Establishment of the Circumferential Actin Filament Network Is a Prerequisite for Localization of the Cadherin-Catenin Complex in Epithelial Cells

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Abstract
With the adhesion molecules, the actin cytoskeleton controls cell-cell and cell-substrate interactions and participates in transmembrane signaling. The relationships between actin and adhesion complexes at the sites of adhesion have been well documented. Here we investigate by a series of studies whether a relationship exists between actin organization and the localization and function of the components of the cadherin-catenin complex (CCC) that participates in the cell-cell adherens junction. Reversible actin depolymerization reversibly affects the peripheral distribution of CCCs. Mutations in adenovirus E1A and the small GTPase rac1, but not Ha-ras, disrupt the circumferential, cortical actin filament (CAF) network and the targeting of CCC components to the cell surface. Disruption of actin stress fibers or microtubules does not interfere with CCC localization and function. Constitutive loss of the apical cortical actin ring results in epithelial cells in which components of the CCCs are found only in intracellular vesicles and never at the surface. A kinetic analysis of the de novo appearance of the CAF network and the CCCs at the cell surface was also conducted. When F-actin was dissolved, surface CCC components were internalized. Reestablishment of CAFs required about 4 h, during which time E-cadherin and α-catenin were found first in a juxtanuclear location and then in intracellular vesicles or post-Golgi carriers, similar to what was observed in cells expressing mutant E1A or rac1. Thus, disruption of preexisting CCCs resulted in their internalization and recycling to the Golgi. It was only after the regeneration of the filamentous actin ring beneath the cell surface that peripheral localization of CCCs was observed. A similar result was observed with dominant negative rac1. These data suggest that the status of cortical actin is assessed and transduced and thereby regulates the transport and delivery of cadherin and catenins to the cell surface.

Introduction
Cells in tissues are involved in a variety of relationships with their environment (reviewed in Refs. 1 and 2). The generation and maintenance of these interactions are important for maintaining tissue organization and for the conductance of the specialized function(s) of each respective tissue. The associations with other cells include specialized adhesion structures, such as TJs (occluding junctions), AJs (zonula adherens), and desmosomes. FAs and hemidesmosomes mediate connections with the physical surroundings, such as the basement membrane or extracellular matrix. Epithelial cells form permeability barriers between different areas in an organism. As a consequence, they are exposed to and can exhibit different interactions with their basal, lateral, and apical environments. This yields an asymmetric cell that is polarized in the apical-basal dimension (reviewed in Refs. 1 and 3). The signal for establishment of polarity starts with a cortical spatial cue: cell adhesion, which is mediated by a surface adhesion receptor. Specialized cytoskeletal and signaling networks assemble into multimolecular complexes at the cytoplasmic side of the receptor. The formation of such multipolypeptide organizations at targeted functional sites seems to be a common theme in signal transduction (reviewed in Ref. 4). In the absence of extracellular contacts, single epithelial cells are not obviously polar.

The AJs contain a classical cadherin that is a transmembrane, Ca\(^{2+}\)-dependent, homophilic, intercellular adhesion receptor that associates with the submembranous (circumferential) CAF network (or cable; reviewed in Refs. 5–8). Cadherins extend beyond the cell surface as lateral dimers that interact with similar molecules on adjacent cells to generate a zipper-like structure. The consequences of such a homotypic interaction include morphological changes that contribute to the establishment of typical tissue architecture. The E-cadherin ectodomain alone cannot accumulate at cell-cell contact sites and is therefore insufficient for AJ function. The intracellular, cytoplasmic domain of cadherin is also required (9, 10). The interaction between cadherin and CAF is not direct; rather, it is mediated by a catenin complex that interacts with the cytoplasmic tail of cadherin. The α- and β-catenins interact with different molecules, and the relative contributions of these interactions to the function of the AJ complex is not clear.

Received 7/8/99; revised 10/26/99; accepted 11/1/99.
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3 The abbreviations used are: TJ, tight junction; CCC, cadherin-catenin complex; CSK, cytoskeleton; AJ, adherens junction; CAF, cortical actin filament; PGC, post-Golgi carrier; FA, focal adhesion; ER, endoplasmic reticulum; SF, stress fiber; EGFR, epidermal growth factor receptor; F-actin, fibrillar actin; ABP, actin-binding protein; WT12S, wild-type E1A 12S.
b-catenins are the major complexed proteins involved in adhesion. Other associated proteins have been identified, but their role in adhesion is unresolved (see Ref. 8). b-Catenin binds to the cytoplasmic tail of cadherin, serving as a linker to a-catenin. The b-catenin binding site on E-cadherin is critical for chaperoning E-cadherin out of the ER and is therefore important for its processing and targeting (11). However, it is the association of a-catenin with the complex that is necessary for functional adhesion. A chimera of E-cadherin and a-catenin is also an ABP and bundling protein (13). It is likely that b-catenin, as the linker, serves to regulate cadherin function (14, 15). The CCC may be a target for various types of signal transduction (reviewed in Refs. 8 and 16).

