Contextual Binding of p120$^{ctn}$ to E-cadherin at the Basolateral Plasma Membrane in Polarized Epithelia*

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E-cadherin-catenin complexes mediate cell-cell adhesion on the basolateral membrane of epithelial cells. The cytoplasmic tail of E-cadherin supports multiple protein interactions, including binding of β-catenin at the C terminus and of p120$^{ctn}$ to the juxtamembrane domain. The temporal assembly and polarized trafficking of the complex or its individual components to the basolateral membrane are not fully understood. In Madin-Darby canine kidney cells at steady state and after treatment with cycloheximide or temperature blocks, E-cadherin and β-catenin localized to the Golgi complex, but p120$^{ctn}$ was found only at the basolateral plasma membrane. We previously identified a dileucine sorting motif (Leu$^{586}$/Leu$^{587}$; termed S1) in the juxtamembrane domain of E-cadherin and now show that it is required to target full-length E-cadherin to the basolateral membrane. Removal of S1 resulted in missorting of E-cadherin mutants (EcadAS1) to the apical membrane; β-catenin was simultaneously missorted and appeared at the apical membrane. p120$^{ctn}$ was not mistargeted with EcadAS1, but could be recruited to the E-cadherin-catenin complex only at the basolateral membrane. These findings help define the temporal assembly and sorting of the E-cadherin-catenin complex and show that membrane recruitment of p120$^{ctn}$ in polarized cells is contextual and confined to the basolateral membrane.

Classical cadherins are part of a family of cell-surface glycoproteins that mediate cell-cell adhesion in most solid tissues of the mammalian body (1, 2). The most extensively studied and prototypical member of the family is E-cadherin, which is required for the establishment and maintenance of cell-cell adhesion and cell polarity in epithelia and has key roles in tissue morphogenesis and tumorigenesis (3–5). E-cadherin is a single transmembrane-spanning protein that functions in adhesion as a multiprotein complex. The extracellular domain of E-cadherin binds in a homophilic Ca$^{2+}$-dependent fashion to juxtaposed E-cadherin molecules on adjacent cells. In addition, β-catenin or plakoglobin binds with high affinity to the C terminus of E-cadherin, and α-catenin binds indirectly via β-catenin to link the complex to the actin cytoskeleton (reviewed in Ref. 6). A number of other cytoplasmic proteins, including Hakai (7), Go$_{12}$ (8), and presenilin-1 (9), have also been shown to interact with E-cadherin. In addition to their roles in cell-cell adhesion, members of the catenin family are also involved in cell signaling and cell dynamics. Notably, β-catenin can alternately act as a transcriptional co-activator in the Wnt signaling pathway (reviewed in Ref. 10).

p120$^{ctn}$ is an Armadillo repeat protein originally identified as Src kinase substrate (11). p120$^{ctn}$ can interact with a number of receptor protein-tyrosine phosphatases at the plasma membrane or translocate to the nucleus, suggesting a role in regulating cell-cell adhesion and gene expression (12–15). Although a series of studies have elucidated roles for p120$^{ctn}$ in stabilizing and regulating the adhesive properties of cadherins, it has not yet been established when and where p120$^{ctn}$ and E-cadherin interact. The p120$^{ctn}$-binding site has been mapped to a core region of 15 amino acids in the juxtamembrane domain (JMD) of the E-cadherin cytoplasmic tail (16).

Delivery of E-cadherin to the basolateral surface and its incorporation into adherens junctions are key events in cell polarization and tissue morphogenesis. E-cadherin itself, or as a presumptive complex, has to be sorted and targeted to the basolateral membrane in polarized cells. Previous studies have demonstrated that β-catenin binds with high affinity to E-cadherin early in the biosynthetic pathway (17) at a core distal region of 30 amino acids (18). Further evidence suggested that this cadherin-catenin pair first forms at the level of the endoplasmic reticulum (19). E-cadherin and β-catenin are therefore structurally and functionally linked from the outset. The temporal sequence for assembly of other components of the complex is currently less clear. Several reports show that α-catenin with its attendant actin does not interact until the complex is delivered to the plasma membrane (17). A recent study now reinvigorates the issue of cadherin-catenin complex assembly by suggesting that N-cadherin binds early to p120$^{ctn}$ and at a later stage to β-catenin (20).

