Recycling of E-Cadherin: A Potential Mechanism for Regulating Cadherin Dynamics

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Abstract. E-Cadherin plays critical roles in many aspects of cell adhesion, epithelial development, and the establishment and maintenance of epithelial polarity. The fate of E-cadherin once it is delivered to the basolateral cell surface, and the mechanisms which govern its participation in adherens junctions, are not well understood. Using surface biotinylation and recycling assays, we observed that some of the cell surface E-cadherin is actively internalized and then recycled back to the plasma membrane. The pool of E-cadherin undergoing endocytosis and recycling was markedly increased in cells without stable cell-cell contacts, i.e., in preconfluent cells and after cell contacts were disrupted by depletion of extracellular Ca²⁺, suggesting that endocytic trafficking of E-cadherin is regulated by cell-cell contact. The reformation of cell junctions after replacement of Ca²⁺ was then found to be inhibited when recycling of endocytosed E-cadherin was disrupted by bafilomycin treatment. The endocytosis and recycling of E-cadherin and of the transferrin receptor were similarly inhibited by potassium depletion and by bafilomycin treatment, and both proteins were accumulated in intracellular compartments by an 18°C temperature block, suggesting that endocytosis may occur via a clathrin-mediated pathway. We conclude that a pool of surface E-cadherin is constantly trafficked through an endocytic, recycling pathway and that this may provide a mechanism for regulating the availability of E-cadherin for junction formation in development, tissue remodeling, and tumorigenesis.

Key words: epithelial junctions • endocytosis • epithelial morphogenesis • clathrin-coated vesicles • biotinylation

The cadherins are a large family of cell surface glycoproteins which mediate cell-cell adhesion in most solid tissues of the body (Takeichi, 1991, 1995; Yap et al., 1997a). Cadherin-based adhesion is a central determinant of cell patterning during development and is necessary for the preservation of established tissue architecture in mature organisms. This is exemplified by E-cadherin, the prototypical epithelial cadherin. Genetic studies have shown that E-cadherin is essential for epithelial integrity during development in flies, X. laevis, and mice (Levine et al., 1994; Hermiston and Gordon, 1995; Tepass, 1996; Uemura et al., 1996). At the cellular level, E-cadherin facilitates assembly of other specialized intercellular junctions (desmosomes, gap, and tight junctions) necessary to link epithelial cells into functional monolayers (Gumbiner et al., 1988; Musil et al., 1990; Wheelock and Jensen, 1992). Cadherin-based adhesive contacts also participate in establishing apical-basal polarity (Vega-Salas et al., 1987; Nelson et al., 1990; Yap et al., 1995), possibly through a role in the targeted delivery of membrane components to the basolateral cell surface (Mays et al., 1995; Grindstaff et al., 1998). Finally, cellular studies have suggested that E-cadherin may influence cell locomotion (Hermiston and Gordon, 1995; Hermiston et al., 1996) and population dynamics (Watabe et al., 1994; Hermiston and Gordon, 1995), properties which could contribute to its morphogenetic influence. Taken together, these findings indicate a central role for E-cadherin in many aspects of epithelial biogenesis.

It is becoming increasingly clear that cadherin function is a dynamic process. Morphogenetic events associated with cellular rearrangements, movements, and wound healing are reported to involve regulated changes in cadherin function. For example, compaction of blastomeres in the early mouse embryo is associated with the activation of E-cadherin by protein kinase C signaling pathways (Vestweber et al., 1987; Winkel et al., 1990), whereas physiological reduction in the adhesiveness of C-cadherin triggered by the mesoderm-inducing factor, activin, is necessary for morphogenetic movements to occur in early X. laevis development (Brieher and Gumbiner, 1994;
Zhong et al., 1999). In postnatal life as well, morphogenetic movements in tissues with rapid turnover rates, such as the gut epithelium, are likely to involve the continual breaking and reforming of cell-cell adhesive contacts (Gumbiner, 1992).

In contrast to the signals which may regulate cadherins, much less is known about the effector mechanisms which cells utilize to modulate cadherin adhesive function (Y ap et al., 1997a). One potential mechanism is dynamic turnover of cadherin molecules on the cell surface. The amount of cadherin protein expressed on the cell surface clearly influences cadherin adhesiveness (A ngres et al., 1996; Y ap et al., 1997b). Several observations suggest that cadherins may undergo regulated trafficking to and from the surface. Isolated epithelial cells commonly display a prominent intracellular pool of E-cadherin which appears to be recruited to the cell surface upon cell-cell contact (M CNeill et al., 1993; A dams et al., 1996; M yat et al., 1998) implying the existence of a trafficking pathway for targeted delivery of E-cadherin. Conversely, chelation of extracellular calcium (Ca^{2+}) induced the internalization of intact adherens plaques or large plaque fragments, including molecules such as E-cadherin (K artenbeck et al., 1982; D uden and Franke, 1988; K artenbeck et al., 1991). The developmentally regulated uptake of cadherins from the surface, with accumulation within intracellular vesicles, has also been observed in some instances of epithelial-to-mesenchymal transformation (M iller and M Clay, 1997). These observations suggest that surface expression of E-cadherin may be influenced by the balance between transport processes that deliver proteins to the surface and endocytic mechanisms that mediate uptake and recycling of membrane components.

In this study we have investigated the endocytosis and recycling of detergent-soluble surface E-cadherin in MDCK cells. Our studies indicate that E-cadherin at the cell surface is not automatically incorporated into stable junctional complexes. Instead, even at steady-state in confluent monolayers, at least one pool of surface E-cadherin remains subject to endocytosis and is recycled to the cell surface via a post-Golgi endosomal pathway. The proportion of E-cadherin in this recycling pool is increased in the absence of stable cell-cell contacts—in preconfluent cells and after cell-cell contacts are disrupted by chelation of extracellular Ca^{2+}. We suggest that the regulated uptake and recycling of surface E-cadherin provides a mechanism for the dynamic modulation of cadherin expression and cell adhesion.