The actin CSK controls cell-cell and cell-substrate interactions with the adhesion molecules, and it participates in transmembrane signaling (reviewed in Refs. 8, 17, and 18). CCCs interact with actin at the sites of cell-cell contact (19), and this association has been thought to be the mechanism by which the catenins contribute to the adhesive function of the complex (6). It has been postulated that cadherin adhesive strength is regulated by CCC interactions with the CSK. Cytochalasin inhibits cadherin-mediated adhesion (20, 21). However, because the CSK is involved in so many cellular processes, it could also be influencing adhesion indirectly. The best evidence for a role for actin is the association of a-catenin with actin (13). Additionally, regulators of actin have been shown to affect cell-cell adhesion (22–31). CCCs can also organize actin locally at the sites of cell-cell contact (22, 32, 33). We have previously shown that specific mutations in genes that encode proteins that affect the circumferential actin network affect a loss of CCC from the cell surface, with a concomitant loss of adhesion (23, 34, 35). This supports the hypothesis that CCCs are dependent on the actin CSK for their localization and function. Additionally, cadherin and catenin stability is decreased when CAFs are disturbed (36), suggesting the possibility that actin association is important for their stability and perhaps for targeting.

In the studies described herein, we have directly addressed this issue by examining the effects of genes and substances that affect actin structure on the localization of CCCs. In all cases, disruption of cortical actin interferes with CCC localization. We have also conducted a kinetic analysis of the de novo appearance of the cortical actin network and the CCCs at the cell surface. We have found that when F-actin is dissolved, CCCs are internalized to a juxtanuclear, Golgi location. Once actin starts to repolymerize, CCC proteins are found in PGCs (37). However, it is only after the presence of a filamentous actin ring beneath the cell surface that peripheral localization of CCCs is observed. This suggests that the status of circumferential actin is sensed and transduced and thereby regulates transport and delivery of cadherin and catenins to the cell surface.

Results

Hypertransforming Mutants of Adenovirus E1A Are Defective in Their Actin Structures and Localization of E-Cadherin. The E1A 12S gene (WT12S) of adenovirus is able to immortalize primary epithelial cells and to promote epithelial characteristics (34, 35, 38–42). This includes not only morphology, dependence on attachment to substrate, but also the expression and functioning of many epithelial cell-specific markers, including junctional complexes such as AJs. E-cadherin and the catenins are appropriately localized to the membrane at cell-cell junctions (Fig. 1; data not shown) in WT12S-immortalized epithelial cells. Such cells also exhibit a strong circumferential actin filament network as the dominant actin structure, with minor fine SFs visible at the level of substrate attachment. They very much resemble Madin-Darby canine kidney cells, which exhibit three actin CSK arrangements: (a) an apical ring that includes and circumcribes the microvilli; (b) a cortical network; and (c) SFs at the base of cells. TJs and AJs are at the same level as
the apical ring, at the junction of the apical and lateral surfaces (43, 44). Whereas E1A-immortalized cells have acquired infinite proliferative potential, they are not transformed. E1A-immortalized cells cannot grow in soft agar or form tumors in nude mice. Constitutively activated ras oncoprotein, V12ras, usually induces loss of AJ function (28). However, the prodifferentiation property of WT12S is so strong that even in the presence of V12ras, the WT12S + V12ras-expressing cells retain CAFs and appropriately localized and functioning AJs (Fig. 2) and TJs, similar to cells immortalized by WT12S that express only the endogenous WTras (Fig. 1; Refs. 23, 34, and 35). However, WT12S + V12ras cells have lost the expression of actin SFs and FAs (23, 34, 45). In contrast to the WT12S-immortalized cells, WT12S + V12ras-transformed cells are able to grow in soft agar and form tumors at the site of inoculation in nude mice but do not metastasize (46, 47).

The COOH terminus of WT12S is involved in modulating the extent of transformation by V12ras. Mutations in the COOH terminus of 12S result in V12ras-transformed cells that are hypertransformed in vitro and in vivo. They have acquired the ability to metastasize in nude mice (46–49). These mutant 12S + V12ras-hypertransformed cells exhibit drastically altered morphologies and very different growth and social properties in culture (34, 35, 45–49). Interestingly, cells that are immortalized but not transformed by such an E1A hypertransforming mutant are also different from cells immortalized by WT12S, even in the absence of oncogenic V12ras (34). Epithelial cells immortalized by one such 12S COOH-terminal mutant, HBdl12, are shown in Fig. 1. In contrast to cells immortalized by WT12S, the morphology of the HBdl12 cells no longer appears epithelial. The HBdl12 cells exhibit a deranged actin CSK, and E-cadherin is no longer observed at the cell surface but rather appears intracellularly in vesicular structures. As expected, HBdl12 cells can no longer form a permeability barrier (34). Thus, hypertransforming mutants of E1A interfere with the differentiation program of epithelial cells, resulting in aberrant actin structures. The data support a relationship(s) between the state of differentiation and actin structural manifestations and CCCs.

Constitutively Active but not Dominant Negative rac Results in the Loss of Surface E-Cadherin. We have previously shown that the coexpression of a constitutively activated rac protein, V12rac, with WT12S + V12ras can also bring about a hypertransformed phenotype, which can be suppressed by the expression of dominant negative rac, N17rac (45). Thus, the prodifferentiation effects of WT12S can be disrupted by V12rac. rac is a member of the Rho GTPase subfamily, which is a subset of the large ras-related GTPase superfamily. Like the several other members of the Rho GTPase subfamily, rac is involved in the regulation of actin structures (reviewed in Refs. 50–53). Consistent with this function of rac, actin in WT12S + V12ras + V12rac cells is aberrantly arranged compared to cells expressing WTrac (WT12S + V12ras + neo) or dominant negative rac (N17rac; WT12S + V12ras + N17rac; Fig. 2; Ref. 45). Cells expressing

