the cytoplasmic and membrane domains (Wheelock et al., 1987). We suggest that the interaction of E-cadherin with the cytoskeleton provides strength to the contact and initiates remodeling the cell (see below).

The main candidates for mediating interactions between E-cadherin and the cytoskeleton are the catenins (Ozawa et al., 1989; Herrenknecht et al., 1991; Nagafuchi et al., 1991; McCrea et al., 1991). However, the catenins bind to newly synthesized E-cadherin before transport to the cell surface, and do not dissociate from E-cadherin once the complex has formed (Ozawa and Kemler, 1992). It is unlikely, therefore, that changes in the distribution of the catenins are responsible for the change in the ability of E-cadherin to interact with

the cytoskeleton. It is possible, however, that posttranslational modifications of the catenins alter the association of E-cadherin with the cytoskeleton.

Actin and fodrin have both been suggested to be responsible for the pool of E-cadherin^{TXI}. Previous studies have shown that E-cadherin co-localizes with actin (Hirano et al., 1987; Ozawa et al., 1990; Matsuzaki et al., 1990; Balsamo et al., 1991; Takahashi et al., 1992) and ankyrin and fodrin (Nelson et al., 1990; Itoh et al., 1991) in a variety of cell types, and complexes of E-cadherin, ankyrin, and fodrin have been identified in extracts from MDCK cells (Nelson et al., 1990; Itoh et al., 1991). To determine if changes in the availability of these proteins could account for changes in the amounts of E-cadherin^{TXI} we followed their distributions as a function of cell contact.

Actin was detected at the edge of both contacting and noncontacting membranes during all times examined, and we could not detect any significant increase in the concentration of actin at cell contacts during the time when the increase in E-cadherin^{TXI} was observed. Analysis of fodrin also demonstrated no significant changes in its distribution during this time. Together, these results indicate that the increase in E-cadherin^{TXI} does not correlate with a recruitment of either actin of fodrin to the contact. However, cell contact may result in alterations in E-cadherin, or associated proteins, that allow it to attach to the pre-existing cortical actin meshwork. In previous work we demonstrated that ~30% of Triton X-100-soluble E-cadherin is present in a complex with fodrin and ankyrin (Nelson et al., 1990). Based upon the results presented here, we suggest that the initial interaction of E-cadherin with the Triton X-100-insoluble cytoskeleton is independent of these E-cadherin-fodrin complexes, and that these complexes are recruited later in the development of cellular polarity.

Diffusion-mediated trapping of E-cadherin at cell contacts, resulting in localized increases in the concentration of E-cadherin, has been proposed as a mechanism for stabilizing cell adhesion and inducing interactions with cortical cytoskeleton (Gingell and Owens, 1992; reviewed in Singer, 1992; and McNeill and Nelson, 1992). However, our finding that there is no significant increase in the concentration of E-cadherin at the contact, at the time at which there is an increase in the amount of E-cadherin^{TXI}, are inconsistent with this mechanism.

Since cells can form stable contacts independent of the interaction of E-cadherin with the Triton X-100-insoluble cytoskeleton, yet binding of E-cadherin to the Triton X-100-insoluble cytoskeleton does eventually occur, what is the function of this cytoskeletal interaction? One possibility is that E-cadherin binding to the cytoskeleton nucleates cytoskeleton assembly at the contact, providing a mechanism for the subsequent long-term remodeling of the membrane. We have suggested previously that interactions between E-cadherin and the cortical cytoskeleton drives the assembly of the cytoskeleton at cell contacts, which results in the recruitment to those sites of specific membrane proteins, such as Na,K-ATPase (Nelson and Veshnock, 1987; Morrow et al., 1989; Davis et al., 1990), that also bind to the cortical cytoskeleton (Nelson et al., 1990). This is supported by the isolation of protein complexes from MDCK cells that contain E-cadherin, Na,K-ATPase, ankyrin, and fodrin (Nelson and Hammerton, 1989; Nelson et al., 1990). More directly, expression of E-cadherin in fibroblasts results in the recruitment of fodrin and Na,K-ATPase to points of cell-cell contact (McNeill et al., 1990). In addition, cytoskeletal assembly could recruit kinases and phosphatases to the contact, resulting in localized modifications of E-cadherin and the cytoskeleton (Tsukita et al., 1991; Yang and Tonks, 1991). With the methods developed in this study, an extensive analysis of the timing of recruitment of such signaling molecules can now be performed.

Role of Signaling in Formation of Cell Contacts

The most remarkable finding of this study is the rapid association of E-cadherin^{TXI} specifically at the contact point after a lag time of 10 min (Figs. 3, *e–h*, and Fig. 4). The prompt and localized nature of these responses suggests that a form of signaling is occurring. This signal is not due to a change in the concentration of E-cadherin at the contact (see above), and cannot be due simply to a global activation of a second messenger system, since these events are restricted to the contact site.

Homotypic interactions between E-cadherin leading to conformational changes in E-cadherin, or posttranslational modifications in E-cadherin and/or associated proteins could account for these events. Posttranslational modifications of components of the membrane skeleton have been shown to modify their affinities (Bennett, 1990), and posttranslational modification of E-cadherin has been correlated with alterations in the ability of E-cadherin to mediate adhesion (Sefton et al., 1992). Local activation of second messengers induced by cadherin-cadherin binding could lead to these modifications. Antibody binding to neuronal cell adhesion molecule (an Ig superfamily adhesion molecule) has been demonstrated to induce calcium fluxes, alterations in intracellular pH, and inositol phospholipid turnover within the first 20 min of interaction (Schuch et al., 1989). Recent studies have shown that induction of neurite outgrowth by N-cadherin depends upon activation of G proteins and L- and N-type Ca^{2+} channels (Doherty et al., 1991). In those studies the endpoint was measured 24-48 h after induction. The present study provides first insights into immediate and early events in epithelial cell adhesion and demonstrates that an important signaling event occurs within minutes of cell contact in a spatially restricted manner, providing a precise spatial and temporal framework for future studies.

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