**Materials and Methods**

**Cell Culture**

MDCK cells, strain II, were grown and passaged as described previously (N arula et al., 1992) in DMEM with 10% FCS and 2 mM glutamine in 5% CO_{2} and 95% air. Cells used in experiments were plated on semipermeable polycarbonate filters (Transwell; Corning Costar) as confluent monolayers for immunofluorescence experiments. For immunoblotting we used a mouse monoclonal antibody against human E-cadherin (Transduction Laboratories) or a rat monoclonal E-cadherin antibody (D ECA M A-1; Sigma Chemical Co.). Other primary antibodies used include mouse monoclonal against β-catenin (Transduction Laboratories), mouse monoclonal antibody 6H directed against the Na^{+} K^{+}ATPase subunit (a generous gift of Dr. M. Caplan, Y ale U niversity, New H aven, CT), mouse anti-TFR (Zymed Laboratories), rabbit anti-rab 5, and rabbit anti-rab 7 (both provided by Dr. C. haver, E uropean M olecular B iology L aboratory, H eidelberg, G ermany). Secondary antibody conjugates used were sheep anti-mouse IgG-Cy3 (J ackson ImmunoR esearch L abs), goat anti-rabbit IgG-Cy3 (Sigma Chemical Co.), goat anti-rabbit IgG-A LE X A 488 (M olecular Probes), HRP-labeled goat anti-mouse IgG (B io-R ad L aboratories), and HRP-labeled sheep anti-rat IgG (A mersharn L ife S cience).

**Immunofluorescence Staining**

Cells were fixed in 4% paraformaldehyde in PBS for 90 min and then permeabilized for 5 min in PBS containing 0.1% Triton X-100. Cells were incubated with primary antibodies followed by incubation in secondary antibodies using PBS containing BSA as a blocking buffer. Cells were mounted in 50% glycerol/1% n-propyl-gallate in PBS and viewed by confocal microscopy on a Bio-Rad M RC-600 confocal laser scanning microscope mounted on a Zeiss A xioskop or by epifluorescence on an O lympus Provis X-70 microscope. Images were collected with an Olympus C CD 300E T-R CX camera using NIH image software. Intensity measurement values were obtained from analysis of multiple fields in duplicate images on the confocal microscope. Image analysis was performed by measuring the total and relative fluorescence intensities using SO M software.

**Cell Surface Biotinylation**

MDCK cells grown on filters were incubated with 1.5 mg/ml sulfosucinimidyl 2-(biotinamido) ethyl-dithiopropionate (sulfo-NHS-SS-biotin) (Pierce Chemical Co.) applied to the basal side of the filter, followed by washing with sulfosuccinimidyl blocking reagent (50 mM NH_{4}Cl in PBS containing 1 mM MgCl_{2} and 0.1 mM CaCl_{2}) to quench free sulfosuccinimidyl-biotin, followed by several further washes in PBS. Cells were then scraped off filters and lysed in 500 μl of RIPA buffer (20 mM Tris-HCl, pH 7.4, with 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA) with protease inhibitors. Cell extracts were centrifuged to obtain a detergent-insoluble pellet and a detergent-soluble supernatant which was incubated with streptavidin beads (Sigma Chemical Co.) to collect bound, biotinylated proteins. These samples were then analyzed by SDS-PAGE and immunoblotting to identify E-cadherin. In all cases, immunoblot membranes were stained with 0.1% Coomassie brillian blue to ensure even protein transfer and protein loading. Different luminescence exposures were collected and exposures in the linear range were used.

**Biotinylation Assay for Endocytosis and Recycling**

Confluent MDCK cells grown on Transwell filters were biotinylated as above at 0°C followed by washing and quenching free biotin. Cells were then incubated in normal media at 18°C or 37°C. The 18°C temperature block has been used to accumulate internalized proteins in early or sorting endosomes by preventing them from progressing further into the endocytic or recycling pathways (Galloway et al., 1983; Dunn et al., 1989; 1. Abbreviations used in this paper: sulfosuccinimidyl 2-(biotinamido) ethyl-dithiopropionate; TFR, transferrin receptor.)
Czekay et al., 1997). Monolayers were glutathione stripped essentially as described by Graeve et al. (1989). Cells were incubated in two 20-min washes of glutathione solution (60 mM glutathione, 0.83 M NaCl, with 0.83 M NaOH and 1% BSA added before use) at 0°C which removed all cell surface biotin groups. Remaining biotinylated proteins were subsequently inside cells by endocytosis and were therefore protected from glutathione stripping.

To measure recycling of endocytosed proteins accumulated at 18°C, cells were glutathione stripped at 0°C and then returned to 37°C for various times in normal medium. Cells were then washed quickly in PBS and incubated with 0.01% trypsin (type IV; Sigma Chemical Co.) in Ca²⁺-free PBS for 20 min followed by addition of 100-fold excess soybean trypsin inhibitor to inhibit further protease digestion. Trypsinized, biotinylated cell surface proteins were recovered from the supernatant by incubation with streptavidin beads, and the cells were then lysed in RIPA buffer and remaining cell-associated biotinylated proteins recovered on streptavidin beads. The extracellular fragment of E-cadherin released by trypsin and cell-associated E-cadherin were analyzed by SDS-PAGE and immunoblotting.

**Potassium (K⁺) Depletion**

Cells were depleted of K⁺ to selectively block clathrin-mediated endocytosis essentially as described by Larink et al. (1983). Cultures were rinsed three times with K⁺-free buffer (140 mM NaCl, 20 mM Hepes, pH 7.4, 1 mM CaCl₂, 1 mM MgCl₂, and 1 mg/ml α-glucose), and hypotonically shocked by a brief rinse followed by incubation at 37°C for 5 min in K⁺-free buffer diluted 1:1 with distilled water. Next, cells were rinsed three times and incubated in K⁺-free buffer at 37°C for 15 min and finally incubated with 1.5 mg/ml biotin as described previously. Biotinylated E-cadherin was recovered with streptavidin beads and detected by immunoblotting.