Recently, we described a dileucine targeting signal required for the sorting and polarized trafficking of E-cadherin chimeras to the basolateral surface in epithelial cells (21). A chimeric Tac/E-cadherin reporter molecule is sorted by utilizing the cytoplasmic tail of E-cadherin for basolateral trafficking. Mu-
tation of the so-called S1 motif in the tail results in mis-sorting and delivery to the apical membrane (21). We have now gone on to explore the sorting function of the S1 motif in the context of full-length E-cadherin. Dileucine motifs are commonly found as basolateral or sometimes endocytic sorting signals (22). Mechanically, they bind to adaptor subunits such as β-adaptin at the level of the trans-Golgi network (TGN) to sort proteins during polarized trafficking (23). High proximity of the S1 motif to the p120β−− binding site in the JMD of E-cadherin prompted us to jointly investigate sorting, polarized trafficking, and the temporal binding of p120β−− based on the assumption that steric hindrance might possibly preclude simultaneous binding of a putative sorting adaptor and p120β−−. The results presented herein suggest that the assembly and sorting of E-cadherin with catenins are indeed orchestrated in both time and space.

EXPERIMENTAL PROCEDURES

Cell Culture—Madin-Darby canine kidney (MDCK) cells (strain II) were grown and passaged as described previously (21) in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal calf serum and 2 mM l-glutamine in 5% CO2 and 95% air. For experiments, cells were plated at subconfluent density on glass coverslips or at confluent density on semipermeable polycarbonate filters (Transwell, Corning Costar, Cambridge, MA) and maintained for 1–3 days before being used. Chinese hamster ovary (CHO) cells were grown and passaged in Ham’s F-12 medium with l-glutamine and 10% fetal calf serum in 5% CO2 and 95% air. Cells were plated onto glass coverslips and dishes and maintained for 2–3 days before being used.

In some experiments, cells were incubated in medium containing 10 μM cycloheximide (Sigma) to prevent protein synthesis and to deplete proteins from the biosynthetic pathway. Some cells were incubated in CO2-independent medium (Invitrogen) at either 18 or 20 °C for 3 h prior to use. The 18 °C temperature block accumulates proteins in early or recycling endosomes (24). The 20 °C temperature culture block prevents exit of proteins from the TGN, causing accumulation of proteins within the Golgi complex (25).

Antibodies—Mouse monoclonal antibody HECD1 (provided by Dr. M. Takeichi, Kyoto University) and a rabbit polyclonal antibody raised against the extracellular domain of human E-cadherin (provided by R. G. Ali, S. Verma, and A. S. Y., University of Queensland) were used to detect human E-cadherin. A mouse monoclonal antibody that recognizes dog E-cadherin (3B8, obtained from Dr. W. Gallin, University of Alberta) was used in experiments using MDCK cells. Other primary antibodies used were a mouse anti-human p120β−− monoclonal antibody and an anti-p290 polyclonal antibody (Transduction Laboratories, Lexington, KY) and rabbit polyclonal antibodies raised against catenin (Sigma) and green fluorescent protein (GFP, Molecular Probes, Inc., Eugene, OR). Secondary antibodies used included Cy3-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes, Inc.), and horse radish peroxidase-conjugated sheep anti-mouse IgG and horseradish peroxidase-labeled goat anti-rabbit IgG (Amrad, Victoria, Australia). Alexa 488 and Texas Red-conjugated phalloidin were used to stain F-actin (Molecular Probes, Inc.).

Vector Construction—The molecular cloning techniques used were from Sambrook et al. (26), whereas all reagents were obtained from New England Biolabs Inc. (Beverly, MA). A human E-cadherin cDNA in the pcDNA1 expression vector has been described previously (27). The S1 dileucine mutant E-cadherin was created by replacing the extracellular and transmembrane regions of pCMV-Tac/Ecad (578–728)ΔS1 (21) with the corresponding region of E-cadherin (residues 1–577). The extracellular and transmembrane regions of E-cadherin were amplified by PCR with specific primers from pcDNA-Ecad-GFP (21). The oligonucleotides contained restriction enzyme sites generated in-frame fusions with the dileucine-mutated cytoplasmic domain present in pCMV-Tac/Ecad (578–728)ΔS1. Cloning the PCR product into pcDNA-Tac/Ecad (578–728)ΔS1 using NheI and HindIII sites generated pcDNA-EcadΔS1, which was confirmed by DNA sequencing. The EcadΔS1 cDNA was then subcloned into the pcDNA vector to produce the resistance gene construct pcDNA-Ecad-GFP (21) containing a GFP tag fused in-frame to the C terminus of the E-cadherin cytoplasmic domain. pcDNA-Ecad-GFP was digested with KpnI (unique site 5′ of S1) and XbaI (unique site at the end of GFP), releasing E-cadherin cytoplasmic and GFP cDNAs. pcCMV-EcadΔS1 was also digested with KpnI and XbaI, releasing an equivalent fragment minus GFP, but containing the ΔS1 mutation. This ΔS1 fragment was then ligated into the KpnI- and XbaI-digested pcDNA-Ecad-GFP plasmid, creating pcDNA-EcadΔS1. Next, a GFP tag was fused to the cytoplasmic tail of pCMV-EcadΔS1. pcDNA-Ecad-GFP was digested with SgrA1 and XbaI, releasing GFP cDNA. This was cloned into pCMV-EcadΔS1 digested with the same enzymes, creating pCMV-EcadΔS1-GFP. The p120β−− plasmid (GFP-tagged p120β−−, pEGFP-C1/mtx1A) (28) was generously provided by Dr. Albert Reynolds (Vanderbilt University).

