# A dileucine motif in its cytoplasmic domain directs β-catenin-uncoupled E-cadherin to the lysosome

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## Summary

**The E-cadherin-catenin complex regulates $Ca^{2+}$-dependent cell-cell adhesion and is localized to the basolateral membrane of polarized epithelial cells. Uncoupling β-catenin from E-cadherin by deletion or substitution mutations causes accumulation of these proteins in intracellular compartments, including the trans-Golgi network and early endosomes, and degradation in lysosomes. Expression of a dominant-negative dynamin did not change the pattern of the mutant E-cadherin localization, indicating that the endocytosis of the protein from the cell surface does not contribute significantly to the accumulation of the protein in the intracellular compartments. Alternatively, E-cadherin lacking its entire cytoplasmic domain (tail-less E-cadherin) was detected on the surface of cells and targeted to the basolateral membrane. We found that 20 amino acid residues within the juxtamembrane region contain the signal responsible for intracellular accumulation and the lysosomal targeting of E-cadherin. A dileucine motif within this region seems crucial, because substitution of these residues to alanines resulted in efficient surface expression of the protein. The tail-less E-cadherin construct and the dileucine-substitution construct were detected on the basolateral membranes. Thus, the dileucine motif of E-cadherin is not required for its basolateral targeting.**

Key words: Cadherin, Catenin, Lysosome, Trans-Golgi, Transport

## Introduction

Cadherins are a family of cell-surface glycoproteins that mediate $Ca^{2+}$-dependent cell-cell adhesion in tissues (Takeichi, 1988). E-cadherin, a prototypical member of this family, is required for the establishment and maintenance of cell-cell adhesion and cell polarity in epithelia, and plays key roles in tissue morphogenesis and tumorigenesis. E-cadherin is a transmembrane (TM) protein that functions in adhesion as a multiprotein complex. The extracellular domain of E-cadherin binds in a homophilic, $Ca^{2+}$-dependent manner to juxtaposed E-cadherin molecules on adjacent cells. In addition, β-catenin or plakoglobin binds with high affinity to the C-terminus of the cytoplasmic domain, and α-catenin binds indirectly via β-catenin to link the complex to the actin cytoskeleton (Gates and Peifer, 2005). Recent studies have suggested that the linkage between the E-cadherin-catenin complex and actin filaments is more dynamic than previously thought (Yamada et al., 2005). p120, like β-catenin, is an armadillo-repeat protein that can interact with cadherins (Reynolds et al., 1994). In addition to regulating adhesion and actin cytoskeletal organization, recent studies revealed that p120 regulates the steady-state levels of cadherins in cells (Davis et al., 2003; Xiao et al., 2003).

Epithelial cells are morphologically and functionally polarized with distinct complements of cell surface proteins and lipids at their apical and basolateral poles. Maintenance of this polarity requires that newly synthesized proteins are sorted and targeted to specific membrane domains. Specific amino-acid sequence motifs act as signals for membrane proteins to be targeted to the basolateral cell surface (reviewed in Rodriguez-Boulan and Musch, 2005). For example, tyrosine-based motifs target the low-density lipoprotein receptor to the basolateral domain of polarized cells (Matter et al., 1992), whereas a dileucine motif is used by other proteins, such as the Fc receptor (Hunziker and Fumey, 1994). These amino-acid motifs act also as targeting signals to endosomes and lysosomes (Bonifacino and Traub, 2003). Delivery of E-cadherin to the basolateral surface and its incorporation into adherens junctions are key events in cell polarization and tissue morphogenesis. Previous studies have demonstrated that β-catenin binds with high affinity to E-cadherin early in the biosynthetic pathway of E-cadherin (Hinck et al., 1994). Further evidence suggested that this cadherin-catenin pair first forms in the endoplasmic reticulum (ER) (Chen et al., 1999). E-cadherin is, therefore, structurally and functionally linked to β-catenin from the earliest stages of E-cadherin synthesis. Analysis of the exocytic transport of E-cadherin revealed that it is transported from the trans-Golgi network (TGN) to the Rab11-positive recycling endosome on its way to the cell surface (Lock and Stow, 2005). Furthermore, golgin-97 was identified as a selective and essential component of the carriers transporting E-cadherin out of the TGN (Lock et al., 2005).

Frequent cytoplasmic (perinuclear) rather than cell-surface localization of E-cadherin in carcinoma cells has been reported (Carpenter et al., 2002). This suggests that in some carcinomas, there is a defect in transport of E-cadherin to the cell surface. While carrying out experiments to establish MDCK cell clones expressing different E-cadherin constructs – with deletions and amino-acid substitutions in the cytoplasmic domain or the

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extracellular domain – we found that uncoupling the binding of β-catenin from E-cadherin by introducing the deletion and substitution mutations in E-cadherin resulted in the accumulation of the proteins in intracellular compartments, which include the TGN and early endosomes, and degradation in lysosomes. By contrast, E-cadherin lacking the entire cytoplasmic domain was detected on the surface of cells. To define the region of the E-cadherin cytoplasmic domain responsible for the intracellular accumulation and targeting to lysosomes of the β-catenin-uncoupled E-cadherin constructs, we made a series of constructs with deletions or amino-acid substitutions. Our results showed that the 20 amino-acid-long juxtamembrane (JM) region is responsible for the intracellular accumulation and lysosomal targeting of the β-catenin-uncoupled proteins. A dileucine motif in this region is crucial because substitution of these residues with alanine (LA substitution) resulted in efficient surface expression of the protein. Contrary to previous reports, which show that this juxtamembrane motif targets E-cadherin to the basolateral domain (Miranda et al., 2001; Miranda et al., 2003), we found both E-cadherin lacking its entire cytoplasmic domain (the tail-less construct) and its substitution constructs localized at the basolateral membrane.

## Results

### E-cadherin constructs that cannot bind to β-catenin accumulate in intracellular compartments in MDCK cells

A wild-type E-cadherin construct tagged with a hemagglutinin (HA) epitope (hereafter referred to as Ecad) (Fig. 1A) was introduced into MDCK cells, and stable transfectants were isolated. Ecad was targeted to the basolateral surface of polarized MDCK cells (Fig. 1B). Substitution of the eight conserved serine residues for alanine (SA substitution) in the catenin-binding site of E-cadherin has been shown to eliminate the interaction with β-catenin and inactivate the mutant protein (Stappert and Kemler, 1994). We introduced the same SA substitution in the full-length E-cadherin construct to yield a mutant protein hereafter referred to as ESA (Fig. 1A). This protein was exclusively detected intracellularly (Fig. 1B). Deletion of the 70 C-terminal amino-acid residues of E-cadherin – including the β-catenin-binding site – yielded construct EC81, which also accumulated intracellularly (Fig. 1B).

We next quantified biochemically the surface-expression levels of the above constructs at in MDCK cells. Cell-surface E-cadherins cannot be digested by trypsin in the presence of $Ca^{2+}$ but are degraded in the absence of $Ca^{2+}$. Thus, in the presence of EGTA (TE treatment) intracellular E-cadherin remains undigested, whereas in the presence of $Ca^{2+}$ (TC treatment) the remaining E-cadherin after trypsinization represents the amount total E-cadherin. Although EGTA induces internalization of E-cadherin, the rate of E-cadherin endocytosis is so slow compared with that of E-cadherin digestion that the cell-surface E-cadherin is almost completely removed after TE treatment (data not shown). Immunoblot analysis of cells after TC or TE treatment using anti-HA antibodies revealed that a significant amount (~90%) of Ecad was digested following TE treatment (Fig. 1C). By contrast, more than 80% of ESA and EC81 remained undigested after TE treatment (Fig. 1C). Thus, the steady-state distribution of these protein constructs is intracellular, and less than 20% of ESA and EC81 is expressed on the cell surface.

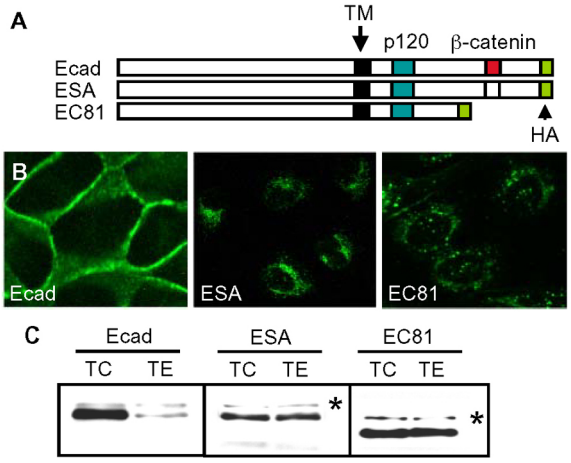


**Fig. 1.** E-cadherin constructs that do not bind β-catenin accumulate in intracellular compartments in MDCK cells. (A) Schematic representations of HA-tagged wild-type E-cadherin (Ecad) and two Ecad mutants: ESA, full-length E-cadherin in which eight conserved serine residues within the catenin-binding site are substituted with alanine; EC81, E-cadherin in which 70 amino acids of the C-terminal are deleted. (B) Immunofluorescence staining of MDCK cells immunostained for Ecad, ESA and EC81 using anti-HA mAbs. Analysis of two to four other clones expressing each construct gave essentially the same results. (C) Trypsin digestion of cells expressing Ecad, ESA or EC81 with or without free $Ca^{2+}$. Cells were incubated with 0.01% trypsin for 10 minutes at 37°C in the presence of 2 mM $Ca^{2+}$ (TC) or 1 mM EGTA (TE) and immunostaining with anti-HA mAb showed that a significant percentage of ESA and EC81 remain inside the cells. Protein bands of high molecular mass (marked by asterisks) correspond to the intracellular, incompletely processed proteins that retain the precursor segment.