**Ricin Uptake**

The uptake of FITC-ricin was used to measure non-clathrin-mediated endocytosis essentially as described previously (Wilson and Colton, 1997). Confluent MDCK cells grown on Transwell filters were incubated for 1 h at 4°C in 0.1 mg/ml ricin-FITC (Sigma Chemical Co.) in serum-free or under conditions of K⁺ depletion. Cells were warmed to 37°C for 2 h before fixation and staining. A 18°C temperature block has been shown to cause the accumulation of endocytosed proteins in early or sorting endosomes (Czekay et al., 1997). The 18°C temperature block did not adversely affect cell morphology or cell-cell contacts as shown by the F-actin staining pattern (Fig. 1, e and f), nor was there a detectable change in the cell surface staining of E-cadherin at 18°C. However, the vesicular staining of E-cadherin was more pronounced in cells incubated at 18°C (Fig. 1 b). Notably, at 18°C the location of the vesicular E-cadherin staining within the cells was now more prominent in the perinuclear region rather than being at the cell periphery. E-Cadherin staining was largely unaltered in cells pretreated with cycloheximide to stop protein synthesis (Fig. 1 d), indicating that both the cell surface staining and the intracellular staining represent stable pools of E-cadherin. Thus, while the majority of E-cadherin is present on the lateral plasma membrane, the presence of intracellular staining suggests that a pool of E-cadherin may be internalized from the cell surface, where it accumulates at 18°C.

**Endocytosis of Basolateral Surface E-Cadherin**

To establish whether surface E-cadherin can be internalized we developed an assay to track the uptake of E-cadherin labeled by biotinylation on the basolateral surfaces of MDCK cells. As described in Materials and Methods, cells were surface-biotinylated at 0°C then returned to 37°C for 1 h to allow trafficking to resume. Cells were then incubated in several washes of glutathione solution at 0°C to remove covalently bound biotin groups from amines exposed on the cell surface. Biotinylated E-cadherin internalized at 37°C should be sequestered and, therefore, protected from glutathione stripping. Cycloheximide-treated cells were included in all experiments to eliminate the pool of newly synthesized E-cadherin from consideration. In control experiments, cells were surface-biotinylated for 1 h at 0°C, then immediately washed in glutathione at 0°C. No E-cadherin was recovered in the biotinylated fraction (Fig. 2 a, lane 3), confirming that under these conditions glutathione efficiently stripped all biotinylated groups from surface proteins. In contrast, after 1 h at 37°C a biotinylated pool of E-cadherin was detected in cells following glutathione stripping of surface proteins (Fig. 2 a, lanes 6 and 7). This pool represented ~13% of the total E-cadherin biotinylated at the beginning of the experiment, indicating that upon return to physiological temperature, a small amount of surface E-cadherin was internalized and hence protected from glutathione stripping. In contrast, the basolateral membrane protein Na⁺-K⁺-ATPase did not undergo internalization under the same conditions, since a glutathione-resistant pool of Na⁺-K⁺-ATPase was not detected after incubating biotinylated cells at 37°C for 1 h (Fig. 2 a, lanes 6 and 7).

To assess the kinetics of internalization, we allowed surface-biotinylated E-cadherin to internalize for various times (5-180 min) at 37°C in cells preincubated with cycloheximide (Fig. 2 b). A non internalized pool of E-cadherin was detected after 5 min, but for the remainder of the 3-h chase period, the amount of intracellular biotinylated E-cadherin did not change, indicating that at physiological temperature, the relative size of the endocytosed pool of E-cadherin was kept constant in confluent monolayers (Fig. 2 b, lanes 3–7, and Fig. 2 d). The fact that this pool did not decrease in cycloheximide-blocked cells shows that the internalized E-cadherin is not generally fated for degradation after endocytosis, while the lack of accumulation suggests that there may be constant recycling of the intracellular pool of E-cadherin.

In light of the immunofluorescence observation that E-cadherin accumulates intracellularly at 18°C, surface biotinylation was also used to assay the effect of low temperature on the internalization and accumulation of E-cadherin. Whereas at 37°C there was a constant internalized
pool of biotinylated E-cadherin (Fig. 2b), at 18°C the internalized pool of E-cadherin showed progressive accumulation (Fig. 2, c and d). After 20 min at 18°C, 35% of the surface-biotinylated E-cadherin was internalized (Fig. 2, lane 5) and by 2 h the majority (80%) of the surface-biotinylated E-cadherin had accumulated inside cells (Fig. 2, lane 6). After prolonged accumulation (3 h) some apparent degradation products of E-cadherin were noted on gels (Fig. 3, lane 7). The absence of such bands at 37°C (Fig. 2b) further suggests that E-cadherin is normally recycled. Overall these results show that there is active internalization of E-cadherin from the cell surface and that its uptake is selective, since other basolateral cell surface proteins, such as Na⁺K⁺-ATPase, are not undergoing the same process.

**Endocytosed E-Cadherin Is Actively Recycled to the Basolateral Membrane**

To study possible recycling of E-cadherin, we then developed an assay to identify recycling of internalized biotinylated E-cadherin back to the cell surface. Surface proteins were biotinylated at 0°C, then cells were incubated at 18°C to allow the internalization and accumulation of E-cadherin. After treatment with glutathione to strip remaining biotinyl groups from cell surface proteins, cells...
were then released at 37°C to resume trafficking. Endocytosed E-cadherin that was returned to the cell surface was collected by surface trypsinization under conditions which cleave the ectodomain of E-cadherin, releasing a soluble fragment (Takeichi, 1977). The supernatants of trypsin-treated cells were then incubated with streptavidin beads, separated by SDS-PAGE, and immunoblotted with specific antibodies. (a) The top shows that internalized E-cadherin accumulated at 18°C (lane 3) gradually disappeared from the internal pool over 15 min (lanes 4–7). The bottom shows that the 82-kD ectodomains of biotinylated E-cadherin was initially detected after 5 min at 37°C (lane 5) and maximal amounts were detected after 15 min at 37°C (lane 7) showing that the internalized E-cadherin was recycling and reappearing on the cell surface. (b) Surface-biotinylated (lane 1) TfR was also internalized (lane 4) but disappeared from the internal pool (lanes 5–7) more rapidly under the same conditions. Results are representative of four experiments.