Vector Transfection and Expression—MDCK or CHO cells were plated at subconfluent density 24 h before transfection. The LipofectAMINE™ Plus system in combination with Opti-MEM (both from Invitrogen) was used for transfections according to manufacturer’s guidelines. Cells were typically used 24–48 h later, in some cases after reseeding on filters at confluent density. Stably expressing cell lines were generated as described previously (21).

Indirect Immunofluorescence—Cells grown on filters or glass coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and then permeabilized in 0.1% Triton X-100 for 5 min and stained as described previously (21). Cells were viewed using an Olympus Provis AX-70 microscope, and images were captured on a CCD300ET-RCX camera (Dage-MTI, Inc., Michigan City, IN) using NIH Image software or a Bio-Rad Radiance 2000 confocal system (Bio-Rad, Hercules, CA) mounted on a Nikon E600 microscope. All confocal hardware was driven by a workstation running LaserSharp 2000 imaging software (Bio-Rad). Images were collected using a ×60 objective (numerical aperture of 1.4), giving an optimal section depth of 0.6 μm. Cy3-conjugated secondary antibodies were excited using a 543-nm helium/neon laser, whereas Alexa-488-conjugated secondary antibodies were excited with a 488-nm argon laser. Post-capture image analysis and processing of confocal image stacks were performed using the LaserVox software package (Bio-Rad).

Immunoprecipitation and Immunoblotting—Cells were solubilized in cold precipitation buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100) containing Complete™ protease inhibitors (Roche Applied Science, Mannheim, Germany) on ice and extracted for 30 min at 4 °C. Post-nuclear supernatants were obtained by centrifugation at 17,000 × g for 10 min. For immunoprecipitation, extracts were precleared with protein G or protein A for 30 min, followed by incubation with anti-human E-cadherin antibody complexed to UltraLink-immobilized protein G (Pierce) or anti-GFP antibody complexed to protein A (Pierce) for 2 h. Precipitates were recovered by centrifugation and then washed several times with cold precipitation buffer and with 10 mM Tris-HCl (pH 7.4) prior to solubilization in concentrated SDS-PAGE sample buffer. Immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting with Supersignal West Pico (Pierce) as described previously (21).

RESULTS

E-cadherin Complexes with β-Catenin and p120β−− at the Lateral Plasma Membrane in MDCK Cells—E-cadherin, β-catenin, and p120β−− are known members of cadherin-catenin complexes at adherens junctions in polarized epithelia (6). Con-focal microscopy of MDCK cell monolayers confirmed the co-location of all three molecules on lateral cell membranes in these cells (Fig. 1A). Antibodies to E-cadherin co-immunoprecipitated both β-catenin and p120β−− (Fig. 1B), confirming that, at steady state, all three proteins form a stable complex at adherens junctions.

E-cadherin-GFP and β-Catenin, but Not p120β−−, Are Found on Intracellular Membranes—In MDCK cells, transiently expressed GFP-tagged E-cadherin (Ecad-GFP) (21) produced the same staining pattern as endogenous E-cadherin; it was found predominantly at the basolateral membrane, and newly synthesized Ecad-GFP was also seen in a perinuclear Golgi compartment (Fig. 2A). Co-localization with the TGN marker p230 confirmed the Golgi localization of the intracellular pool of Ecad-GFP. Both endogenous β-catenin and p120β−− co-localized with Ecad-GFP at the basolateral cell surface (Fig. 2A). Co-staining of β-catenin with Ecad-GFP also occurred at the level of the Golgi complex, consistent with previous studies showing that β-catenin associates with E-cadherin early in the biosynthetic pathway (19). In contrast, we noted that p120β−− staining...
sites.

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in confluent cells, the immunolabeling process, we performed experiments

transiently transfected with Ecad-GFP and co-immunolabeled for p230,

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co-localized with p120GFP at the lateral membrane; however, there was no co-staining of p120GFP on intracellular membranes. Notably, p120GFP-transfected cells showed increased E-cadherin staining on lateral membranes, an observation consistent with the demonstration that increased p120ctn levels lead to increased stabilization of E-cadherin at the cell surface (30). Thus, in MDCK cells at steady state, β-catenin and Ecad-GFP appear together in the Golgi complex, but p120ctn is found with Ecad-GFP only at the plasma membrane.