Confocal analysis revealed that Ecad primarily accumulates in the basolateral membrane and co-localizes with a basolateral marker, $Na^+$,$K^+$-ATPase, in polarized MDCK cells (Fig. 2). By contrast, ESA and EC81 accumulate in an intracellular membrane compartment of the cells and do not overlap the marker (Fig. 2). To define the site where the β-catenin-uncoupled constructs accumulated, stable transfectants expressing EC81 were labeled with antibodies against GRP78, GM130, golgin-97 and EEA1, or a LysoTracker probe; these reagents mark the ER, cis-Golgi, TGN and early endosomes, or lysosomes, respectively. EC81 was localized in the Golgi region and vesicular structures, which include early endosomes and lysosomes (Fig. 3). As expected, EC81 did not colocalize with the ER marker GRP78 (Fig. 3), suggesting that uncoupling of β-catenin-binding causes accumulation of the constructs in intracellular compartments, including the post-Golgi compartments.

### Intracellular accumulation of the β-catenin-uncoupled E-cadherin does not depend on endocytosis from the cell surface

The observed accumulation of ESA and EC81 in intracellular compartments may have been the result of their specific retention in these compartments. Alternatively, these proteins were first delivered to the surface and then subsequently recycled back to these compartments. It has been shown that E-cadherin endocytosis occurs via a clathrin-dependent

pathway and can be inhibited by dominant-negative dynamin, a GTPase that is a key regulator of clathrin-mediated endocytosis (Miyashita and Ozawa, 2007). To distinguish between these possibilities, we expressed a dominant-negative form of dynamin and blocked clathrin-dependent endocytosis of E-cadherin. MDCK cells stably expressing EC81 were transfected with expression vectors for wild-type or dominant-negative (K44A) dynamin II tagged with green fluorescent protein (GFP) (Kasai et al., 1999), and the effects on EC81 localization were determined. Intracellular staining of EC81 did not change upon overexpression of either wild-type dynamin II (Fig. 4A), or dominant-negative dynamin II (Fig. 4A). Incubation of cells expressing EC81 or ESA in hypertonic solution, which also inhibits clathrin-dependent endocytosis (Ivanov et al., 2004), did not change the intracellular staining of these proteins, although weak membrane staining appeared (data not shown, but see below). From these data, we conclude that the intracellular accumulation of EC81 and ESA does not depend on endocytosis from the cell surface.

To investigate the possibility that EC81 appears on the surface at any time during its biosynthesis, we treated cells with 10 μM cycloheximide for 12 hours to deplete all newly synthesized proteins from the protein synthetic pathway and then analyzed the immunolabeling patterns after washing out the drug (Fig. 4B). After incubation with cycloheximide, there was a significant decrease in plasma-membrane Ecad, and intracellular labeling of Ecad completely disappeared (Fig. 4B, 0 hours). Two hours after the cycloheximide was washed out, Ecad labeling reappeared in the cytoplasm, most probably in the Golgi complex. By 4 hours, there was prominent Golgi and cell-surface labeling (Fig. 4B, 2 hours and 4 hours). Throughout this recovery, labeling of E-cadherin on the plasma membrane steadily increased. These results show that Ecad can

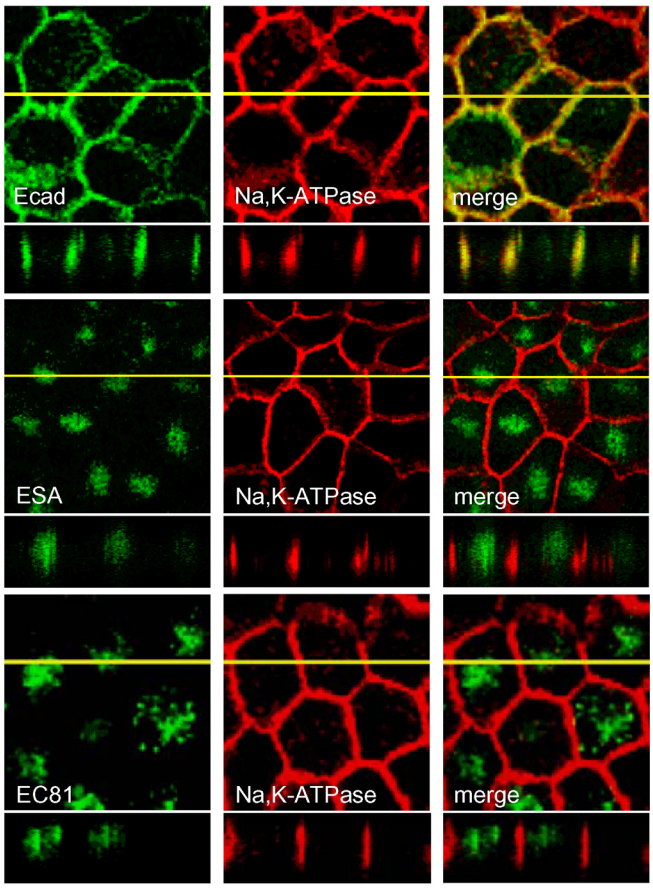


**Fig. 2.** Localization of E-cadherin constructs. Immunofluorescence images of stably transfected cells expressing E-cadherin constructs (anti-HA antibody) and endogenous $Na^+,K^+$-ATPase. (Top panels) Cross sections of monolayers. (Bottom panels) *xz* sections of cells. Yellow lines in the top panels indicate the planes from which the *xz* images were optically reconstructed.

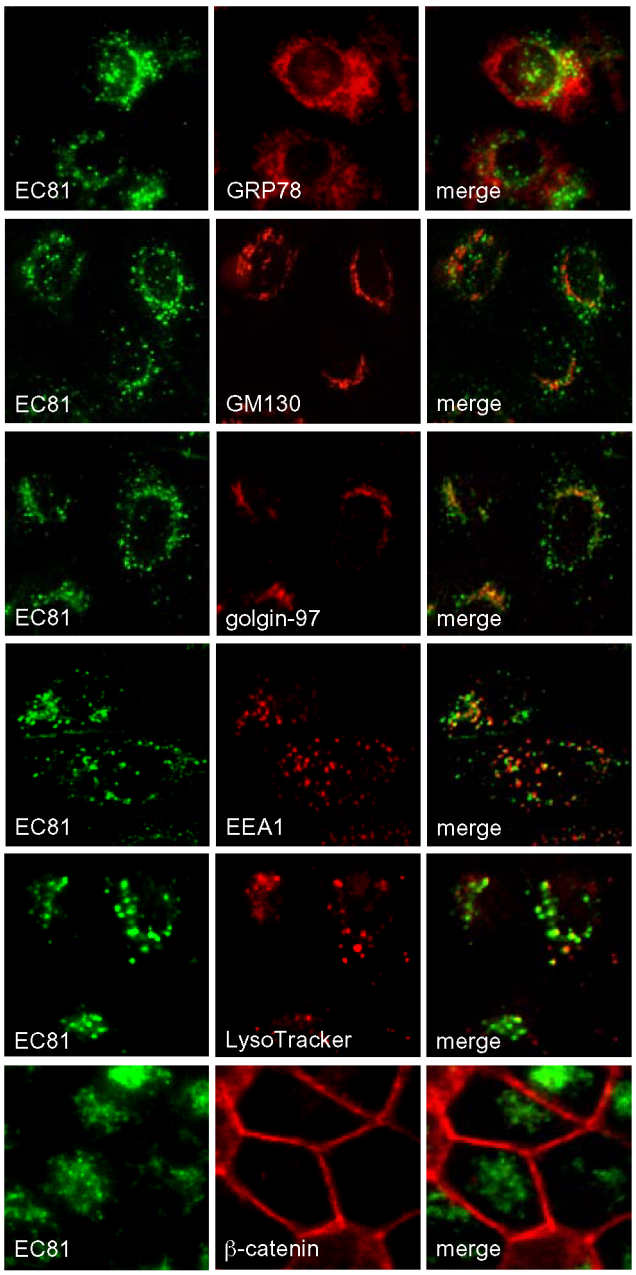


**Fig. 3.** EC81 colocalizes with Golgi and post-Golgi markers. MDCK cells stably expressing EC81 were fixed and incubated with rat monoclonal antibodies against HA, the ER marker GRP78, the Golgi markers GM130 or golgin-97, post-Golgi compartment markers EEA1 or LysoTracker Red DND-99, or β-catenin.