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Le et al. E-Cadherin Recycling

Figure 2. Internalization of surface-biotinylated E-cadherin. (a) Confluent MDCK cells treated with (+) or without (–) cycloheximide (CHX) were surface-biotinylated at 0°C. Detergent-soluble, surface-biotinylated proteins were recovered on streptavidin beads and analyzed by SDS-PAGE. E-Cadherin and Na⁺K⁺ATPase in these fractions were detected by immunoblotting as depicted. Biotinylated cell surface E-cadherin was recovered from the cell extracts (lanes 1 and 2). Glutathione stripping (g.s.) immediately after biotinylation at 0°C completely removed biotin from surface E-cadherin (lane 3). Surface-biotinylated cells were then incubated at 37°C and after 1 h, total biotinylated E-cadherin was recovered (lanes 4 and 5). E-Cadherin sequestered in an internal pool was recovered after glutathione stripping showing that some of the surface E-cadherin was endocytosed (lanes 6 and 7). Na⁺K⁺ATPase was biotinylated (lanes 1 and 2) but was not found in an internal pool under the same conditions (lanes 6 and 7). (b) A time course of E-cadherin uptake. CHX-treated cells were surface-biotinylated at 0°C (lane 1) and then incubated at 37°C for periods of 0–180 min (lanes 2–7). A constant amount of surface-biotinylated E-cadherin was sequestered from glutathione stripping at chase times from 5–180 min (lanes 3–7), consistent with a steady-state flux of E-cadherin into and out of this intracellular pool. (c) When the same experiments were carried out on MDCK cells incubated at 18°C, internalized E-cadherin now accumulated, resulting in increasing amounts of E-cadherin in the internal pool over 180 min (lanes 3–7). Fainter, lower molecular weight bands (lane 6 and 7) may be due to some degradation of internalized E-cadherin after prolonged incubations. (d) Relative amounts of internalized E-cadherin, quantitated by densitometry and expressed as a percentage of the total initial surface-biotinylated pool in cells incubated at 37°C or 18°C. Data are means ± SEM from three separate experiments.
of the total unbiotinylated fraction were loaded. Unbiotinylated E-cadherin from preconfluent cells (lane 1) and confluent cells (lane 3) and unbiotinylated (supernatant) fractions were analyzed by SDS-PAGE and immunoblotting. All of the biotinylated fraction and 20% were surface-biotinylated; biotinylated E-cadherin in detergent-soluble cell extracts was recovered by streptavidin affinity, biotinylated tentative fields of cells were photographed with similar exposures. (b) Surface biotinylation of E-cadherin. Preconfluent and confluent cells which includes the intracellular pool. (c) The relative amounts of E-cadherin in detergent-soluble (biotinylated and unbiotinylated) and detergent-insoluble (TX-insoluble) fractions were compared by immunoblotting and densitometry in preconfluent and confluent cells. Detergent-insoluble E-cadherin, which is likely to represent protein incorporated into cytoskeleton-stabilized junctional complexes, increases in confluent cells. Within the detergent-soluble pool, biotinylated cell surface E-cadherin increases (from ~10% to ~50%) in confluent monolayers. Data are means ± SEM from three separate experiments.

Thus, biotinylated E-cadherin on the basolateral surface of MDCK cells can be followed through endocytic and recycling pathways.

To further test the influence of recycling on the internal pool of E-cadherin, we treated MDCK cells with bafilomycin A1. Bafilomycin A1 inhibits recycling by blocking transport of endocytosed material back to the cell surface at a late endosomal stage (Johnson et al., 1993; Presley et al., 1997). Cells were surface biotinylated at 0°C then incubated in the presence or absence of bafilomycin A1 (1 μM) at 37°C, followed by glutathione stripping. In the presence of bafilomycin A1, there was a threefold increase in the glutathione-resistant pool of E-cadherin (Fig. 4, lane 6) in comparison to the normal amount of internalized E-cadherin (Fig. 4, lane 5). Bafilomycin A1 was therefore effective in accumulating the endocytosed E-cadherin, consistent with a block in the recycling of E-cadherin.

Preconfluent Cells Have a Large Pool of Internalized E-Cadherin

In light of reports that cell-cell contact may influence recruitment of E-cadherin to the cell surface, we compared the immunofluorescence localization of E-cadherin in MDCK cells grown and maintained at different densities. In confluent cell monolayers, E-cadherin staining was found predominantly at the cell surface, as shown in Fig. 1 a. In contrast, preconfluent cells which were not yet polarized and had not yet formed extensive adherens junctions showed relatively little E-cadherin staining at the cell surface but there was a concomitantly larger intracellular pool of labeled E-cadherin (Fig. 5 a). Some of the intracellular staining in the perinuclear Golgi region disappeared after cycloheximide treatment and is thus likely to represent newly synthesized E-cadherin in the biosynthetic pathway. There was also prominent vesicular staining of

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**Figure 4.** Endocytosis of E-cadherin in the presence of bafilomycin A1 (BAF). Cells were surface-biotinylated at 0°C and then incubated at 37°C in the presence (+) or absence (−) of 1 μM BAF. Surface-biotinylated E-cadherin (lane 1) was recovered from total cell extracts (lanes 3 and 4) and from the internal pool (lanes 5 and 6) sequestered from glutathione stripping. A greater amount of internalized biotinylated E-cadherin was recovered after BAF treatment (lane 6) in comparison to the normal amount of internal E-cadherin (lane 5), consistent with BAF-induced accumulation of E-cadherin. The result is representative of three independent experiments.