Fig. 2. WT12S supports epithelial differentiation in the presence of V12ras, but constitutively activated rac (V12rac) disrupts circumferential actin and results in the accumulation of E-cadherin in PGCs. Primary epithelial cells were transfected with expression plasmids containing the genes indicated on the left. At confluence, the cells were fixed and processed for indirect immunofluorescence using antibodies to E-cadherin and phalloidin-FITC. Bar, 25 μm.
V12rac also have very altered morphologies and have almost a total loss of epithelial characteristics (23, 45). The CCCs in WT12S + V12ras + V12rac cells are very unstable, E-cadherin is not found on the surface, and no cell-cell junctions are observed (Fig. 2; Refs. 23 and 36). This is consistent with the observation that activated rac is required for hepatocyte growth factor/scatter factor-induced AJ disruption (28). E-cadherin is found in vesicular structures in the cytoplasm of WT12S + V12ras + V12rac cells (Fig. 2), similar to those observed above in HBd12-immortalized cells (Fig. 1). In cells expressing N17rac (WT12S + V12ras + N17rac), the actin CSK resembles that seen in WT12S + V12ras cells. The CCCs are even more stable in the presence of N17rac than in WT12S-immortalized cells (36), and they are correctly localized at cell-cell interfaces (Fig. 2). The levels of rac expression are similar in V12rac- and N17rac-expressing cells (36). Thus, the localization and stability of E-cadherin seem to be affected by rac, presumably via its regulation of actin. Whereas rac seems to affect the localization of E-cadherin, other surface receptors are not thus affected, such as the EGFR (Fig. 3).

**Disruption of Cortical Actin by Cytochalasin Reversibly Disrupts Circumferential CCCs.** Cytochalasins B and D are actin capping drugs that depolymerize F-actin into G-actin and compromise the permeability barrier, allowing an increase in the diffusion of small molecules (54). When WT12S-immortalized epithelial cells were treated with cytochalasin, the SFs were lost first (within 30 min), at which time the CAFs began to depolymerize into discrete puncta or aggregates along the former cell-cell interfaces (Fig. 4, arrow 2). This differs from the ATP depletion of Madin-Darby canine kidney cells, in which cortical actin was the most sensitive to depolymerization (43). Wherever CAFs were disrupted, circumferential staining of E-cadherin was also lost. Interestingly, E-cadherin colocalized with actin in the newly formed puncta at the cell perimeters (Fig. 4, arrow 2). Wherever cortical actin was still present, the localization of E-cadherin was also unaltered (Fig. 4, arrow 1). The time frame of actin depolymerization and AJ disruption seemed to be coordinated. Similar kinetics of E-cadherin and Na⁺K⁺-ATPase internalization and cortical actin depolymerization and apical ring dissolution by ATP depletion have been observed (55). With increased incubation time with cytochalasin, the CAFs ultimately were completely lost, concomitant with an increase in the number and size of the actin-containing aggregates. At this time, all of the E-cadherin colocalized with actin in the large puncta that formed (Fig. 4, 2h, arrow 2). These actin and E-cadherin-containing aggregates could even be visualized by phase microscopy as phase-dense structures (Fig. 4, 2h, arrow 2). Whether the puncta/aggregates observed here are analogous to the previously described puncta, which are spatially discrete microdomains at cell-cell contacts that contain AJ proteins that associate with a bundle of actin filaments to initiate cell contacts (56), is not known. By 2 h, cell morphology was compromised, with the nuclei confined to a small area with cytoplasm. The effects of cytochalasin on actin and consequently E-cadherin in WT12S + V12ras cells were similar to that seen in WT12S-immortalized-only cells (Fig. 5), indicating that the status of actin SFs did not contribute to the observed E-cadherin phenotypes, because WT12S + V12ras cells do not express SFs or FAs (Fig. 5; Refs. 34 and 45), presumably due to the activation of rho by V12ras. The results from cytochalasin disruption of actin suggest that E-cadherin distribution at the cell perimeter is intimately linked to the presence of the circumferential actin ring. However, the effect of cytochalasin differs from that of the oncogenes described above. This probably reflects differences between transient versus constitutive alterations of actin and/or the consequences of targeted versus more pleiotropic effects on the cell. The effects of cytochalasin are reversible, and in a very short time frame after removal, the puncta disappeared, and circumferential actin reformed. The redistribution of E-cadherin along the intercellular interfaces was subsequent to the regeneration of apical actin rings. That is, no surface E-cadherin was observed in those areas where the apical cortical ring had not already been reestablished (Fig. 4, bottom Fig. 3. The localization of the EGFR is unaffected by the expression of wild-type or mutant rac. The indicated cell lines were processed for indirect immunofluorescence using antibodies to the EGFR. Bar, 25 μm.
Areas with cortical actin but no surface E-cadherin could be observed (Fig. 4, arrow 3), indicating that actin reallocation was not dependent on E-cadherin. Thus, it seems as if the redistribution of E-cadherin, even from E-cadherin-containing foci at the surface, occurred after the submembranous actin network was in place.

Nocodazole binds to and destabilizes microtubules. Treatment of cells with nocodazole did not disrupt either actin structures or E-cadherin localization (Fig. 6) in the time frame examined, even after the accumulation of mitotic cells could be detected microscopically (Fig. 6, arrows). Although the mitotic cells demonstrated the typical rounded appearance and were lifted up from the monolayer, they did not dissociate from their neighbors, further indicating the extent of adhesion due, at least in part, to maintenance of AJs. Disruption of apical cortical actin and AJs due to ATP depletion is also not dependent on the microtubule network (43, 55).