To further investigate whether endogenous p120ctn staining ever appears in the biosynthetic pathway, we treated cells with cycloheximide to deplete all newly synthesized proteins from the biosynthetic pathway and then analyzed staining patterns after washout of the drug, when newly synthesized proteins reappear inside cells (Fig. 3A). Immediately after incubation with cycloheximide, there was a significant decrease in E-cadherin plasma membrane staining and a complete disappearance of any intracellular staining; the cells also became flattened and more irregular in shape (Fig. 3A, 0hr). This corresponded to diminished p120ctn plasma membrane staining and increased cytoplasmic staining. Two hours after washout of the cycloheximide, E-cadherin staining reappeared in the Golgi complex as determined by co-localization with β-COP (data not shown). By 4 h, there was prominent Golgi and vesicular staining, representing E-cadherin accumulation in secretory or endocytic compartments (Fig. 3A, 2hr and 4hr). Throughout this recovery, staining of E-cadherin on the plasma membrane also increased steadily, as did staining of p120ctn, which increased on the plasma membrane while decreasing in the cytoplasm. At no time did p120ctn staining appear on intracellular membranes other than on the plasma membrane. These results suggest that p120ctn does not appear in biosynthetic compartments on its way to the cell surface, whereas E-cadherin complexes can be stained in transit through the Golgi complex and secretory pathway. The staining in these cells suggests that p120ctn is translocated directly to the plasma membrane from the cytoplasm.

As another approach to investigate whether p120ctn can be found on intracellular compartments, we used temperature blocks to accumulate E-cadherin in the TGN or in endosomes. Cells subjected to a 20 °C temperature block for 3 h typically accumulate newly synthesized proteins in the TGN (25). In keeping with this, E-cadherin staining of the perinuclear Golgi region was increased in cells incubated at 20 °C (Fig. 3B). Under the same conditions, there was no staining of p120ctn in the perinuclear area. We have previously shown that recycling

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To rule out the possibility that p120ctn was not detected at intracellular sites because of epitope masking or any deficiencies of the immunolabeling process, we performed experiments on cells overexpressing p120GFP (28). In confluent cells, p120GFP localized to the lateral plasma membrane; it was not on intracellular membranes and appeared as faint diffuse staining only in the cytoplasm (Fig. 2B). Endogenous E-cadherin co-localized with p120GFP at the lateral membrane; however, there was no co-staining of p120GFP on intracellular membranes. Notably, p120GFP-transfected cells showed increased E-cadherin staining on lateral membranes, an observation consistent with the demonstration that increased p120ctn levels lead to increased stabilization of E-cadherin at the cell surface (30). Thus, in MDCK cells at steady state, β-catenin and Ecad-GFP appear together in the Golgi complex, but p120ctn is found with Ecad-GFP only at the plasma membrane.

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Cadherin-Catenin Binding and Basolateral Targeting

Fig. 3. Staining of E-cadherin in the biosynthetic pathway: lack of p120\textsuperscript{ctn} co-localization. A, MDCK cells were incubated with cycloheximide for 12 hr to deplete E-cadherin from the biosynthetic pathway and the cell surface. Cycloheximide was washed out, and cells were returned to normal medium for various times before being fixed. Cells were then immunostained for E-cadherin (antibody 3B8) or p120\textsuperscript{ctn}. In cycloheximide-treated cells, staining of both proteins appeared only on the plasma membrane. At 2 and 4 hr of recovery, E-cadherin staining reappeared inside cells as Golgi staining (arrowhead), and vesicular p120\textsuperscript{ctn} staining still appeared only on the plasma membrane. B, MDCK cells were incubated at 20°C to block exit from the TGN or at 18°C in the presence of cycloheximide (CHX) to accumulate proteins in endosomes. Cells were fixed and labeled for either E-cadherin (antibody 3B8) or p120\textsuperscript{ctn}. Under both conditions, E-cadherin accumulated in intracellular compartments, particularly the Golgi complex (arrow); but p120\textsuperscript{ctn} did not. p120\textsuperscript{ctn} remained as diffuse cytoplasmic or plasma membrane staining.

Surface E-cadherin is accumulated in endosomes in cells treated with cycloheximide (to deplete biosynthetic proteins) and then incubated at 18°C for 3 hr (31). This treatment resulted in enhanced vesicular endosomal staining of E-cadherin, but not of p120\textsuperscript{ctn} (Fig. 3B). This staining provides further evidence to suggest that p120\textsuperscript{ctn} does not co-accumulate with endogenous E-cadherin complexes in the biosynthetic pathway or in endosomes. In all of these experiments in polarized cells, the only membrane domain that p120\textsuperscript{ctn} localized to was the lateral plasma membrane, regardless of the presence of E-cadherin complexes on other membranes.