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**Figure 5.** Distribution of E-cadherin in preconfluent MDCK cells. (a) Immunofluorescence staining of E-cadherin in preconfluent cultures treated with or without cycloheximide (+/− CHX). At steady state (−CHX), there is a small amount of cell surface staining, prominent perinuclear staining over the Golgi complex, and bright vesicular staining throughout the cytoplasm. When protein synthesis is blocked (+CHX), the Golgi staining disappears but the vesicular staining remains. Representative fields of cells were photographed with similar exposures. (b) Surface biotinylation of E-cadherin. Preconfluent and confluent cells were surface-biotinylated; biotinylated E-cadherin in detergent-soluble cell extracts was recovered by streptavidin affinity, biotinylated and unbiotinylated (supernatant) fractions were analyzed by SDS-PAGE and immunoblotting. A 20% of the total unbiotinylated fraction that was compared to the amounts of surface-biotinylated E-cadherin in preconfluent cells (lane 2) and confluent cells (lane 4). There is an increased amount of biotinylated cell surface E-cadherin in confluent cells with a concomitant decrease in the unbiotinylated fraction, which includes the intracellular pool. (c) The relative amounts of E-cadherin in detergent-soluble (biotinylated and unbiotinylated) and detergent-insoluble (TX-insoluble) fractions were compared by immunoblotting and densitometry in preconfluent and confluent cells. Detergent-insoluble E-cadherin, which is likely to represent protein incorporated into cytoskeleton-stabilized junctional complexes, increases in confluent cells. Within the detergent-soluble pool, biotinylated cell surface E-cadherin increases (from ~10% to ~50%) in confluent monolayers. Data are means ± SEM from three separate experiments.
E-cadherin in the peripheries of preconfluent cells. As in confluent cells, staining of this vesicular pool was not altered by cycloheximide treatment suggesting that it represents E-cadherin in an endocytic pathway, a pool which is enhanced in preconfluent cells.

Surface biotinylation experiments confirmed the relative difference in E-cadherin distribution between confluent and preconfluent cells. In preconfluent cells, ~10% of the detergent-soluble E-cadherin was biotinylated on the basolateral surface, whereas in confluent cells, ~47% of the E-cadherin was biotinylated on the cell surface (Fig. 5, b and c). Taken together, these results suggest that E-cadherin redistributes from a predominantly intracellular pool to sites of cell-cell contact as MDCK cells grow to confluence. Insofar as a significant proportion of the intracellular E-cadherin in preconfluent cells represents a stable cycloheximide-resistant pool that is capable of undergoing recycling, this suggested that cell-cell contact may influence the recycling of E-cadherin.

**E-Cadherin Recycling Mediates Restoration of Cell-Cell Contact after Chelation of Extracellular Ca\(^{2+}\)**

To further investigate the influence of cell-cell contact on E-cadherin recycling, we examined the effect of EDTA on epithelial morphology and E-cadherin localization in confluent MDCK monolayers. Chelation of extracellular Ca\(^{2+}\) disrupts epithelial cohesion, at least partly through inhibition of the adhesive binding activity of the E-cadherin ectodomain (Takeichi et al., 1981; Kemler et al., 1989; Kartenbeck et al., 1991). As shown in Fig. 6, exposure of MDCK cells to EDTA (2.5 mM) induces a rapid and reversible change in monolayer organization. Within 10-15 min cells began to retract from one another, and by 45 min there were mostly isolated cells lacking cell-cell contacts (Fig. 6 b). Restoration of extracellular Ca\(^{2+}\) rapidly restored cell-cell contacts, and by 1 h most cells had spread to reform extensive regions of confluence (Fig. 6 d). Staining for E-cadherin showed that EDTA induced a rapid internalization of surface E-cadherins with intense intracellular punctate labeling apparent after Ca\(^{2+}\) chelation (Fig. 7 a and Fig. 8 b) compared to the predominant cell surface staining of E-cadherin in contact zones in untreated cells (Fig. 7 a and Fig. 8 a). Quantitative immunofluorescence analysis indicated that whereas in control cells the majority of E-cadherin staining was at the cell surface, after treatment with EDTA, E-cadherin was now predominantly intracellular (Fig. 7 b). Upon restoration of extracellular Ca\(^{2+}\), E-cadherin staining reappeared at sites of cell-cell contact, with a concomitant reduction in intracellular staining (Fig. 8 d).
Figure 7. Endocytosis of E-cadherin after depletion of extracellular Ca²⁺. Internalization of E-cadherin was compared in untreated confluent MDCK cells and in cells incubated in medium containing EDTA (−EDTA) and media containing 2.5 mM EDTA (+EDTA) and cycloheximide (10 μM) for 30 min. In the presence of EDTA cells show prominent intracellular vesicular staining of E-cadherin. (a) Immunofluorescence localization of E-cadherin in control MDCK cells (−EDTA) and MDCK cells incubated in DMEM containing 2.5 mM EDTA (+ EDTA) and cycloheximide (10 μM) for 30 min. In the presence of EDTA cells show prominent intracellular vesicular staining of E-cadherin. (b) Quantification of relative fluorescence intensities at the cell surface and inside cells measured by SOM software showed that chelation of Ca²⁺ resulted in a dramatic increase in intracellular staining accompanied by a concomitant decrease in plasma membrane staining, indicative of E-cadherin endocytosis stimulated by EDTA. Data are means ± SEM (n = 4). (c) Surface biotinylation. Cells were surface-biotinylated at 0°C (lane 1) and then incubated at 37°C in normal medium (lanes 3 and 5) or in medium containing EDTA (lanes 4 and 6) for 30 min to allow for internalization. Total biotinylated E-cadherin was unchanged in the total cell extracts under both these conditions (lanes 3 and 4). After glutathione stripping there was a significantly increased pool of internalized biotinylated E-cadherin after Ca²⁺ depletion (lane 6) compared to control cells (lane 5). Thus, EDTA treatment increased internalization of surface-biotinylated E-cadherin. (d) Surface trypsinization. Cells were incubated in normal media or in medium containing 2.5 mM EDTA for 30 min. Cell surface proteins were removed by trypsinization and the remaining E-cadherin in cell extracts was analyzed by SDS-PAGE and immunoblotting with a NH₂-terminal antibody (388). Total cellular E-cadherin remained unchanged in the absence (lane 1) or presence (lane 2) of Ca²⁺ chelation. A small pool of internalized E-cadherin was detected after trypsin treatment in cells incubated in normal media (lane 3), but this pool was dramatically increased in the presence of EDTA (lane 4), showing increased internalization of surface E-cadherin. Results shown are representative of three independent experiments.
to specifically inhibit clathrin-coated pit uptake of the low density lipoprotein receptor (Larkin et al., 1983) and other receptors. MDCK cells were preincubated with K⁺-free media, then gently rinsed and exposed to hypotonic K⁺-free media followed by incubation in K⁺-free media. Cells were then surface-biotinylated, incubated at 37°C for internalization, and then the surface was glutathione stripped.