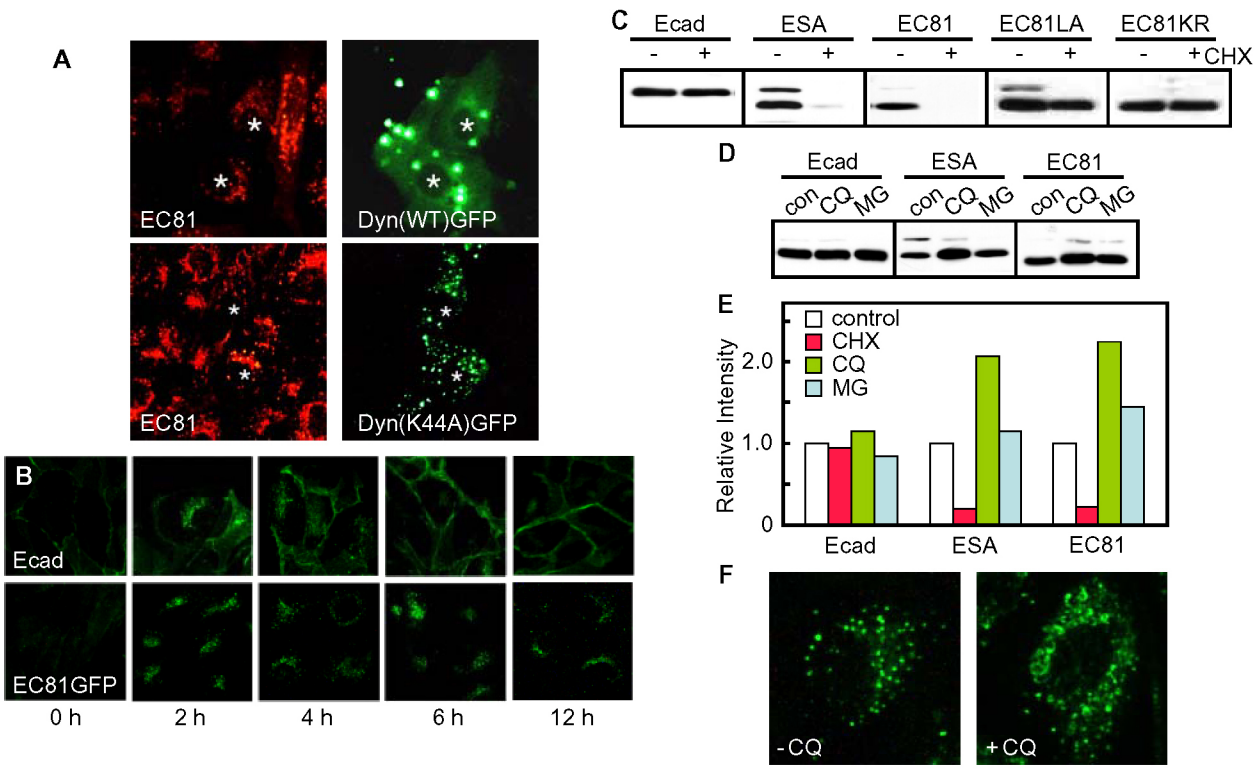


**Fig. 4.** β-catenin-uncoupled E-cadherin is targeted to lysosomes. (A) Intracellular accumulation of EC81 does not depend on clathrin-mediated endocytosis. MDCK cells stably expressing EC81 were transfected with (upper panel) wild-type or (lower panel) dominant-negative dynamin and analyzed for their transient expression. Asterisks indicate cells expressing GFP-tagged dynamin. (B) Newly synthesized Ecad is expressed at the cell surface but EC81GFP is not. MDCK cells stably expressing Ecad or EC81GFP were incubated with 10 μM cycloheximide for 12 hours to deplete Ecad or EC81GFP from the protein synthesis pathway and the cell surface. Cycloheximide was washed out, and cells were returned to normal medium for the indicated times before fixation. Cell-surface localization of Ecad is already detectable after 4 hours, increasing over time. EC81GFP was never detected at the surface. (C) ESA and EC81 decreased upon treatment with cycloheximide. Cells were incubated for 2 hours in the presence or absence of 10 μM cycloheximide (CHX) and then subjected to immunoblot analysis using anti-HA antibody. (D) Effect of lysosome and proteasome inhibitors on ESA and EC81 stability. Cells were incubated for 3 hours with the lysosome inhibitor chloroquine (CQ, 200 μM) or the proteasome inhibitor MG132 (MG, 10 μM). Cell lysates were prepared and analyzed by western blotting using anti-HA antibodies to detect ESA and EC81. (E) Quantitative analysis of C and D, indicating that ESA and EC81 levels are increased in lysosomes in cells treated with chloroquine. (F) Accumulation of EC81 in lysosomes after chloroquine treatment. Cells were incubated for 3 hours in the presence (+CQ) or absence (–CQ) of chloroquine and processed for immunofluorescence staining with anti-HA. Images show a marked accumulation of EC81 after treatment.

be labeled as it is transported through the Golgi complex. Cycloheximide treatment of MDCK cells expressing EC81 tagged with GFP (EC81GFP) completely eliminated the intracellular labeling of EC81GFP (Fig. 4B). Two hours after the cycloheximide was washed out, EC81GFP reappeared in the Golgi complex; this labeling pattern did not change over the following 24 hours (Fig. 4B and data not shown). Unlike Ecad, at no time was EC81GFP detected on the plasma membrane by this method.

During this series of experiments, we noticed that the β-catenin-uncoupled E-cadherins were much more sensitive to cycloheximide than was Ecad. Ecad staining was unaltered in cells pretreated for several hours with cycloheximide, indicating that the cell-surface staining represents stable pools of E-cadherin. Immunoblot analysis revealed that incubation of cells with 10 μM cycloheximide for 2 hours resulted in a reduction of the ESA and EC81 proteins, whereas Ecad did not change significantly (Fig. 4C and quantified data in Fig. 4E). Thus, the turnover rate of ESA and EC81, which are both accumulated in intracellular compartments, is much faster than Ecad, which is targeted to the cell surface. Although we did not analyze it in detail, the half-life of these proteins appeared to be to be ~1 hour. These results are surprising, because the half-life of E-cadherin in MDCK cells has been reported to be ~5 hours (Shore and Nelson, 1991).

### The β-catenin-uncoupled E-cadherins are degraded in the lysosomes

The faster turnover and the accumulation of EC81 in lysosome-related organelles described above prompted us to test whether EC81 is degraded by lysosomes. EC81 levels in cells treated with the various inhibitors were analyzed by western blotting. In cells expressing ESA and EC81, chloroquine, an inhibitor of lysosomal functions, induced accumulation of these proteins, but MG132, a proteasomal inhibitor, did not (Fig. 4D). Chloroquine treatment increased ESA and EC81 expression ~2.0- to 2.2-fold (Fig. 4E). By contrast, little effect on Ecad was observed with the same drugs, indicating that the sensitivity to