From these data, it appears that appropriate E-cadherin surface expression is not dependent on the microtubular or actin SF components of the CSK but is dependent on the presence of a CAF network.

Kinetics and Structural Intermediates of F-Actin Reform after Dissolution. The previous data have demonstrated a strong correlation between the status of the CAFs and the localization of E-cadherin along the intercellular interfaces. To more directly determine the relationship between these two, the kinetics of de novo formation of CAFs and E-cadherin surface localization were determined. To disrupt their cell-cell interactions and actin structures, suspensions of single cells, which are round and nonpolar, were generated. Confluent monolayers of WT12S-immortalized cells were treated with TE (0.05% trypsin-0.5 mM EDTA in PBS), WT12S + V12ras cells were treated with TE or E (0.5 mM EDTA in PBS). This also allowed a comparison between the effect of the removal of surface cadherin by proteolysis and the effect of E-cadherin endocytosis, as mediated by

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**Fig. 4.** Cytochalasin disruption of actin results in the accumulation of E-cadherin in actin-containing aggregates at former cell-cell interfaces. On reversion, actin reorganization precedes the redistribution of E-cadherin. Confluent WT12S-immortalized epithelial cells were treated with cytochalasin B for the times indicated at the left. After 2 h, the cytochalasin was removed, and the cells were incubated for an additional hour (R 1 h). The cells were processed for immunofluorescence using antibodies to E-cadherin and phalloidin-FITC. Arrow 1, the colocalization of E-cadherin and F-actin at cell-cell interfaces. Arrow 2, the colocalization of E-cadherin with actin in aggregates at the cell-cell interface after a short incubation with cytochalasin. These can be seen as phase-dense structures in the phase-contrast micrograph. Arrow 3, sites where CAFs have been reestablished but E-cadherin has not been reestablished after the removal of cytochalasin. Bar, 25 μm.
Ca²⁺ depletion (57). WT12S-immortalized cells could not be disrupted by treatment with E alone, even after 60 min at 37°C. The single cells were then plated on naive tissue culture dishes and analyzed at different time points after plating. A time course of attachment to substrate is shown in Fig. 7. Approximately 30–40% of the cells were resistant to PBS wash and judged adherent after 1 h. It required nearly 6 h for almost all of the plated cells to attach to the dish. There were no significant differences between treating the cells with TE or E with respect to the profile of adherence or the pattern of actin and E-cadherin subcellular distribution. There were, of course, differences in the levels of E-cadherin at the early time points after TE treatment as compared with E treatment (see below).

The cells that attached after increasing time intervals were examined for the expression and localization of F-actin, using phalloidin conjugated to FITC (Fig. 8, left column) and proteins of the CCC (see below). At early time points after plating (up to 2 h), the WT12S cells were round to irregularly shaped and were relatively apolar. The cells manifested predominantly as nuclei, with very little visible cytoplasm (Fig. 8). Very little F-actin was present in these cells, and what was present was concentrated in a single patch in a juxtanuclear location, where E-cadherin was also found. Thus, disruption of intercellular interactions by EDTA in the presence or absence of trypsin resulted in depolymerization of F-actin inside the cells, consistent with the observation that low calcium disrupts cortical actin (58). Curiously, if two cells were proximal, it seemed as if their respective actin-positive regions were apposed (Fig. 8, 15 min. and 1 hr.). By 2 h after plating, an increase in F-actin was observed, and ring-like actin structures could be seen in those areas of the cell spreading out from the nucleus (Fig. 8; see also Fig. 13, below). The F-actin ring continued to expand in diameter as the cells spread out on the substrate, such that by 4 h after plating, a prominent cortical ring was detectable (Fig. 8, see also Figs. 9 and 13). By 6 h after plating, most of the cells were sitting on the substrate (Fig. 7), and groups of interacting cells could be seen. In these cell clusters, a prominent cortical actin cable formed at the external edges of the cluster, with fainter F-actin present along internal, intercellular interfaces. By 8 h after plating, the cells were well spread and started to resemble untreated cells, including the onset of SFs in the case of WT12S cells. By 24 h after plating, a confluent, interconnected monolayer of well-spread cells was observed. These cells exhibited all of the normal actin structures present in the respective monolayer before disruption (compare Fig. 1 with Fig. 8, 24 h). Similar results were obtained on examination of WT12S + V12ras cells, as shown for the critical 4 h after plating time point in Fig. 9, where the prominent cortical ring is detectable. Thus, SF formation and CAF formation are independent, because no SFs were detectable in WT12S + V12ras cells. These data demonstrate that EDTA-induced depolymerization of F-actin results in the loss of polarity, epithelial morphology, and the collection of actin in a juxtanuclear location. From this site, actin polymer-
izes slowly and then spreads out in circumferential rings to the edge of the cell. Subsequently, intercellular F-actin forms, followed by the formation of SFs.