Cadherin Binding and Membrane Recruitment of p120\textsuperscript{ctn}—The association of the E-cadherin complex with p120\textsuperscript{ctn} was then investigated using transfected cells expressing Ecad-GFP and a targeting mutant of E-cadherin. We have previously identified the dileucine motif (S1) as a basolateral sorting signal required for targeting of an E-cadherin chimera, Tac/Ecad\textsuperscript{(574–724)AS1}, consisting of the extracellular and transmembrane domains of Tac (interleukin-2 receptor α-subunit) fused to the cytoplasmic tail of E-cadherin (21). This S1 mutation (Leu\textsuperscript{586}–Leu\textsuperscript{587} mutated to alanines) was now introduced into full-length human E-cadherin cDNA, creating EcadΔS1, and into GFP-tagged E-cadherin, creating EcadΔS1-GFP (Fig. 4A), which were used to probe the sorting of E-cadherin and its association with catenins; this is also of interest since the S1 motif is closely adjacent (within 15 amino acids) to the p120\textsuperscript{ctn} binding site in the JMD of E-cadherin (16).

CHO cells endogenously express p120\textsuperscript{ctn}, but not E-cadherin (32). Transient expression of Ecad-GFP or Ecad\textsuperscript{ctn} in CHO cells produced plasma membrane staining and resulted in the recruitment of p120\textsuperscript{ctn} and the expression and recruitment of β-catenin to the plasma membrane (Fig. 4B). Transient expression of ΔS1 targeting mutants in the context of full-length E-cadherin (EcadΔS1-GFP) or a chimera (Tac/EcadΔS1) (data not shown) also successfully recruited p120\textsuperscript{ctn} and β-catenin to the plasma membrane, showing that both catenins can interact successfully with the targeting mutants. Immunoprecipitation was used to demonstrate directly that p120\textsuperscript{ctn} can bind to the tail of both wild-type Ecad-GFP and EcadΔS1-GFP (Fig. 4C). An anti-GFP antibody was used to immunoprecipitate protein complexes from extracts of cells transfected with GFP alone, with Ecad-GFP, or with EcadΔS1-GFP. p120\textsuperscript{ctn} coprecipitated efficiently with both human Ecad-GFP and EcadΔS1-GFP. Thus, mutation of the S1 motif does not affect the binding of p120\textsuperscript{ctn} to the E-cadherin tail, providing further definition of the E-cadherin p120\textsuperscript{ctn}-binding site and allowing for speculation about the nature of interactions between E-cadherin and sorting adaptors or p120\textsuperscript{ctn}.

Targeting of E-cadherin and ΔS1 Mutants in Polarized Cells—To study a possible relationship between sorting and p120\textsuperscript{ctn} binding, we constructed and expressed full-length E-cadherin with the ΔS1 mutation. Overexpressed E-cadherin is faithfully sorted and targeted to the basolateral membrane of polarized MDCK cells. This is shown here in cells overexpressing untagged human E-cadherin, which could be detected with a human-specific antibody (Fig. 5), but is also true of Ecad-GFP (21). In contrast, EcadΔS1-GFP or EcadΔS1 expressed in polarized MDCK cells was mistargeted and could be stained on both the apical and basolateral membrane domains (Fig. 5). Missorting of these ΔS1 mutants is not a result of excessive overexpression, as wild-type Ecad-GFP and EcadΔS1-GFP, p120\textsuperscript{ctn} were co-labeled with both human and canine-specific antibodies to detect recombinant and endogenous E-cadherin, respectively (Fig. 6). Immunofluorescence labeling and confocal analysis showed there was no missorting of endogenous E-cadherin; however, trans-
fected cells did have reduced membrane staining of endogenous E-cadherin on the lateral membrane, suggesting that there is competition for membrane binding at this site or compensatory changes in the amount of endogenous E-cadherin synthesized in transfected cells. Wild-type E-cadherin was not missorted along with Ecad/H9004S1, making it unlikely that the two molecules dimerize, suggesting that they are handled separately by the sorting machinery or that the S1 sorting signal is dominant.

**Expression and Mistargeting of Ecad/H9004S1 Change Epithelial Cell Morphology**—Transfected MDCK cells were put under antibiotic selection to produce cell lines stably expressing Ecad/H9004S1 or Ecad-GFP. By immunostaining, the resulting cell lines still showed heterogeneous expression of recombinant proteins. Groups of cells expressing detectable levels of EcadS1 or Ecad-GFP were stained with the anti-human E-cadherin antibody. Cells overexpressing wild-type Ecad-GFP grew as typical cobblestone monolayers, with individual cells maintaining their shape and polarized phenotype. Cells overexpressing EcadS1 showed a markedly altered morphology, taking on a spindle shape and extending cellular processes consistent with a loss of cell polarity and epithelial organization. In a double-blind analysis, cells overexpressing EcadS1 were recognized as having distinctly altered morphology (data not shown). This change in morphology is shown in Fig. 7b, where we noted that even cells adjacent to the highly expressing stained cells also exhibited altered morphology. Cell lines expressing EcadS1 also grew noticeably slower than the parent MDCK cells or Ecad-GFP-expressing cells. Expression of missorted Ecad/H9004S1 on the apical cell surface disrupted cell polarity and epithelial morphology, showing that correct sorting of E-cadherin is essential for these processes.