In control cells some of the surface-biotinylated E-cadherin was recovered in an internal pool (Fig. 9 a, lane 5; see also Fig. 2 a). A similar proportion of E-cadherin was internalized after hypotonic shock alone (Fig. 9 a, lane 6) which did not arrest E-cadherin internalization. However in cells treated with hypotonic shock followed by incubation in K⁺-free media there was no biotinylated E-cadherin internalized (Fig. 9 a, lane 7). Under the same conditions, the internalization of biotinylated TfR, which is known to be taken up by a clathrin-dependent pathway, was also blocked (Fig. 9 a, lane 7), whereas the surface residence of Na⁺K⁺ATPase, which in our hands is not internalized, was unchanged. In contrast, the uptake of FITC-labeled ricin, which occurs via a clathrin-independent mechanism (for review see Sandvig and van Deurs, 1996), was unaffected by hypotonic shock and K⁺ depletion (Fig. 9 b). The same levels of FITC-ricin staining were measured in treated and untreated monolayers. Thus, K⁺ depletion effectively and specifically blocked clathrin-dependent uptake, implicating such a pathway in the internalization of E-cadherin.

Double labeling was also carried out by immunofluorescence staining to colocalize internalized E-cadherin with known markers of compartments in clathrin-mediated pathways. Confluent cell monolayers were temperature-blocked at 18°C in order to accumulate intracellular E-cadherin. In cells at 18°C, internalized E-cadherin was colocalized in some, but not all, vesicles stained for the early endosomal marker, rab 5 (Fig. 10, a and b). In contrast, internalized E-cadherin did not colocalize with the late endosomal protein, rab 7 (Fig. 10, c and d). The vesicular staining pattern of E-cadherin and its partial overlap with rab 5 shows that at 18°C endocytosed E-cadherin accumulates in early endosomal or recycling compartments. The lack of colocalization with rab 7 in late endosomes is further evidence that this pool of endocytosed E-cadherin is not destined for lysosomal degradation.

Internalization of E-Cadherin in Complexes with β-Catenin

At the cell surface classical cadherins exist in macromolecular complexes with cytoplasmic catenins (Takeichi, 1991, 1995; Yap et al., 1997a). A preliminary investigation into whether catenins are internalized we probed for β-catenin in surface-biotinylated fractions containing E-cadherin. β-Catenin was coisolated with surface-biotinylated E-cadherin (Fig. 11). Then we allowed surface-biotin-
biotinylated E-cadherin is internalized under these conditions. As shown previously (Fig. 2c), the majority of surface-
internalized pool on streptavidin beads after surface strip-
ning was obtained in both sets of cells. Inhibition of clathrin-
depletion under the shown condi-
tions (Kartenbeck et al., 1991), it is tempting to speculate
also present in the internalized, biotinylated fraction un-
der these conditions. Insofar as E-cadherin is the major
surface protein known to associate with β-catenin in
MDCK cells, we assume that β-catenin and E-cadherin are
being internalized as a complex. Interestingly, only a rela-
tively small amount of β-catenin is internalized compared
to the initial surface-associated pool. Thus, internalization
may result in altered stoichiometry of cadherin-catenin
complexes. Further studies are now required to extend
these observations.

Discussion

In this paper we have studied the trafficking movements of
cell surface E-cadherin. Our findings indicate that, rather
than being a uniformly stable resident on the cell surface,
some pools of surface E-cadherin are endocytosed and re-
cycled back to the surface. Experiments designed to follow
the fate of surface-biotinylated proteins revealed that a
portion of surface-labeled E-cadherin is endocytosed into
an intracellular compartment. This corresponds to a vesic-
ular pool of intracellular E-cadherin that was detected by
immunofluorescence staining, a significant proportion of
which persisted after inhibition of protein synthesis by
cycloheximide and therefore represents a stable pool that is
not in the biosynthetic pathway. The internalization pro-
cess was somewhat selective for E-cadherin rather than re-
flecting bulk clearance of membrane proteins, since under
certain conditions where E-cadherin showed clear uptake, no sim-
ilar internalization of another basolateral membrane pro-
tein, Na+/K+ ATPase, could be detected. Moreover, at
steady-state the biotinylated E-cadherin was not degraded
after endocytosis, but was instead recycled back to the cell
surface where it could be detected by surface trypsiniza-
tion. Thus, there appears to be a rapid recycling pathway
responsible for constantly internalizing and recycling a
portion of E-cadherin on the cell surface.