Dissolution of CAFs Results in the Accumulation of E-Cadherin in a Juxtanuclear Site, and Regeneration of Surface E-Cadherin Requires Restoration of CAFs. The WT12S cells were also examined for the expression and distribution of CCC proteins at different times after plating. Up to 2 h after plating, during which time no distinct actin structures could be distinguished, E-cadherin was observed in a juxtanuclear, Golgi location (Fig. 8, right column; data not shown), regardless of whether the cells were treated with TE or E. This resembles the observations seen on Ca²⁺ depletion of bovine kidney epithelial cells (57). The E-cadherin-positive region seemed to encompass the actin-positive focus (Fig. 8, compare right and left panels). Once F-actin rings started to appear (at 2 h after plating), E-cadherin was seen to move out from the Golgi and was predominantly in vesicular structures in the cytoplasm, within the actin ring. These vesicles are probably PGCs (37) bringing E-cadherin to the plasma membrane. Even at 4 h after plating, when a distinct cortical actin ring could be detected, no surface E-cadherin was yet observed (Figs. 8 and 9). Thus, F-actin at the cortex of the cell precedes E-cadherin at the surface. Some E-cadherin at the plasma membrane was detectable at 6 h, after the establishment of F-actin at the membranes between adjacent cells, although the predominant form was still vesicular. During the next several hours, the levels of E-cadherin at cell-cell interfaces increased as the numbers and intensity of vesicular E-cadherin decreased. By 24 h after plating, all of the detectable E-cadherin was present at the surface, and vesicular E-cadherin was no longer detectable, at least in WT12S cells. WT12S + V12ras cells synthesize E-cadherin at a higher level than is necessary to accommodate all of the AJs at the surface at any one time. Consequently, vesicular E-cadherin was always detectable (see Fig. 2). Because the turnover rate of CCCs is also higher in these cells (36), it is continually in demand to replace surface E-cadherin. The increased synthesis and degradation rates are a reflection of the overall increased metabolic rate of WT12S + V12ras cells, which could also be detected in the more rapid proteolytic processing of E-cadherin (see below), which occurs in the Golgi (59, 60).

Protein Synthesis Is Not Required at Early Times through Vesicular E-cadherin but Is Required for Membrane Association of Actin and E-cadherin. To ascertain whether any new protein synthesis was required in regenerating CAFs, CCCs, or cell-cell interactions, an experiment was conducted as described above, except cycloheximide was added at the time of plating the cells. As can be seen in Fig. 10, incubation of the cells with cycloheximide at the time of plating did not affect the early appearance of the cells or the actin and E-cadherin distribution to the juxtanuclear site (Fig. 10, inserts). That is, localization of E-cadherin to the perinuclear region and its movement into vesicles occurred without new protein synthesis (Fig. 10, bottom). Actin was also observed to form the early filamentous structures, including the perinuclear ring, but it did not proceed to the submembranous localization. Some filopodial and lamellipodial actin was also detectable. Concomitant with these
observations, some flattening and spreading of the cells did occur. The process seemed able to reach the 4 h postplating stage, but complete flattening, CAFs, surface E-cadherin, and cell-cell interactions were not obtained. Unfortunately, this experiment could not be conducted beyond 8 h because cytotoxicity became obvious.

The Catenins Colocalize with Actin during the Redistribution of CCCs to the Surface. When other CCC components were similarly examined, it could be seen that β-catenin was also observed in vesicles and in a soluble, cytoplasmic form (Fig. 9) at 4 h after plating. α-Catenin is an ABP and bundling protein and is the AJ component respon-
sible for connecting the complex to the actin CSK (13, 61). 
α-Catenin colocalized with E-cadherin at all of the time 
points examined, including the juxtanuclear location at early 
time points after plating and vesicles at later time points, until 
the cortical actin cable had been reestablished, after which 
α-catenin also associated with the plasma membrane (Fig. 
11). An actin ring always preceded the centrifugal migration 
of α-catenin toward the cell periphery (Fig. 12). Examination 
of the localization pattern of a member of another junctional 
complex, TJs, under these same conditions, demonstrated 
that ZO-1 association with the cell surface also occurred 
after the formation of the circumferential actin cable system 
(Fig. 9), consistent with previous observations (62).

Expression of CCC Proteins during Reestablishment of 
an Adherent Epithelial Cell. The status of expression of the 
CCC polypeptides was assayed after treatment of cells with 
TE or EDTA and in the floating and attached populations of 
such treated cells at 1-h intervals after plating by immuno-
blotting lysates with antibodies specific for each polypeptide 
(Fig. 13). When WT12S + V12ras cells were treated with TE, 
the extracellularly disposed E-cadherin was degraded 
(Fig. 13A, row E). Any incompletely glycosylated form (rep-
resented by the slightly more slowly migrating E-cadherin) 
that had not yet reached the surface was protected. Its level 
in the cells varied. Complex carbohydrate addition occurs in 
the late Golgi, just before delivery to the surface (60). At 1 h

Fig. 9. Circumferential F-actin formation pre-
cedes the surface localization of other junctional 
proteins. Single cell suspensions of WT12S + 
V12ras cells were plated on coverslips and fixed 
at 4 h after plating. They were processed for im-
munofluorescent visualization of the indicated 
polypeptides. Bar, 25 μm.

Fig. 10. Protein synthesis is required 
for F-actin and E-cadherin association 
with the membrane, but not for early 
steps in the reestablishment process. 
Single cell suspensions of WT12S cells 
were plated onto coverslips in the pres-
ence (bottom row) or absence (top row) 
of cycloheximide. The cells were fixed at 
2 (inserts) and 6 h after plating. They 
were processed for E-cadherin and F-
actin immunofluorescence. Bar, 25 μm.
after plating, when 30–40% of the cells were attached to substrate (Fig. 7), very little E-cadherin could be detected by immunoblotting lysates from either the attached or floating population. However, new E-cadherin (and other CCC proteins, too) was being synthesized and could be detected by metabolic labeling with a high concentration of [35S]methionine (Fig. 14, left panel). By 2 h after plating, there was a substantial increase in the glycosylation intermediate, which must be due to new synthesis, and a small amount of mature E-cadherin, which probably reflects completion of processing of the remnant immature form, in the attached population. This is reflected in the subcellular localization pattern observed via immunofluorescence (Fig. 8). During the course of subsequent time points, there was initially an increase in the levels of both forms of E-cadherin, with the level of the immature form slowly diminishing as the level of the mature form increased. No completely unprocessed (Mr 135,000) form was detected in the Western blots (Fig. 13), indicating that the early processing events (signal sequence cleavage, simple sugar addition, transport to Golgi, and proteolytic processing) were occurring rapidly (Fig. 14), consistent with the immunocytochemistry data (Fig. 8). However, the addition of complex carbohydrates and generation of the final mature E-cadherin was much slower than is normally observed in undisturbed, intact, adherent monolayers (23, 36).