β-Catenin, but Not p120ctn, Is Mistargeted to the Apical Membrane with EcadΔS1—MDCK cells transiently expressing human E-cadherin or EcadS1 were co-stained to detect β-catenin or p120<sup>ctn</sup>. Introduction of EcadS1 resulted in missorting of β-catenin, which was then found by confocal imaging on the apical membrane, along with EcadS1 (Fig. 8A). The correct basolateral targeting of β-catenin, as part of a preformed cadherin-catenin complex, was also dependent on the S1 sorting signal. This provides a new line of evidence to suggest that...
confirmed that Ecad\text/H9004 the apical membrane, despite the findings in Fig. 6, which p120, a biosynthetic pathway. /H9004 labeled to localize Ecad with mistargeted Ecad remaining localized at the basolateral membrane even in cells /H9252 focal at the cell boundaries of the lateral membrane (cultures. Endogenous E-cadherin labeled with antibody 3B8 was found and recombinant E-cadherins. a either E-cadherin or Ecad MDCK cells were transiently transfected with cadherin localization. /H9004 in transiently transfected MDCK cells. The immunofluorescence labeling of endogenous E-cadherin or recombinant proteins in transiently transfected MDCK cells. a–d show confocal XY cross-sections at various levels of MDCK monolayers grown on filters, whereas a’–d’ represent XZ sections of other areas of the same cultures. Endogenous E-cadherin labeled with antibody 3B8 was found at the cell boundaries of the lateral membrane (a and a’), as was transfected E-cadherin stained with HEC1D1 (b and b’). In contrast, EcadS1 (antibody HEC1D1) was stained on the apical membrane, in addition to being labeled on the basal membrane (c and c’). GFP fluorescence shows both apical and basolateral EcadS1-GFP (d and d’).

**FIG. 6.** Ecad\text/S1 expression does not disrupt endogenous E-cadherin localization. MDCK cells were transiently transfected with either E-cadherin or Ecad\text/S1 and co-labeled to detect both endogenous and recombinant E-cadherins. a–d show confocal XY sections at various levels, whereas the insets show XZ sections of similar regions of each sample. Recombinant human E-cadherin co-localized at the basolateral membrane with endogenous canine E-cadherin (a and b). Ecad\text/S1 co-localized with endogenous E-cadherin only at the basolateral membrane (c and d); no endogenous E-cadherin was missorted to the apical membrane with Ecad\text/S1 (see insets).

\beta-catenin interacts with E-cadherin prior to sorting, early in the biosynthetic pathway.

The same result was not found when cells were double-labeled to localize Ecad\text/S1 and endogenous p120ctn. p120ctn remained localized at the basolateral membrane even in cells with mistargeted Ecad\text/S1 (Fig. 6B). p120ctn never appeared at the apical membrane, despite the findings in Fig. 6, which confirmed that Ecad\text/S1 has the ability to interact with p120ctn. We therefore conclude that p120ctn targeting to the basolateral membrane is not dependent on the Ecad\text/S1 motif. p120ctn arrives independently at the lateral cell surface, or it may bind to the basolaterally destined cadherin-catenin complex after sorting has occurred. Most importantly, the recruitment of p120ctn to the plasma membrane in polarized cells can happen only in the context of the basolateral plasma membrane.

**DISCUSSION**

The cadherin-catenin complex is a large multiprotein unit that functions at adherens junctions to mediate cell-cell adhesion. The cytoplasmic tail of E-cadherin supports many protein interactions necessary for assembly of the complex, for its association with the actin cytoskeleton, and for modulating adhesive function and signaling. Just where and when individual components join the cadherin-catenin complex have profound implications for its function at adherens junctions and for its trafficking to and from the plasma membrane. In this study, we used several approaches to explore the association of \beta-catenin and p120ctn with E-cadherin, and we have shown that they bind in a temporal fashion to E-cadherin, before and after delivery to the cell surface, respectively. Importantly, we found that the sorting and basolateral targeting of the E-cadherin-\beta-catenin complex is mediated by the S1 motif of E-cadherin. S1-mediated sorting is necessary for (but precedes) the binding of p120ctn, which does not occur until at or near the basolateral plasma membrane. Several pieces of evidence support this conclusion, including the lack of intracellular membrane localization of endogenous p120ctn or p120GFP at steady state and then after manipulations to highlight or accumulate E-cadherin inside cells. Finally, the finding that it can bind to, but is not missorted by, Ecad\text/S1 shows that p120ctn recruitment is an obligate process of the basolateral membrane environment.