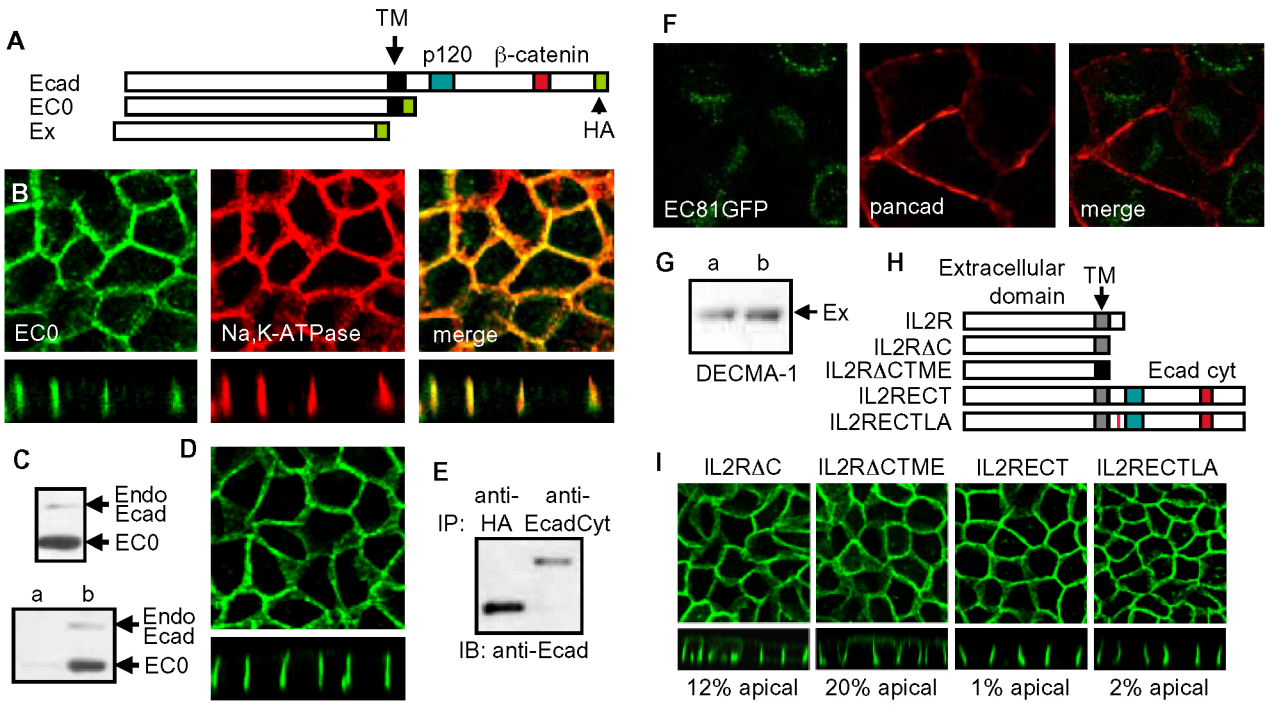


**Fig. 5.** The tail-less construct EC0 is detected on the cell surface. (A) Schematic representation of HA-tagged wild-type E-cadherin (Ecad) and two mutant constructs: EC0, lacking the entire cytoplasmic domain; Ex, consisting of the extracellular domain but lacking the cytoplasmic and TM domains. (B) Confocal imaging of basolateral targeting of the tail-less EC0 construct. E-cadherin and endogenous $Na^+$,$K^+$-ATPase were detected by using DECMA-1and anti-$Na^+$,$K^+$-ATPase mAb. (C) Expression and basolateral targeting of EC0. (Upper panel) Immunoblot analysis of total cell lysates using DECMA-1 revealed that five times more EC0 was expressed than endogenous E-cadherin. (Lower panel) Cells expressing EC0 were grown on Transwell filters and their (a) apical or (b) basolateral membranes were biotinylated. After precipitating the biotinylated proteins using immobilized streptavidin, EC0 and endogenous E-cadherin were detected using DECMA-1. (D) No EC0 labeling was detected on the apical membrane. Cells grown on Transwell filters were fixed and incubated with DECMA-1 without permeabilization to detect E-cadherin on the cell surface. Optical data were obtained as described in B. (E) EC0 does not form lateral dimers with endogenous E-cadherin. Cells expressing EC0 were lysed and incubated with either anti-HA or mAbs against the cytoplasmic domain of E-cadherin (C20820) preabsorbed with protein G-Sepharose. Proteins were separated by SDS-PAGE and detected using DECMA-1. (F) Immunofluorescence labeling of MDCK cells expressing EC81GFP with pan-cadherin antibodies. (G) The Ecad mutant construct Ex is released into both the apical and basolateral media. Cells expressing Ex were grown on Transwell filters. Secretion of Ex in the apical and basolateral media was assessed by immunoprecipitation with rabbit anti-E-cadherin antibodies and immunoblotting with DECMA-1. The barrier function of the transfectants was confirmed by the observation that mouse immunoglobulin was detected in the apical medium when added to the apical medium, but not when added to the basolateral medium (not shown). (H) Schematic representation of the full-length IL2 receptor α chain construct (IL2R) and its derivatives. IL2RΔC: a construct lacking the entire cytoplasmic domain; IRΔCTME: a tail-less construct whose transmembrane domain was replaced with that of E-cadherin; IL2RECT: a construct whose cytoplasmic domain was replaced with that of E-cadherin; IL2RECTLA: a construct whose cytoplasmic domain was replaced with that of E-cadherin with the LA substitution. (I) Detection of IL2RΔC and IL2RΔCTME but not IL2RECT and IL2RECTLA on the apical membrane. Optical data were obtained as described in Fig. 5D except that anti-IL2R antibodies were used.

the drug is linked to the location of the proteins. Essentially identical results were obtained in experiments using 10 mM $NH_4Cl$ to inhibit lysosomal enzymes (data not shown). After chloroquine treatment, large vacuolated lysosomes characteristic of chloroquine-treated cells was observed by phase-contrast microscopy (data not shown). Finally, in cells treated with chloroquine, a marked accumulation of EC81 was observed in these compartments (Fig. 4F). On the basis of these results, it seems that significant fractions of ESA and EC81 are degraded by lysosomal enzymes.

### The tail-less construct EC0 was detected on the cell surface and then targeted to the basolateral membrane

It is possible that the cytoplasmic domain of E-cadherin has a specific surface-membrane-targeting signal that is deleted in the β-catenin-uncoupled constructs. It seemed also possible that the complex formation of E-cadherin with β-catenin resulted in a conformation or motif in the cytoplasmic domain of E-cadherin that allowed the complex to be efficiently targeted to the surface membrane. To test these possibilities, we expressed an E-cadherin construct in which the entire cytoplasmic domain of E-cadherin was deleted (EC0) (Fig. 5A). EC0 was transported to the cell surface (Fig. 5B). More importantly, cell-surface biotinylation of the apical and basolateral membranes of cells cultured on Transwell filters revealed that EC0 was exclusively transported to the basolateral membrane (Fig. 5C).

Because biotinylation may not label all surface proteins (Gottardi et al., 1995), we also assessed the apical surface expression of EC0 by antibody labeling of cells without

permeabilization. After fixation, MDCK cells cultured on Transwell filters were incubated with anti-E-cadherin mAb (DECMA-1) in the apical and basolateral chambers. Although this mAb recognize not only EC0 but also endogenous canine E-cadherin (Fig. 5C), no labeling was detected on the apical membrane (Fig. 5D). Thus some punctate staining found in permeabilized cells (Fig. 5B) was intracellular.

The tail-less EC0 construct was active in aggregation assays and formed so-called lateral dimers on the membrane surface (Ozawa, 2002), raising the possibility that EC0 and endogenous E-cadherin form heterodimers during trafficking. To address this possibility, MDCK cells expressing EC0 were subjected to immunoprecipitation using antibodies against HA and the cytoplasmic domain of E-cadherin. The condition was successfully used to detect the lateral dimmers of E-cadherin (Ozawa, 2002). Immunoblot analysis of the immunoprecipitates with DECMA-1 revealed that EC0 did not co-precipitate with endogenous E-cadherin (Fig. 5E). Incubation of the cells with 20 μg/ml 3,3′-dithio*bis*(sulfosuccinimidylpropionate), a crosslinking reagent, did not result in co-precipitation of these two proteins (data not shown). If EC0 can dimerize with endogenous canine E-cadherin, EC81 should display the same characteristics. This, however, was not the case, because labeling of MDCK cells expressing EC81GFP with pan-cadherin antibodies, which recognize a number of cadherins from different species including canine E-cadherin, revealed no colocalization of EC81GFP and the endogenous cadherins (Fig. 5F). MDCK cells have been reported to express E-cadherin as well as cadherin-6 (K-cadherin) (Stewart et al., 2000). Immunoblot analysis revealed that the pan-cadherin antibodies recognized not only canine cadherin-6 but also canine E-cadherin (data not shown). MDCK cells expressing EC81 that were labeled with anti-HA and anti-β-catenin mAbs revealed no significant intracellular colocalization of EC81 and β-catenin (Fig. 3). Furthermore, the fact that – compared with endogenous E-cadherin – five times more EC0 was observed on the basolateral membranes (Fig. 5C), argues against the idea that endogenous E-cadherin carries EC0 to the surface by forming dimeric complexes. Therefore, it is unlikely that the transport of EC0 to the basolateral membrane depended on its association with endogenous E-cadherin. The correct basolateral targeting of EC0 in the absence of the cytoplasmic domain demonstrates that this domain is not essential for basolateral sorting and targeting in MDCK cells.

To determine whether the E-cadherin TM domain is required for basolateral targeting, an E-cadherin construct lacking the cytoplasmic and TM domains (named Ex) (Fig. 5A) was introduced into cells and stable transfectants were isolated. Cells were cultured on Transwell filters, and the media from the apical and basolateral compartments were collected and analyzed. Ex was secreted and was detected in both the apical and basolateral media (Fig. 5G). These results suggest that, the extracellular domain of E-cadherin alone does not specifically target E-cadherin to the basolateral membrane, and the TM domain of E-cadherin is required for its transport to the basolateral membrane. The addition of the E-cadherin TM domain to the extracellular domain of human IL2 receptor (Fig. 5H), however, did not result in the sorting of the protein to the basolateral membrane, i.e. the construct was detected on both basolateral and apical membranes (Fig. 5I). Thus the E-cadherin TM domain alone is not sufficient for the basolateral localization.