When the cells were treated only with EDTA, no proteolysis of E-cadherin was detected in the time frame of the incubation (Fig. 13B, row E). At 1 h after plating, the amount of E-cadherin was higher in the floating cells, seemingly a simple reflection of the number of cells in each pool (Fig. 7). The level in the floating population decreased quickly, such that by 2 h after plating, when about 25% of the cells were nonadherent, very little remained. This loss from the floating pool was reflected in an increase in the E-cadherin levels in the adherent pool (see also Fig. 14). There probably was also some degradation of E-cadherin in the floating pool. By 3 h after plating, the level of mature E-cadherin was greater than that at the starting point and increased at each subsequent interval examined, reflecting new synthesis.

Examination of α-catenin indicated that it was sensitive to TE (Fig. 13A, row α). This was surprising because it is intracellular. However, α-catenin was also sensitive to treatment with EDTA alone (Fig. 13B, row α), which probably explains its sensitivity to TE. Because α-catenin binds to actin, its sensitivity to EDTA may be a reflection of the dissolution of cortical actin. We have previously shown that when α-catenin cannot bind actin, it is very unstable (23, 36). Barely detectable levels of α-catenin were observed in either attached cells or floating cells at 1 h after plating. No α-catenin was ever detected in the floating population. In the attached population, α-catenin was again detectable at 2 h after plating, and its level continued to increase due to de novo synthesis, although at a much lower rate than E-cadherin, at each later time point.

β-Catenin was insensitive to either TE or EDTA (Fig. 13, A and B, row β). This was not surprising because it is intracellular.
lular and does not interact with actin. β-Catenin did not seem to be made more sensitive to proteolysis by either of these treatments, despite no longer being at its appropriate location. Such insensitivity could be due to the fact that β-catenin can participate in other, nonadhesive functions in other compartments of the cell (reviewed in Refs. 15 and 63–65), although these were not detected in the cell types used here.

β-Catenin could be detected at about the same levels at 1 h after plating in both adherent and floating populations. With each subsequent time point, the levels in the floating pool diminished as the levels in the attached cells increased, reflecting the conversion of the population to adherence (Fig. 7) and probably some additional new synthesis as well, because the level at 6 h after plating was greater than that at the end of the treatments. The control polypeptide analyzed in this study was Grp94, which is an ER chaperone, not an AJ or surface protein. Grp94 exhibited a pattern that resembled β-catenin in that its expression level was unaffected by either treatment, and it was present at high levels in both pools (Fig. 13B, row G).

DISCUSSION

F-Actin Status Regulates the Subcellular Localization of AJ Proteins. CCCs are localized at the junction between the apical and basolateral domains of polarized epithelial cell membranes, participate in AJs, and mediate cell-cell interactions (5, 8). Much information has been obtained concern-
Actin Regulation of CCC Targeting

...ing the components of this adhesion complex, although, additional members and regulators continue to be identified. The synthesis, processing, and interactions among the members of the CCC have been described. Many mechanisms of AJ regulation have been identified. That association of the CCC with actin is required for the adhesive function has long been established. The reciprocal interactions and influences between CCCs and actin at the localized sites of cell-cell interaction leading to compaction (cell colony formation) have been detailed previously (22, 56, 66). These data indicate that before contact formation, actin already exists as a cortical belt circumscirping the cell or colony. At sites where cell-cell contacts are developing, CCCs associate with the actin bundles in puncta. Outside the puncta, the actin bundles are broken and reoriented toward the contact site. In mature contacts, after adhesion, actin bundles are oriented toward E-cadherin. Thus, E-cadherin-mediated adhesion results in reorganization of the cortical actin CSK at the sites of cell-cell adhesion. These studies started with the presence of cortical actin and CCC components and dissected the subsequent interactions as adhesion developed. However, whether there is regulation of the centrifugal transportation of the constituent members of AJs to the plasma membrane and whether there is a role for the actin CSK in this process remain to be determined. The data in this study suggest that the status of the circumferential actin network is communicated to the CCC and/or the Golgi and/or transport machinery/vesicles and that the CAF must be well formed for the CCC to be released from the Golgi and delivered to the plasma membrane. Disruption of CAFs by EDTA (58) results in the endocytosis of preexisting AJ proteins and their recycling to the juxtanuclear Golgi location. We have observed that newly synthesized E-cadherin passes rapidly through its early processing steps such as insertion into the ER, where signal cleavage and the addition of simple sugars occurs, and migration into the Golgi, where the critical cleavage of the NH2-terminal extension occurs (44, 60, 67). The interactions with β-catenin and α-catenin also occur rapidly, as per usual, such that even after a short pulse, all three are seen in a complex. The association of β-catenin with E-cadherin is required for egress from ER, which occurs rapidly after synthesis (11). However, E-cadherin and its associated proteins are held at the Golgi until actin polymerization begins anew. A similar lag was observed for newly made vesicular stomatitis virus G protein-green fluorescent protein on treatment of cells with cytochalasin B (37). On reappearance of a circumferential ring of F-actin, E-cadherin and the catenins are observed moving out from the Golgi in vesicles. These vesicles are more than likely the post-Golgi transport intermediates, or PGCs, that convey protein cargo from the Golgi to the plasma membrane (Ref. 37; reviewed in Ref. 68). Our results, together with those of others (37, 69–71), support a role for the cortical F-actin-based CSK, but not actin SFs or the microtubule-based CSK, to facilitate protein export out of the Golgi. Actin filaments are directly involved in the subcellular localization and morphology of the Golgi (72). Similar to what has been reported for vesicular stomatitis virus G-containing PGCs (37), the E-cadherin-containing vesicles do not contain β-COP.4 In epithelial cells, the catenin complex links the peripheral actin microfilament cable to E-cadherin. α-Catenin is an ABP that is a critical mediator of this (13). α-Catenin could also serve as the sensor of actin status. We have previously shown that inability to interact with CAFs destabilizes α-catenin and, consequently, AJs (23, 36). Thus, α-catenin may be involved in signaling for CCC centrifugal migration as CAFs are assembled.