What proportion of cell surface cadherin is endocytosed
and recycled? Our data indicate that this is influenced by
cell-cell contact. In confluent monolayers with stable cellu-
lar junctions, only a small pool of E-cadherin appears to
recycle. At physiological temperature, the internalized
pool of surface-labeled E-cadherin was consistently ~13% of
the total biotinylated pool. When recycling was inhibited
by bafilomycin A1 or by an 18°C temperature block, the
internalized pool accumulated, finally representing up to
80% of the total biotinylated pool after 2 h at 18°C. How-
ever, under the same conditions, immunofluores-
cence showed that the majority of cellular E-cadherin re-
mained at the cell surface in contact zones. Therefore, it
seems clear that biotinylation detects a relatively small
subset of the total surface E-cadherin in stable monolay-
ers, albeit one that includes the pool capable of undergo-
ing selective recycling. In stable epithelial monolayers,
E-cadherin exists in multiple pools, including classical ad-
herens junctions as well as in extrajunctional regions of the
lateral cell surface (Wang et al., 1990; Nathke et al., 1994;
Yap et al., 1995) that may be differentially extractable in
nonionic detergents (McCrea and Gumbiner, 1991; Shore
and Nelson, 1991). Therefore, while previous studies have
demonstrated uptake of junctional plaques or large frag-
ments (Kartenbeck et al., 1991), it is tempting to speculate

Figure 9. Endocytosis of E-cadherin is inhibited by K+ depletion.
(a) Control cells (lane 1), cells pretreated with hypotonic shock
alone (lane 2), or cells which were pretreated with hypotonic
shock for 5 min then also incubated in K+-free medium for 15
min (lane 3) were surface-biotinylated. Biotinylated proteins
were collected and analyzed by SDS-PAGE and immunoblotted
to detect E-cadherin, transferrin receptor, and Na+/K+ ATPase. A
set of duplicate cells was then treated and surface-biotinylated
and then incubated at 37°C in either K+-free medium (lanes 6
and 7) or normal medium (lane 5) to allow for internalization
of surface proteins. Cells were glutathione-stripped and the internal
pool recovered with streptavidin beads. Biotinylated E-cadherin
and TfR were both internalized in control cells (lane 5) and in
cells treated with hypotonic shock alone (lane 6), but internaliza-
tion of both proteins was effectively blocked in cells depleted of
K+ using hypotonic shock and K+-free medium (lane 7). Na+/K+ ATPase did not internalize under either control or K+ de-
pletion conditions (lanes 5–7). The results are representative of
four separate experiments. (b) Clathrin-independent endocytosis
of FITC-ricin. Cells were incubated either in normal media or
were hypotonically shocked and incubated in K+-free media as
outlined above. Cells were then surface-labeled with FITC-ricin
at 4°C for 1 h and then incubated in normal or K+-free media for
30 min at 37°C to allow for internalization. Nonendocytosed
FITC-ricin was removed by several washes with 0.2 M lactose in
PBS. Cells were then fixed and the amount of internalized FITC-
ricin was viewed and analyzed by confocal microscopy using
SOM software. Similar amounts of intracellular FITC-ricin stain-
ing were obtained in both sets of cells. Inhibition of clathrin-
mediated endocytosis by K+ depletion under the shown condi-
tions in a and b, did not affect FITC-ricin uptake. Data are
means ± SEM (n = 3).
that in mature monolayers the extrajunctional cadherins are more likely to be free to migrate within the plane of the membrane and hence be endocytosed.

In contrast, recycling is significantly increased when cells are unable to make stable cell-cell contacts; i.e., in preconfluent cultures and when productive cadherin-based contacts are disrupted by depletion of extracellular Ca$^{2+}$. The proportion of stable intracellular E-cadherin was found to be considerably greater in preconfluent cells than in confluent monolayers, and as reported by earlier studies (Kartenbeck et al., 1991; Alexander et al., 1998), surface E-cadherin was rapidly internalized when cells were subjected to chelation of extracellular Ca$^{2+}$. Indeed, assessment by quantitative immunofluorescence and surface trypsin susceptibility suggested that the majority (~80%) of total cellular E-cadherin is now internalized upon depletion of extracellular Ca$^{2+}$.

The simplest explanation of these findings is that cell-cell contact regulates E-cadherin trafficking by downregulating the endocytosis of surface E-cadherin. We envisage that in the absence of stable contacts, a large proportion of E-cadherin is constantly recycled to and from the cell surface. Upon formation of productive contacts endocytosis would be downregulated and participating E-cadherin molecules withdrawn from the recycling pathway. Other examples of regulated endocytosis and recycling pathways exist for cellular control of cell surface events; the insulin-responsive glucose transporter, GLUT-4 (Rea and James, 1997), the CFTR chloride channel (Bradbury et al., 1992), and aquaporin water channels (Brown et al., 1998) are all sequestered inside cells under baseline conditions, being released to the cell surface via regulated recycling pathways in response to cytoplasmic signaling events. E-Cadherin may thus be another example of how a repertoire of recycling pathways are used to regulate the function of cell surface proteins.

Our experiments do not yet allow us to identify the molecular mechanism by which cell contact regulates cadherin recycling. It is attractive to speculate that cadherin ligation itself might influence the cytoplasmic machinery responsible for endocytosis. This would be consistent with the central role of E-cadherin in establishing cell-cell contacts (Gumbiner et al., 1988; Musil et al., 1990; Wheelock and Jensen, 1992; McNeill et al., 1993; Yap et al., 1995) and with evidence that ligand binding can influence the clustering activity of the cadherin cytoplasmic tail (Yap et al., 1998). The observation that β-catenin association may be decreased in the internalized pool of E-cadherin further suggests that cytoplasmic interactions mediated by the E-cadherin tail may differ depending on whether E-cadherin is undergoing recycling or is stabilized at the cell surface. However, changes in extracellular Ca$^{2+}$ can also perturb cell junctions via activation of protein kinases (Citi, 1992; Alexander et al., 1998). Various signaling molecules, including heterotrimeric G proteins (de Almeida et al., 1994), are also recruited to sites of contact as cells grow to
Contact-dependent regulation of E-cadherin recycling may therefore reflect not only changes in the state of E-cadherin binding but also cytoplasmic signaling events associated with, and/or independent of, the E-cadherin complex itself.