### The N-terminal repeats of the extracellular domain are required for the basolateral localization of E-cadherin

The results described above suggest that the extracellular domain of E-cadherin, in addition to its TM domain, is also required for the basolateral localization of the protein. The extracellular domain of cadherins consists of five cadherin repeats (EC1-EC5). To find out which repeat is required for the basolateral localization of EC0, we made a series EC0 constructs, each lacking one of the five repeats (Fig. 6A), and expressed them in MDCK cells (Fig. 6B). Cell-surface biotinylation of the apical and basolateral membranes of cells cultured on Transwell filters revealed that small amounts of the constructs lacking either EC1, EC2 or EC3 were detected on the apical membrane (Fig. 6C). The presence of these deletion constructs on the apical membrane was confirmed by antibody labeling of cells as described in Fig. 5D. Although no apical membrane labeling was detected for EC0ΔEC4 and EC0ΔEC5, constructs EC0ΔEC1, EC0ΔEC2 and EC0ΔEC1 were detected on the apical membrane (Fig. 6D,E). Thus, although the deletion of the C-terminal EC4 and EC5 domains did not affect the basolateral localization of the EC0 protein, deletion of the N-terminal EC1, EC2 and EC3 domains resulted in mis-localization of the EC0 protein to the apical membranes. Thus, the extracellular domain of E-cadherin itself has a role in its basolateral transport when anchored to the membrane.

Since the N-terminal domains of cadherins are crucially involved in the activity of the protein, we expressed a variant of the EC0 construct in which the second tryptophan residue (W2) of mature E-cadherin was substituted with alanine (W2A substitution) (EC0WA); this W2A substitution completely abolished lateral dimerization and the adhesion activity of the protein (Ozawa, 2002). Antibody labeling of cells revealed that EC0WA was detected on the apical membrane in addition to the basolateral membrane (Fig. 6E,G). A similar full-length construct, EWA, having the same W2A substitution but having the entire cytoplasmic domain, was not detected on the apical membrane (Fig. 6E,G). Thus, when the cytoplasmic domain is intact, a construct carrying the mutation of the extracellular domain is correctly targeted to the basolateral membrane. Consistent with this idea, addition of the E-cadherin cytoplasmic domain to the extracellular and TM domains of human IL2 receptor (Fig. 5H) results in its sorting of the to basolateral membrane (Fig. 5I).

### A dileucine motif in the JM region of the E-cadherin cytoplasmic domain is involved in the intracellular accumulation of β-catenin-uncoupled proteins and their targeting to the lysosome

We observed that two β-catenin-uncoupled proteins which contained their membrane-proximal region remained in the intracellular compartments and were then targeted to lysosomes, whereas the tail-less mutant proteins without this region were not. This suggests that the membrane-proximal region of the E-cadherin cytoplasmic domain is involved in the intracellular accumulation and subsequent lysosomal targeting of cadherins. To exactly identify the region responsible for the correct protein transport, we made three additional constructs: EC81ΔMP14, EC81ΔMP39 and EC20 (Fig. 7A), all of which

lack the p120-binding site. None of these deletion mutants co-precipitated with p120 in immunoprecipitation assays using anti-HA antibodies (data not shown). Stable transfectants expressing these constructs were isolated and the location of each construct was determined by immunofluorescence labeling using anti-HA antibodies (Fig. 7B). We found could only detect EC81ΔMP39 on the cell surface. TC or TE treatment followed by immunoblot analysis revealed that a significant percentage of EC81ΔMP14 and EC20 remained inside the cells (Fig. 7C). These results indicate that the 20 amino-acid residues behind the TM domain are sufficient to retain the proteins in the intracellular compartments. Incubation of EC20-expressing cells with chloroquine resulted in increased expression of the protein (data not shown). To identify residues that contain this protein-transport signal, we introduced amino-acid substitutions within this 20-amino-acid region, yielding mutant constructs EC81KR, EC81EPA, EC81LA, EC81PA and EC81DA (Fig. 7D). EC81LA was detected at the cell surface, whereas other constructs were detected in the intracellular compartments (Fig. 7E,F). Consistent with our observation that the cell surface E-cadherin represents a stable pool of the protein, cell surface EC81LA levels did not change significantly after incubation of cells with 10 mM cycloheximide for 2 hours (Fig. 4C). To our surprise, the same treatment did not resulted in a reduction of cell surface EC81KR levels, because the construct seemed to be retained in intracellular compartments (Fig. 4C). Incubation of cells in hypertonic medium, which has been shown to inhibit clathrin-dependent endocytosis (Heuser and Anderson, 1989), did not change the intracellular pool of EC81, EC81PA and EC81DA but reduced that of EC81KR and EC81EPA, and almost eliminated that of EC81LA (Fig. 7F). Thus a portion of EC81KR and EC81EPA seemed to be transported to the cell surface and then internalized. Introduction of the LA substitution in another β-catenin-uncoupled E-cadherin of full-length (ESA), which yield ESALA, resulted in increased cell surface expression of the protein (Fig. 7E). Some intracellular staining of ESALA suggests that there may be other putative targeting motifs in the C-terminal within a region of 70 amino acid.

Our results show that the dileucine motif within cytoplasmic domain of E-cadherin is crucial for the accumulation of the

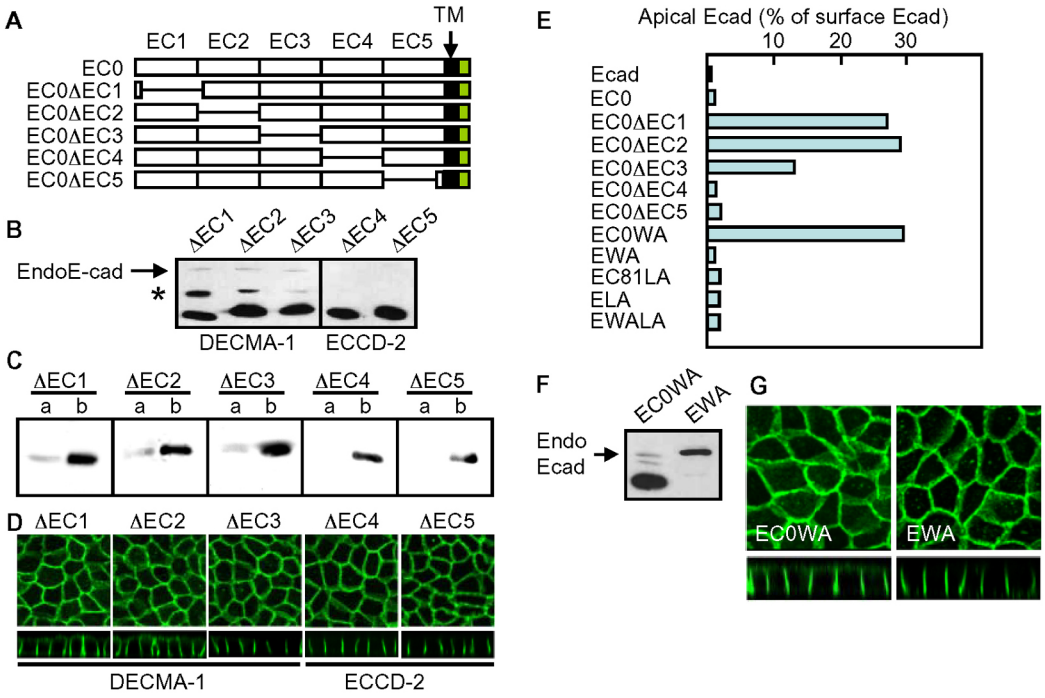


**Fig. 6.** The N-terminal repeats of the E-cadherin extracellular domain are required for the basolateral localization. (A) Schematic representation of EC0 and its derivatives each carrying a deletion of one of the five extracellular domains. (B) Expression of the EC0 derivatives. Constructs were detected using antibodies as indicated. (C) Basolateral localization of the EC0 derivatives. Cells were grown on Transwell filters, and either the (a) apical or (b) basolateral membranes were biotinylated. After the biotinylated proteins were precipitated using immobilized streptavidin, the constructs were detected using DECMA-1 except for EC0ΔEC4, which was detected with ECCD-2. Constructs lacking either EC1, EC2 or EC3 were detected on the apical membrane. (D) Detection of ΔEC1, ΔEC2 and ΔEC3, but not ΔEC4 and ΔEC5 on the apical membrane. Cells grown on Transwell filters were fixed and incubated with DECMA-1 without permeabilization to detect E-cadherin on the cell surface. To detect the EC0 deletion constructs EC0ΔEC4 and EC0ΔEC5, ECCD-2 was used because DECMA-1 cannot recognize these constructs on the cell surface. Constructs EC0ΔEC1, EC0ΔEC2 and EC0ΔEC3 were detected on the apical membrane. Optical data were obtained as described in Fig. 5D. (E) Quantitative analysis of the apical membrane targeting. Relative amounts of the pool of E-cadherin at the apical membrane (not shown) and of its derivatives as described in Fig. 5D, Fig. 6D,G, and Fig. 8D were quantified using NIH Image and expressed as a percentage of the total cell surface pool of the protein. In addition to EC0 derivatives lacking EC1, EC2 or EC3, another EC0 construct containing a W2A substitution (EC0WA) was detected on the apical membrane. (F) Immunoblot detection of EC0WA and EWA. Total cell lysates were analyzed by using DECMA-1. (G). Detection of EC0WA but not EWA on the apical membrane. Optical data were obtained as in Fig. 5D.