Role of Scaffolds in Actin Organization and CCC Localization. In the analyses conducted, the actin CSK was dissolved or disrupted to varying extents and, in some cases, allowed to reorganize. This was a time-dependent, orderly morphogenetic process. The EDTA treatment allowed an almost complete loss of structure and shape and the subsequent visualization of the de novo generation of both. The actin CSK is involved in many morphogenetic processes and cellular functions. Therefore, actin must be able to remodel rapidly in response to changing conditions and signals. Actin remodeling is accomplished by assembling multimolecular complexes of structural and regulatory proteins at specific sites, where actin filaments are polymerized and assembled into superstructures (reviewed in Refs. 18 and 73–75). F-actin polymerization results primarily from the addition of monomers to preexisting filaments or requires nucleation sites. Small, monomeric ABPs are essential parts of the actin polymerization machinery and cooperate to regulate actin assembly (76). The assembly of multiprotein complexes at sites of dynamic actin activity is mediated by a class of proteins that serve as molecular scaffolds (adapters, integrators, and recruiters). These scaffolding proteins are composed of a series of protein-interacting domains that enable them to engage actin itself, ABPs, and proteins of extracellular or intracellular signaling cascades. A major ABP associated with these scaffolds is profilin, which is essential for actin polymerization (77). Profilin binds to pro-rich sequences on the scaffold polypeptide to provide actin monomers to growing filament ends. As would be expected for an actin assembly plant, members of the Rho GTPase family also have binding sites on these scaffolds. The Rho family members are key regulators in signal transduction pathways that lead to actin assembly/reorganization (16, 18, 52). Sometimes there are also binding sites for other signal transducers.

Rac Regulates Actin Structures and Hence CCC Subcellular Distribution. The association of members of the Rho family with the scaffold polypeptides provides a molecular mechanism by which Rho proteins can effect actin rearrangements. As such, scaffold polypeptides can be considered Rho effectors. Rac controls the formation of lamellipodia and membrane ruffles (78). Polymeric actin in rac-induced lamellipodia is associated with small focal complexes that are different from classical FAs but do contain integrins, vinculin, paxillin, and FA kinase. In epithelial cells, Rho and Rac are required for the establishment of cadherin-based cell-cell adhesion and the actin reorganization necessary to stabilize receptors at sites of intercellular junctions.

4 M. P. Quinlan, unpublished data.
(22). Our observations are consistent with those. V12rac disrupts and destabilizes AJs, and N17rac represses and restabilizes AJs (23, 36). Ras hypertransforming E1A mutants, such as HB512, shown here, also disrupt CAFs and AJs, in contrast to WT12S, which maintains AJs even in the presence of oncogenic ras (23, 35). It is likely that E1A mediates its effects through rac. Several downstream molecules mediating the effects of GTPases on actin rearrangements have been identified (reviewed in Ref. 79). In addition to scaffold/adaptor proteins, there are several other potential candidates. Cortactin is an ARP whose redistribution from cytosol to ruffles is regulated by rac1 (80). POR1 binds rac and cdc42 and is found at ruffles (81). LIM kinase 1 participates in rac-mediated actin reorganization (82, 83). In the presence of V12rac, LIM kinase 1 phosphorylates cofilin, inactivating it, resulting in actin filament depolymerization. IQGAP and p140Sra-1 interact with rac1 (and cdc42) and actin, inducing CAF formation (84). Importantly, IQGAP1 is involved in rac regulation of E-cadherin-mediated cell-cell adhesion (27). Consistent with our observations with V12rac, the GTP-dependent interaction of rac with IQGAP results in AJ disruption. These data are also consistent with our observation that V12rac, in contrast to WTrac or N17rac, does not allow stable CAF formation; consequently, E-cadherin is blocked in PGCs and does not reach the cell surface. Interestingly, rac and rho have also been implicated in regulating endocytosis and secretory vesicle trafficking (reviewed in Ref. 16), in addition to the members of the Rab subfamily. Vesicular transport processes require coordinated interactions between membranes and the CSK. This may be via the effects of these GTPases on phospholipid metabolism or scaffold protein complexes. Thus, rac may be involved in multiple steps in the targeting of CCAFs to the surface.