The localization of endogenous E-cadherin and overexpressed untagged human E-cadherin or Ecad-GFP was consistent in all our experiments with synthesis and trafficking through the biosynthetic pathway and polarized delivery to the basolateral plasma membrane. At steady state, \beta-catenin...
staining coincided with that of cell-surface or newly synthesized E-cadherin or Ecad-GFP on the basolateral membrane and in the perinuclear Golgi compartment, suggesting that it, too, is jointly trafficked via the biosynthetic pathway. Our results concur with those derived from pulse-chase labeling and immunoprecipitation experiments in previous studies, which established that β-catenin binds to a C-terminal domain of E-cadherin soon after biosynthesis (17, 19, 34), forming the nexus of a complex that is trafficked to the cell surface. Early binding of β-catenin was deemed necessary for the efficient endoplasmic reticulum exit and processing of some overexpressed E-cadherin chimeras (19); although more recently, using different chimeras, we found that, although β-catenin may facilitate processing of E-cadherin, it is not essential for its trafficking (21). Expression of the E-cadherin targeting mutant EcadΔS1 and the conjoint mistargeting of β-catenin to the apical cell surface now further demonstrate that the two molecules are trafficked together. The joint targeting and efficient binding of β-catenin, shown by membrane recruitment and immunoprecipitations (data not shown), with EcadΔS1 or with EcadΔS1-GFP serve to verify that the cytoplasmic domains of both recombinant proteins are correctly folded. The polarized trafficking of β-catenin, a cytoplasmic protein, is thus wholly dependent on its association with E-cadherin, which provides the necessary sorting signal in the form of the S1 dileucine motif. This is a prime example of how a single sorting signal dictates the polarized trafficking of multiple complexed proteins, and it provides further insight into one of the many possible fates of β-catenin in epithelial cells.

p120<sup>ctn</sup> is another cytoplasmic catenin that binds to E-cadherin, but that appears to function as part of cadherin-catenin complexes only at the plasma membrane, rather than at intracellular sites. The reported roles of p120<sup>ctn</sup> focus mainly on its positive or negative modulation of adhesive strength and on stabilization of surface E-cadherin (reviewed in Ref. 35). Indeed, our current evidence, together with previous studies, indicate that p120<sup>ctn</sup> is only loosely associated with E-cadherin while it is at the cell surface and that it dissociates from the cadherin-catenin complex during trafficking to and from the plasma membrane. For instance, surface E-cadherin induced to internalize by Hakai binding and ubiquitination does so at the expense of p120<sup>ctn</sup> binding (7). In our current study, although β-catenin staining was frequently seen in association with E-cadherin at the level of the Golgi complex, similar co-staining of p120<sup>ctn</sup> was not found, prompting the further analysis of p120<sup>ctn</sup> localization during experimental manipulations of the biosynthetic traffic. De novo synthesized E-cadherin was localized in the perinuclear Golgi complex and in vesicles in cells pretreated with cycloheximide, without any similar staining of p120<sup>ctn</sup>. Under these conditions, p120<sup>ctn</sup> clearly translocated between diffuse cytoplasmic staining and lateral plasma membrane staining, implying that it moves to the cell surface directly from the cytoplasm. p120<sup>ctn</sup> could not be accumulated along with E-cadherin in the TGN at 20 or 18 °C in endosomes, further suggesting that it is not associated with intracellular compartments.

E-cadherin expression was previously found to be necessary and sufficient for plasma membrane recruitment of p120<sup>ctn</sup> (16). Expression of Ecad-GFP or EcadΔS1-GFP in CHO cells produced the same result, efficiently recruiting endogenous p120<sup>ctn</sup> to the cell surface. However, in polarized MDCK cells, expression of E-cadherin randomly on the cell surface was not sufficient to recruit p120<sup>ctn</sup> to the membrane. Instead, p120<sup>ctn</sup> was recruited only by E-cadherin that was targeted to the lateral cell surface. In MDCK cells overexpressing EcadΔS1, p120<sup>ctn</sup> was not missorted or recruited to the apical membrane, but bound only to the lateral cell membrane. This finding is clear in showing that the membrane association of p120<sup>ctn</sup> is highly regulated and occurs only in the presence of E-cadherin within a defined polarized subdomain of the plasma membrane. One of two scenarios could produce this effect: first, a cofactor or modification of p120<sup>ctn</sup> required for its recruitment may be present at the lateral membrane, but not at the apical membrane; or second, p120<sup>ctn</sup> is actually tethered to or constrained at the lateral membrane. Further studies will now have to address these issues. Recently, members of the p120<sup>ctn</sup> family of Armadillo proteins (other than p120<sup>ctn</sup> itself) such as p0071 (plakophilin-4), δ-catenin, and ARVFC have been shown to have PDZ-binding domains, which can mediate interaction with the LAP proteins such as ERBIN (36, 37). ERBIN localizes to the lateral membrane in MDCK cells and may have a role in promoting the final assembly of cadherin-catenin complexes at