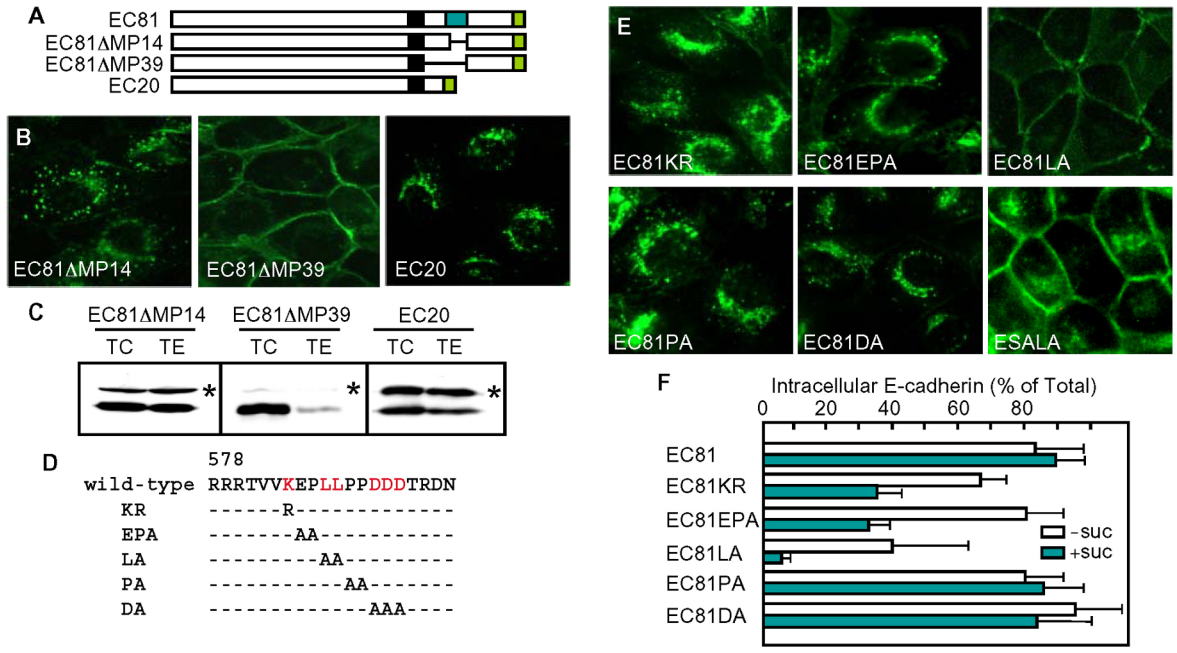


**Fig. 7.** The dileucine motif is crucial for the lysosomal targeting of β-catenin-uncoupled E-cadherin constructs. (A) Schematic representations of construct EC81 and its derivatives: EC81ΔMP14 and EC81ΔMP39, EC81 derivatives with additional internal deletions of residues 602-615 or 578-616, respectively; EC20: a construct lacking 131 amino acids at its C-terminal. (B) Immunofluorescence images reveal that that the 20 amino-acid residues within the JM region of the E-cadherin cytoplasmic domain contain the signal for lysosomal targeting of β-catenin-uncoupled E-cadherin constructs. Cells expressing the indicated constructs were labeled with anti-HA mAbs. (C) TC and TE treatment revealed that a significant percentage of EC20 and EC81ΔMP14 remained inside the cells. Cells expressing the indicated constructs were incubated with 0.01% trypsin for 10 minutes at 37°C in the presence of 2 mM $Ca^{2+}$ (TC) or 1 mM EGTA (TE). Proteins were detected with anti-HA mAbs. Protein bands of high molecular mass (marked by asterisks) correspond to intracellular, incompletely processed proteins that retain the precursor segment. (D) The sequence of the 20 amino-acid residues in the JM region of the E-cadherin cytoplasmic domain and of those substituted in the mutant constructs. (E) Immunofluorescence images showing localization of the constructs. Cells expressing the indicated constructs were labeled with anti-HA mAb. EC81LA was detected at the cell surface, whereas other constructs were detected in the intracellular compartments. (F) Quantification of the intracellular pool of EC81 and its derivatives. Relative amounts of the intracellular pools obtained after TE treatment were quantified using NIH Image and expressed as a percentage of the pool of total protein obtained after TC treatment. Before trypsinization, cells were incubated for 30 minutes at 37°C in medium with or without 0.45 M sucrose (+suc or –suc, respectively). Inhibition of endocytosis by hypertonic medium did not change the intracellular pool of EC81, EC81PA and EC81DA but reduced that of EC81KR, EC81EPA and EC81LA.

protein in intracellular compartments and the subsequent targeting to lysosomes. The dileucine motif has been shown to be crucial for the targeting of human E-cadherin to the basolateral membrane of MDCK and LLC-PK1 epithelial cells (Miranda et al., 2001; Miranda et al., 2003). As described above, EC0, the tail-less protein lacking this motif, localized to the basolateral membrane because it contained the extracellular domain. Consistent with this idea, the cell-surface biotinylation assay revealed that EC81LA is exclusively localized at the basolateral membrane (Fig. 8A). Confocal analysis revealed that EC81LA primarily accumulates in the basolateral membrane and colocalizes with $Na^+$,$K^+$-ATPase in polarized MDCK cells (Fig. 8B). The basolateral localization of EC81LA is not restricted to MDCK cells, it was also observed in DLD1 cells (Fig. 8A, right panel), a human colon-cancer cell line with the ability to polarize and form junctions (Taniguchi et al., 2005). EC81LA did not colocalize with the apical membrane marker protein gp135 (Ojakian and Schwimmer, 1988) (Fig. 8C) – antibody labeling of cells did not stain the apical membrane (Fig. 8D and Fig. 6E). A full-length construct with the same amino acid substitution (ELA) was targeted to the basolateral membrane (Fig. 8C,D). As described above, although introduction of the W2A substitution in the tail-less construct resulted in mis-localization of the protein, the same substitution in the full-length construct did not (Fig. 6G). The W2A substitution in ELA, also had no effect on its sorting (Fig. 8D). Consistent with this result, the LA substitution in a chimeric protein composed of the extracellular and TM domains of human IL2 receptor and the E-cadherin cytoplasmic domain of did not result in mis-sorting of the protein (Fig. 5I).

## Discussion

Here, we have shown that E-cadherin constructs that had been uncoupled from β-catenin binding accumulated in intracellular compartments, including the Golgi complex and early endosomes, and were then targeted to lysosomes. Because the 20 amino-acid residues within the juxtamembrane region were sufficient for accumulation and targeting, and because substitution of two leucine residues for two alanine residues resulted in cell surface expression of the protein, we conclude that these 20 residues comprise a positive signal for the accumulation of the β-catenin-uncoupled E-cadherin in the intracellular compartments and subsequent targeting to

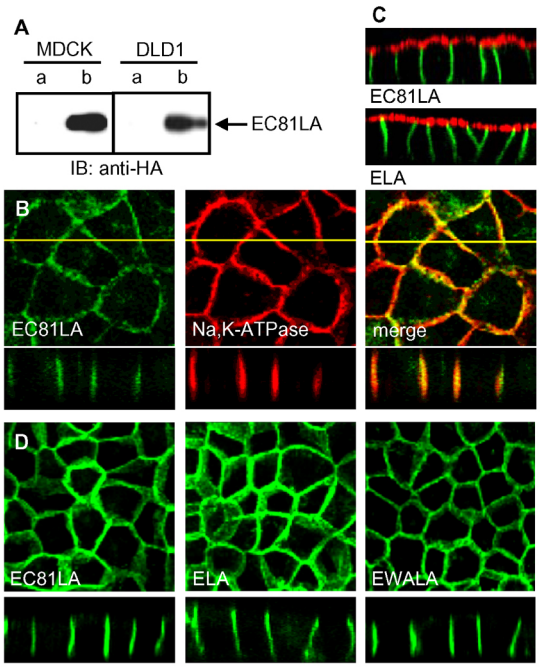


**Fig. 8.** The dileucine motif is not required for basolateral targeting. (A) Basolateral targeting of EC81LA in MDCK and DLD1 cells. Cells were grown on Transwell filters and either the (a) apical or (b) basolateral membranes were biotinylated. After precipitation of the biotinylated protein using immobilized streptavidin, EC81LA was detected using anti-HA mAbs. (B) Confocal imaging of basolateral targeting of EC81LA. EC81LA and endogenous $Na^+$,$K^+$-ATPase were detected using anti-HA antibody and antibody against $Na^+$,$K^+$-ATPase, respectively. (C) EC81LA and ELA do not colocalize with the apical membrane marker gp135. EC81LA and ELA were detected using anti-HA antibody, whereas endogenous gp135 was detected using 3F2 mAb. (D) EC81LA, ELA and EWALA are detected at the basolateral membrane but not the apical membrane. Optical data were obtained as described in Fig. 5D.

lysosomes. Substitution of the preceding residues (lysine or glutamic acid and proline) moderately inhibited intracellular accumulation. These residues could play a role as part of the dileucine signal, either in stabilizing a turn structure or in modulating the affinity of the interaction with adaptor complexes (Bonifacino and Traub, 2003).