MATERIALS AND METHODS

Cell Culture and Adherence Assays. Primary BRK cells were prepared from 5-day-old rats (Fisher F344; Harlan, Indianapolis, IN). The cell lines derived from these cells have been described previously (45). Briefly, they were obtained by cotransfecting primary neonatal rat kidney epithelial cells with expression plasmids containing the individual genes, except for the cells coexpressing N17rac. The N17rac cells were obtained by transfecting already established cell lines with an N17rac expression plasmid and selection with 400 μg/ml G418. Cells were harvested at different times after addition, as detailed in this study. Neither cytochalasin nor nocodazole affected protein synthesis levels, which were determined by incubating cells with drugs or their solvents and 35S-labeled methionine for the same time periods and comparing the levels of incorporation. For the cytochalasin reversal experiments, confluent cells were incubated with cytochalasin for 2 h at 37°C. At the end of this time period, the drug was removed, and the cells were rinsed once with media without drug and then incubated with drug-free media for the indicated time points.

Immunoprecipitations and Western Blotting. For the pulse-chase analyses of attaching cells, adherent cells in 100-mm dishes were starved for 30 min in methionine-free DMEM. The cells were then washed with warm PBS and tryptpsinized or treated with EDTA. The cells were pooled in methionine-free DMEM with 50 μCi of [35S]methionine (ICN Pharmaceuticals, Costa Mesa, CA) per original 107 cells. The cells were divided into equal-cell number aliquots and plated onto 60-mm tissue culture dishes. They were pulsed for 30 min at 37°C. After the pulse, unlabeled methionine was added to 2 mM, and additional DMEM with serum was added. One set of cells was collected after the pulse, and the adherent and floating cells were collected as described above. The other dishes were returned to chase for the indicated time points. At the end of each time point, the adherent and floating cells were collected as described above. The cell pellets collected were lysed in 1% NP40, 50 mM Tris (pH 7.6), 1 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride on ice for 30 min. Lysates were precleared by centrifugation at 16,000 × g for 30 min at 4°C. The supernatant lysates immunoprecipitated with E-cadherin antibody at 4°C, with rocking. Immune complexes were collected with 0.3% protein A-Sepharose CL-4B and washed three times. The final pellet was resuspended in sample buffer (125 mM Tris-HCl, 5% glyceral, 2% SDS, 1% β-mercaptoethanol, and 0.003% bromphenol blue). The samples were resolved by 7.5% SDS-PAGE, fluorographed, and autoradiographed. For Western analyses, cell pellets were lysed with boiling sample buffer. Western analyses were performed as described previously (85). Samples were analyzed on a 7.5% or 15% SDS-PAGE. The primary antibodies for E-cadherin (C20820 for immunoprecipitation and C20020 for immunoblotting) and β-catenin (C19200) were purchased from Transduction Laboratories, (Lexington KY), the primary antibodies for α-catenin were purchased from Zymed (71-1200; San Francisco, CA) and Transduction Laboratories (C21620). The GRP94 antibody was provided by L. Hendershot (St. Jude Children’s Research Hospital, Memphis, TN). The secondary antibodies were conjugated to horseradish peroxidase and purchased from either Sigma Chemical Co. (St. Louis, MO) or Cell Growth & Differentiation 851

5 R. S. Fischer and M. P. Quinlan. While E1A can facilitate epithelial transformation by several dominant oncogenes, but not all, the C-terminus only synergizes with rac/cdc42, in both epithelial and fibroblastic cells, submitted for publication.
Bio-Rad Laboratories (Hercules, CA). Detection was accomplished by chemiluminescence or autoradiography.

**Immunofluorescence Analysis.** Cells were plated on gelatin-coated coverslips for various times in DMEM/FCS. At the time of interest, they were rinsed with PBS or a CSK stabilizing buffer (10 mM HEPES (pH 6.1), 150 mM NaCl, 5 mM EGTA, 5 mM MgCl2, and 5 mM glucose; Ref. 86) at room temperature. Cells were fixed with 1,0% paraformaldehyde in PBS for 15 min at room temperature and washed twice in PBS. To decrease background, quenching was done by incubating cells with 20 mM ammonium chloride in PBS for 20 min at room temperature, followed by three washes in PBS. The cells were then permeabilized with 0.2% Triton X-100 in PBS for 3 min and washed with PBS three times. Blocking was done by incubating coverslips in PBS with 1% serum or BSA for 15 min at room temperature. All antibodies were diluted in PBS with 1% serum/BSA and incubated with the coverslips for 30 min at 37°C in a humidified chamber. After each antibody step, the cells were washed three times with PBS. Once incubated with the secondary conjugated antibodies, coverslips were kept in the dark. They were rinsed once with distilled water just before mounting with Arrol (Air Products, Allentown, PA). The primary antibodies for E-cadherin (C20282) and β-catenin (C19220) were purchased from Transduction Laboratories, vinculin (cl IVIN-1) and FITC-conjugated phalloidin were purchased from Sigma, and ZO-1 (61-7300) and α-catenin (71-1200) were purchased from Zymed. Secondary antibody bodies were conjugated with either Texas Red X or Oregon Green (Molecular Probes, Eugene, OR). Photomicrographs were taken with a Zeiss Axioshot epifluorescence microscope using Kodak Tri-X Pan ASA 400 film. Equal magnification (×80) was used for all panels in all figures.

**ACKNOWLEDGMENTS.** Critical evaluation of the manuscript by S. Sharma is greatly appreciated. We thank L. Hendershot for the Grp94 antibody. RAL20 is very much appreciated for patience and helpful graphics.

**References**


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