Irrespective of the precise molecular mechanism involved, contact-dependent inhibition of recycling has potential implications for understanding the dynamics of E-cadherin expression at the cell surface. For example, contact-dependent inhibition of endocytosis may contribute to the stabilization of E-cadherin expression at the cell surface that has been commonly documented to occur as cells grow to confluence (Shore and Nelson, 1991). This model may also account for the observation that when migrating MDCK cells make nascent productive contacts, cytoplasmic E-cadherin appears to be recruited specifically to the regions of contact (McNeill et al., 1993; Adams et al., 1998). In light of our findings, it is plausible that this site-specific accumulation of cadherins is due to inhibition of endocytosis and consequent stabilization of cadherins at the cell surface in regions of contact, rather than solely through directed transport to the site of contact.

Regulated cadherin recycling may also act to remodel adhesive contacts in dynamic situations where contacts must be rapidly broken and remade, such as during gastrulation movements or wound healing (Gumbiner, 1992; Miller and McClay, 1997). Thus, we found that when cellular contacts were experimentally disrupted by removal of extracellular Ca\(^{2+}\), recycling of preexisting surface E-cadherin was sufficient and necessary to restore epithelial monolayers. Even when protein synthesis was blocked by cycloheximide, cells were able to reform stable contacts and monolayers within 1–2 h after restoration of extracellular Ca\(^{2+}\). Importantly, however, restitution of epithelial integrity was severely compromised when recycling of E-cadherin was blocked with bafilomycin. Therefore, despite the replacement of extracellular Ca\(^{2+}\), the E-cadherins remaining on the surface of the isolated cells did not suffice to restore stable contacts; instead recycling of endocytosed E-cadherin to the surface was necessary for stable contacts to be reestablished. By extension, in tissues undergoing dynamic remodeling, endocytosis could act to rapidly clear cadherin molecules from regions where adhesion must be reduced or broken, with these endocytosed cadherins then redeployed by the recycling pathway to sites where new contacts are being formed. Indeed, endocytosis of cadherin was observed to accompany some instances of epithelial-to-mesenchymal transformation during sea urchin gastrulation (Miller and McClay, 1997), a context where cell-cell contacts are expected to be undergoing rapid remodeling. Furthermore, integrin recycling has been documented in locomoting neutrophils (Lawson and M. axfield, 1995), suggesting that cadherins may exemplify more general mechanisms for modulating surface adhesive activity.

Cellular proteins may be endocytosed via clathrin-mediated or non–clathrin-mediated pathways. Several lines of evidence in the present study point to a clathrin-dependent pathway for selective E-cadherin recycling: (a) E-cadherin uptake was disrupted by hypotonic shock and K\(^{+}\) depletion, a maneuver which has been previously used to specifically inhibit clathrin-coated pit uptake of the low density lipoprotein receptor (Larkin et al., 1983) and other receptors; (b) some of the intracellular pool of stable E-cadherin colocalizes with rabs, a marker which is typically located in early or recycling endosomes of clathrin-mediated trafficking pathways (Chavrier et al., 1990); (c) recycling of surface E-cadherin was significantly reduced by bafilomycin A\(_1\), which is characteristic of a pH-dependent, clathrin-mediated recycling pathway (Johnson et al., 1993; Presley et al., 1997); and (d) recycling was also inhibited by an 18°C temperature block, which has been observed to inhibit trafficking beyond early or sorting endosomes (Gallagher et al., 1983; Dunn et al., 1989; Czeczek et al., 1997). In addition, it is interesting to note that the published sequence of mouse E-cadherin contains several signal sequences in its cytoplasmic domain which are known to specify clathrin-coated endocytosis. A di-leucine motif is found in the juxtamembrane region of the mouse E-cadherin cytoplasmic tail at amino acid position 743–744 (SWISS-PROT accession number P09803) (Ringwald et al., 1987), 10 amino acids from the predicted border with the transmembrane region. Also a motif (Y D S L L) is found at amino acids 829–833 of the cytoplasmic tail. This corresponds to a classical tyrosine-based targeting signal (Y XX X O) overlapping with a di-leucine motif. Identical sequences are found in the cytoplasmic tails of human and Xenopus E-cadherin. Tyrosine-based signals are required for clathrin-coated pit recruitment (Trowbridge et al., 1993) and di-leucine motifs act as potential endocytic signals (Johnson and Kornfeld, 1992; Letourneau and Klausner, 1992). A though mutation of these sites did not affect exocytic trafficking of E-cadherin (Chen et al., 1999), their roles in endocytosis have not yet been assessed. Taken together these findings suggest that E-cadherin is endocytosed via a clathrin-coated pathway and subsequently re-
cycled to the surface principally via an early endosomal compartment. Immunoelectron microscopy studies are currently underway in our laboratories to definitively confirm this possibility.

In conclusion, our findings identify a post-Golgi recycling pathway for E-cadherin that is regulated by cell-cell contact. Recycling appears to be capable of influencing cadherin expression and, by implication, adhesive function, at the cell surface in both stable cell monolayers and during dynamic remodeling. Such a pathway has broad functional implications. Recycling could be utilized, or corrupted, to alter adhesion and tissue patterning during development or in tumorigenesis. Internalization of E-cadherin may also be relevant to its pathogenetic role as a cell surface receptor for cellular invasion by Listeria monocytogenes (Mengaud et al., 1996), a pathway which has yet to be fully elucidated. Understanding the cellular pathway for cadherin recycling and its regulation is therefore likely to provide important insights into the morphogenetic mechanisms of cadherins in development and disease.

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