Although the dileucine motif has been reported to be required for the basolateral targeting of E-cadherin (Miranda et al., 2003), three constructs lacking this dileucine motif – EC0 (lacking the entire cytoplasmic domain), EC81LA (partially truncated) and ELA (full-length but with the LA substitution) – were all detected exclusively on the basolateral membrane. Although a construct containing the extracellular and TM domains of α chain of the IL2 receptor was detected on basolateral and apical membranes, addition of the E-cadherin cytoplasmic domain to this construct yielded exclusively basolateral-membrane targeting. Thus, the cytoplasmic domain indeed contains the targeting signal as reported (Miranda et al., 2001). However, the dileucine motif in the cytoplasmic domain is not required for basolateral targeting because the LA substitution in the chimeric protein does not cause mis-sorting of the protein. Identification of the basolateral targeting signal in the cytoplasmic domain of E-cadherin needs further detailed studies.

Although EC0 was detected exclusively on the basolateral membrane, further deletion of the TM domain resulted in the release of the new construct (Ex) into both the apical and basolateral media. Therefore, it seems that the TM domain is required for the basolateral targeting of the tail-less E-cadherin. However, at present we cannot exclude the possibility that this polarized trafficking is a result of EC0 piggybacking other basolaterally targeted proteins. It is also possible that the basolateral targeting of EC0 is mediated by a default mechanism that requires the TM domain. Although EC0 is localized at the basolateral membrane, EC0WA, EC0ΔEC1, EC0ΔEC2 and EC0ΔEC3 are detected at the apical membrane in addition to the basolateral membrane. These mutant proteins may be transported from the basolateral to the apical membrane by transcytosis (Mostov and Deitcher, 1986). Removal of these EC0 mutants by transcytosis might be explained by the lack of homophilic binding of the proteins. Consistent with this idea, addition of DECMA-1, a function-blocking mAb, to the basolateral medium of EC0-expressing cells induces the appearance of EC0 on the apical membrane (data not shown).

It is known that the ER is a quality-control site during protein synthesis (Trombetta and Parodi, 2003). Misfolded proteins are usually retained in the ER and subsequently targeted for degradation in either lysosomes or the 26S proteasome. However, it has recently been shown that mutant proteins of cystic fibrosis transmembrane conductance regulators escape ER quality control, but are rapidly cleared from the secretory pathway and degraded in lysosomes (Sharma et al., 2004). Since the β-catenin-uncoupled E-cadherins used in the present study are all inactive as cell-adhesion molecules (Ozawa, 2003), the mechanism by which these proteins accumulate in intracellular compartments and, subsequently, degrade in lysosomes is cell-physiologically relevant. Another mutated E-cadherin construct, EWA, is also inactive (Ozawa, 2002) but is, however, transported to the surface of MDCK cells, showing that not all mutant E-cadherin proteins accumulate in intracellular compartments. Colocalization of the retained constructs with the markers of the post-Golgi compartments and inhibition of their degradation by lysosomal inhibitors indicated that the proteins reside in post-Golgi transport carriers and that retention in these compartments is not perfect. Eventually, these proteins were targeted to lysosomes, a process for which the dileucine motif is responsible. It has recently been shown that golgin-97 and Rab11 are important factors in the regulation of post-Golgi transport of E-cadherin to the cell surface. Golgin-97 is one of the earliest regulators identified to be involved in post-Golgi trafficking of E-cadherin, acting at the point where newly synthesized protein emerges from the TGN (Lock et al., 2005). Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin (Lock and Stow, 2005). Partial colocalization of EC81 with golgin-97 suggests it is also involved in targeting to lysosomes.

The assembly of the E-cadherin/β-catenin complex occurs in the very early stages of trafficking (Ozawa and Kemler, 1992; Hinck et al., 1994). Therefore, our observation that inhibiting β-catenin binding resulted in the failure of the mutants to be targeted to the cell surface is consistent with the

idea that the assembly of the E-cadherin/β-catenin complex is an obligatory step in the efficient export of E-cadherin from the intracellular compartments and subsequent delivery of the protein to the basolateral membrane of polarized cells. Like E-cadherin, an N-cadherin construct that cannot bind β-catenin also accumulated in the intracellular compartments of MDCK cells (data not shown). Thus, complex formation with β-catenin seemed to be a prerequisite for the exit of cadherins from the intracellular compartments. Recently, an F9 cell line lacking both β-catenin and plakoglobin was established (Fukunaga et al., 2005). In these cells, E-cadherin was detected in intracellular compartments, suggesting that these proteins were required not only for cell adhesion activity but also for the cell-surface expression of E-cadherin. Together, these data suggested that β-catenin is required for E-cadherin transport to the cell surface.

Recently, it has been shown that type I γ phosphatidylinositol phosphate kinase (PIPKIγ) directly binds to E-cadherin and modulates E-cadherin trafficking (Ling et al., 2007). Depletion of PIPKIγ or disruption of PIPKIγ binding to E-cadherin resulted in defects in E-cadherin transport. The region of E-cadherin required for the PIPKIγ-binding was mapped to ten amino acids overlapping the β-catenin-binding site. PIPKIγ binding is not, however, an absolute requirement for basolateral targeting of E-cadherin because the tail-less E-cadherin is still transported to the basolateral membrane. Although deletion of the last 35 amino acids from the E-cadherin cytoplasmic domain has been shown to eliminate β-catenin-binding but has no effect on PIPKIγ association (Ling et al., 2007), we found that the same construct was detected in intracellular compartments (data not shown). It has also been shown that an E-cadherin germline mutation (V832M) identified in hereditary diffuse gastric cancer (Yabuta et al., 2002) lies in the PIPKIγ-binding region, and that the mutant protein had lost PIPKIγ binding and showed disrupted basolateral membrane targeting (Ling et al., 2007). In contrast to this observation, we found that the mutant protein was mainly present on the surface membrane, and the intracellular accumulation was detected only when cells were plated at low density and, therefore, could not make contact (data not shown).

We identified two leucine residues in the JM region of the E-cadherin cytoplasmic domain as being essential for the protein to accumulate in post-Golgi compartments and for subsequent lysosomal targeting. Dileucine motifs are known to mediate sorting of transmembrane proteins to endosomes and lysosomes (Bonifacino and Traub, 2003). The dileucine motif of E-cadherin might mediate binding to adaptors, such as AP1 and GGAs, and thereby facilitate sorting and targeting to lysosomes (Bonifacino and Traub, 2003). Although the dileucine motif and the β-catenin-binding site are not close to each other, the simplest explanation is that the dileucine motif is obstructed by β-catenin binding, thereby preventing interaction with those adaptors involved in its targeting of E-cadherin to the lysosomes. The cytoplasmic domain of E-cadherin has been shown to unfold, without assuming its correct structure, when β-catenin is not bound. Moreover, it is protected from proteolysis when being in a complex with the β-catenin, whereas it is digested in the absence of β-catenin (Huber et al., 2001). Therefore, another possibility is that, upon β-catenin binding, the cytoplasmic domain of E-cadherin adopts a conformation that prevents interaction of the dileucine motif with the targeting adaptors. In this way, β-catenin binding to E-cadherin might direct transport to the cell surface.