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**Fig. 8. β-Catenin (but not p120<sup>ctn</sup>) is sorted to the apical membrane in EcadΔS1-expressing cells.** Shown is the immunofluorescence labeling of MDCK cells transiently overexpressing EcadΔS1 (panels a–f) or E-cadherin (panels g–l). Recombinant proteins were co-labeled with β-catenin (A) or p120<sup>ctn</sup> (B). Panels show confocal XY sections taken at apical (AP), lateral (LT), and basal (BA) levels of monolayers grown on filters. There was basolateral membrane staining of all proteins. In cells overexpressing EcadΔS1, β-catenin staining was found on the apical membrane along with EcadΔS1 (A, panels a–d), but not in cells overexpressing E-cadherin (A, panels g–l). In EcadΔS1-overexpressing cells and in E-cadherin-expressing cells, there was no apical staining of p120<sup>ctn</sup> (B, a–d and g–l).
this location (38). Finally, confining p120coren to the lateral domain in epithelial cells may be uniquely important for controlling its function in the context of polarized cells.

A recent study (20) on the complexing of catenins to N-cadherin found temporal relationships among N-cadherin, β-catenin, and p120coren quite different from those emerging from our current data and previous studies on E-cadherin. β-Catenin was found to bind later in the biosynthetic pathway after phosphorylation of pro-N-cadherin, whereas p120coren bound to the cadherin both before and after these modifications (20). These conflicting results might reflect differences in the formation of cadherin-catelin complexes stemming from the different cadherin molecules themselves or from their expression and function in epithelial versus non-epithelial cells.

The JMD of E-cadherin supports multiple functions associated with adhesive strength and cadherin clustering (39) and multiple protein interactions, including those of p120coren (16), Hakai (7), and presenilin-1 (9). In addition, by showing that the disruption binding of p120coren to E-cadherin undergoes three major sorting events in its trafficking life. First, in the biosynthetic pathway, E-cadherin is sorted in the TGN for transport directly to the basolateral membrane (40). Second, from there it can be endocytosed with several possible fates, one of which is a third sorting event for recycling back to the plasma membrane (31, 41). Confocal analysis over a time course beginning soon after transfection showed that newly synthesized EcadS1 is transported directly, separately, and simultaneously from the TGN to the apical membrane and to the basolateral membrane (data not shown); and thus, unlike some other membrane proteins in MDCK cells (e.g., the polymeric IgA receptor) (42), there is no transcytosis of newly synthesized Ecad-GFP or of EcadS1. Thus, S1-dependent sorting for basolateral trafficking occurs in the TGN, which is now further supported by the lack of p120coren missorting to date, diencephalon-based signals have been found to bind to γ-adaptin (43); μ1, μ2, and μ3 adapter subunits (44); and to β-adaptin (25). The sorting adaptor responsible for binding to S1 in the TGN has yet to be identified.

As expected, mutation of S1 in the context of full-length E-cadherin resulted in missorting to the apical membrane; and in addition, there was also basal and lateral localization of EcadS1. This is not a surprising result, as other diencephalon-dependent basolateral proteins are similarly distributed when their diencephalon signals are removed. For example, mutation of the diencephalon sorting signals in both the basal cell adhesion molecule and nucleotide pyrophosphatase-1 results in 70% apical and 30% basolateral distributions (45, 46). This non-polarized trafficking could be a result of overloading and spillover from the apical into the basolateral pathways, piggybacking of mutant proteins to other basolateral proteins, the presence of other basolateral signals, or translocation from apical to basolateral membranes. Our data generally discount most of these possibilities, leaving general, bulk, default, or unsorted membrane flow to all areas of the cell as the likely explanation for the presence of EcadS1 in both membrane domains. The mis-sorting of EcadS1 had striking effects on the morphology and growth of epithelial cells, more so in fact than equivalent over-expression of wild-type Ecad-GFP. We would predict that EcadS1 is adhesion-competent, to some degree, when expressed on the apical membrane; and its participation in aberrant homophilic cadherin interactions or in cadherin-based cell motility could account for the loss of cell polarity and epithelial organization. These morphological changes highlight the importance of S1 function in the patently polarized targeting of E-cadherin and suggest that S1-dependent sorting may be particularly critical for epithelial patterning and morphogenesis during development. Our data now show another important consequence of polarity and that is the correct and complete assembly of the full E-cadherin-catelin complex including p120coren.

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Cadherin-Catenin Binding and Basolateral Targeting