Recently we have shown that the dileucine motif is required for clathrin-mediated endocytosis of E-cadherin (Miyashita and Ozawa, 2007). This process is regulated by p120 because uncoupling of p120 binding by amino acid substitutions in the p120-binding site or p120 depletion by specific siRNA against p120 enhanced the dileucine-motif-dependent internalization of E-cadherin. p120 binding, however, seems to have no influence on the interaction of the dileucine motif with the lysosome targeting adaptors, because mutant E-cadherins that are able to bind p120, such as ESA and EC81, as well as other mutant E-cadherins that cannot interact with p120, such as EC20 and EC81ΔMP14, equally accumulate in intracellular compartments and are targeted to the lysosomes. Hypertonic treatment of cells to inhibit clathrin-dependent endocytosis results in reduction of the amount of intracellular EC81KR, EC81EPA and EC81LA, raising the possibility that their accumulation in part depends on endocytosis. The same treatment, however, does not reduce the amount of intracellular EC81, EC81PA and EC81DA. Therefore, accumulation of EC81PA and EC81DA, as in the case of EC81, does not depend on endocytosis of these proteins.

E-cadherin is downregulated in a broad range of epithelial tumors, and its loss is associated with an infiltrating phenotype and poor prognosis (Guilford, 1999). Frequent cytoplasmic (perinuclear) rather than cell-surface localization of E-cadherin in carcinoma cells has been reported (Carpenter et al., 2002). The failure of E-cadherin transport causes decreased cell-surface expression of E-cadherin and permits the dissociation and widespread dissemination of carcinoma cells. E-cadherin missense mutations, including mutations found in the cytoplasmic region involved in interaction with β-catenin, have been identified in various cancers (Concolino et al., 2004). Furthermore, tyrosine phosphorylation of β-catenin induces its dissociation from E-cadherin (Xu et al., 2004). Increased tyrosine phosphorylation of β-catenin in cancer cells has been documented (Birchmeier et al., 1993). Therefore, our observation that uncoupling of β-catenin from E-cadherin results in accumulation of E-cadherin in the post-Golgi compartments and subsequent degradation in the lysosomes might be relevant in how some cancer cells acquire reduced adhesiveness.

## Materials and Methods

### cDNA construction

The following constructs containing mutant variations of E-cadherin cDNA have been described previously (Ozawa, 2002; Ozawa, 2003): HA-tagged full-length E-cadherin (Ecad); full-length E-cadherin in which eight conserved serine residues within the catenin-binding site had been substituted for alanine residues (ESA); E-cadherin truncated C-terminally of 70 amino acids (EC81); E-cadherin lacking the entire cytoplasmic domain (EC0); E-cadherin lacking the entire cytoplasmic domain and containing an additional substitution of the second tryptophan residue for alanine (EC0WA); E-cadherin lacking the cytoplasmic and TM domains (Ex).

Three EC81-derived constructs with additional deletions (Fig. 7A) encoding EC81ΔMP14, EC81ΔMP39 and EC20 were constructed by PCR using the following combinations of primers: EC81ΔMP14 (5′-AGTCAGCTGCACAGGGGCCTG-3′ and 5′-ATAGTAATACACATTGTCCCGG-3′), EC81ΔMP39 (5′-CAGCTGCACAGGGGCCTGGAT-3′ and 5′-CAGAAACAGTAGGAGCAGCAGGAT-3′), and EC20 (5′-TATACCGCTCGAGAGCCG-3′ and 5′-ATCATTGTCCCGGGTATCATCAT-3′). the following primer pairs were used for the following mutations: Lys584 to Arg (KR mutation), 5′-GCGAGCCCCTGCTGCCACCAG-3′ and 5′-GAACCACCGTTCTCCTCCGTAG-3′; Glx585-Pro586 to Ala-Ala (EPA

mutation), 5′-CGCTGCTGCCACCAGATGATGAT-3′ and 5′-CTGCTTTGACCACCGTTCTCCTCC-3′; Leu587-Leu588 to Ala-Ala (LA mutation), 5′-CGCGGGCTCTTTGACCACCGTTCT-3′ and 5′-GCGCCACCAGATGATGATACCCGG-3′; Pro589-Pro590 to Ala-Ala (PA mutation), 5′-TGCAGATGATGATACCCGGGACA-3′ and 5′-GCCAGCAGGGGCTCTTTGACCA-3′; Asp591-Asp592-Asp593 to Ala-Ala-Ala (DA mutation), 5′-GCGGCTGGTGGCAGCAGGGGCTC-3′ and 5′-GGCCACCCGGGACAATGTGTATTAC-3′ (see Fig. 1 and Fig. 6C).

Chimeric constructs of IL2 receptor (IL2R) (Nikaido et al., 1984) and E-cadherin, IL2RΔC, IL2RΔCTME, IL2RECT, and IL2RECTLA were constructed using the following combinations of primers: IL2RΔC (5′-GAAGATCTGGTCCCAAGGGTCAGGAA-3′ and 5′-ATCCCAGGTGAGCCCACTCAGG-3′), IL2RΔCTME (5′-GAAGATCTGGTCCCAAGGGTCAGGAA-3′ and 5′-GCCGGCCACTGCTACCTGGT, 5′-GCCATCCTCGGAATCCTTGGA-3′ and ATCTAGAAACAGTAGGAGCAGC-3′), IL2RECT and IL2RECTLA (5′-GAAGATCTGGTCCCAAGGGTCAGGAA-3′ and 5′-GCCAGGTGAGCCCACTCAG-3′, 5′-GCCGCAGAACGGTGGTCAAAGAGCC-3′ and 5′-CCGCTCGAGGATATCGTCGTCCTCGCCAC-3′).

Expression vectors for GFP-tagged wild-type and dominant-negative dynamin IIaa (pEGFPNDyn2a and pEGFPNDyn2a(K44A), respectively) (Kasai et al., 1999) were kindly provided by Kazuhisa Nakayama (Kyoto University, Japan).

## Cells and transfection

MDCK cells and their transfectants were grown in DME supplemented with 10% FCS. To obtain transfectants, the appropriate expression vectors (5-10 μg) were introduced into cells (grown to $5\times10^5$ cells per dish) using the calcium phosphate method (Ozawa et al., 1989). To determine the effects of dynamin expression on the distribution of EC81, MDCK cell clones stably expressing EC81 were transiently transfected with expression vectors for either wild-type or mutant dynamin using LipofectAmine Plus (GibcoBRL/Invitrogen) as described (Kasai et al., 1999).

## Antibodies

DECMA-1 (provided by Rolf Kemler, Max-Planck Institute for Immunobiology, Freiburg. Germany), mAb against the distinct extracellular domain of E-cadherin (ECCD-2) (Takara Bio Inc, Shiga, Japan), and mAb against the cytoplasmic domain of E-cadherin (C20820) (BD Transduction Laboratories, Lexington, KY) were used to detect E-cadherin. Rat mAb against HA (3F10) was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Pan-cadherin mAb was obtained from Sigma-Aldrich Japan (Tokyo, Japan). Mouse mAbs against β-catenin, GM130, Rab11 and EEA1 were purchased from BD Transduction Laboratories, whereas mAb against GFP were obtained from Clontech (Palo Alto, CA). Another mAb against Rab11 and one against $Na^+$,$K^+$-ATPase were obtained from Upstate (Lake Placid, NY). Mouse mAb against golgin-97 was purchased from CytoStore (Calgary, Alberta, Canada). Anti-GRP78 mAb was obtained from Stressgen Bioreagents (Ann Arbor, MI). LysoTracker Red DND-99 was obtained from Molecular Probes, Inc (Eugene, OR). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Mouse mAb against gp135 was kindly provided by George K. Ojakian (State University of New York Downstate Medical Center).

## Immunoblotting and immunoprecipitation

Immunoprecipitation and immunoblot analyses were carried out as described (Ozawa, 2002). In brief, cells ($2\times10^6$) were lysed in a buffer (25 mM Tris-HCl pH 7.4, containing 1% Triton X-100, 2 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM $Na_3VO_4$, 1 mM PMSF, 10 μg/ml leupeptin, and 25 μg/ml aprotinin). Proteins were collected with mAbs that had been preabsorbed to protein G-Sepharose.

## Fluorescence microscopy

Immunofluorescence labeling of cells was performed as described (Ohkubo and Ozawa, 1999). In brief, cells were fixed with 3% paraformaldehyde in PBS for 20 minutes at room temperature. Cells were permeabilized with 0.1% Triton X-100, and then incubated with primary and secondary antibodies. Cells were analyzed on a conventional Olympus fluorescence microscope (Tokyo, Japan) equipped with a CoolSNAP CCD camera (Nippon Roper) or a confocal laser scanning microscope (Olympus FV500).

## Apical and basolateral biotinylation

Cells ($5\times10^5$) were seeded on Transwell filters, and tight cell monolayers were incubated twice with 0.5 mg/ml sulfo-NHS-biotin (Pierce Chemical Co., Rockford, IL) in either the apical or basolateral chamber at 4°C. Cells were washed with 50 mM $NH_4Cl$ in PBS at 4°C and then stripped with 20 mM Tris-HCl (pH 8.0) containing 1% SDS, boiled for 3 minutes, passed 4-5 times through a 23-G needle and then added to nine volumes of 2% Triton X-100. Biotinylated proteins were collected using streptavidin-conjugated beads.

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