A Dileucine Motif Targets E-cadherin to the Basolateral Cell Surface in Madin-Darby Canine Kidney and LLC-PK1 Epithelial Cells*

Received for publication, March 2, 2001, and in revised form, April 11, 2001
Published, JBC Papers in Press, April 18, 2001, DOI: 10.1074/jbc.M101907200

Kevin C. Miranda‡, Tatiana Khromykh‡, Perpetina Christy‡, Tam Luân Le‡, Cara J. Gottardi‡, Alpha S. Yap‡, Jennifer L. Stow‡, and Rohan D. Teasdale‡

From the ‡Institute for Molecular Bioscience, the §Department of Biochemistry, and the ¶Department of Physiology & Pharmacology, University of Queensland, Brisbane, Queensland 4072, Australia and the ¶Memorial Sloan-Kettering Cancer Center, New York, New York 10021

E-cadherin is a major adherens junction protein of epithelial cells, with a central role in cell-cell adhesion and cell polarity. Newly synthesized E-cadherin is targeted to the basolateral cell surface. We analyzed targeting information in the cytoplasmic tail of E-cadherin by utilizing chimeras of E-cadherin fused to the ectodomain of the interleukin-2α (IL-2α) receptor expressed in Madin-Darby canine kidney and LLC-PK1 epithelial cells. Chimeras containing the full-length or membrane-proximal half of the E-cadherin cytoplasmic tail were correctly targeted to the basolateral domain. Sequence analysis of the membrane-proximal tail region revealed the presence of a highly conserved dileucine motif, which was analyzed as a putative targeting signal by mutagenesis. Elimination of this motif resulted in the loss of Tac/E-cadherin basolateral localization, pinpointing this dileucine signal as being both necessary and sufficient for basolateral targeting of E-cadherin. Truncation mutants unable to bind β-catenin were correctly targeted, showing, contrary to current understanding, that β-catenin is not required for basolateral trafficking. Our results also provide evidence that dileucine-mediated targeting is maintained in LLC-PK1 cells despite the altered polarity of basolateral proteins with tyrosine-based signals in this cell line. These results provide the first direct insights into how E-cadherin is targeted to the basolateral membrane.

E-cadherin is expressed on the lateral membranes of epithelial cells where it accumulates as a major component of the adherens junction. The cadherins in adherens junctions have central roles in establishing and maintaining cell-cell adhesion and cell polarity in epithelia and participate in morphogenesis during development (1–4). The continual expression and function of E-cadherin is important in its role as a tumor suppressor during development (1–4). The continual expression and function of E-cadherin is important in its role as a tumor suppressor during development (1–4).

The continual expression and function of E-cadherin is important in its role as a tumor suppressor during development (1–4).

E-cadherin is expressed on the lateral membranes of epithelial cells where it accumulates as a major component of the adherens junction. The cadherins in adherens junctions have central roles in establishing and maintaining cell-cell adhesion and cell polarity in epithelia and participate in morphogenesis during development (1–4). The continual expression and function of E-cadherin is important in its role as a tumor suppressor during development (1–4).

The continual expression and function of E-cadherin is important in its role as a tumor suppressor during development (1–4).
with tyrosine-based signals, a defect that can be overcome by expression of recombinant μ1B protein (30). Cadherin staining in LLC-PK1 cells appears to be basolateral (31), although the trafficking of these proteins in this cell line has not been studied in detail.

The correct placement of E-cadherin on the plasma membrane is required from an early stage to help establish and maintain cell polarity (32). The mechanisms that mediate the sorting and polarized delivery of E-cadherin to the surface have not been elucidated. Previous studies have shown that β-catenin binds to E-cadherin early in the biosynthetic pathway, implying that the two proteins, and perhaps others, are transported to the cell surface together as a complex (33). More recently, it was suggested that β-catenin plays an essential role in the trafficking of E-cadherin, based on observations that mutated proteins, with reduced binding to β-catenin, were not efficiently delivered to the surface (34). It has also been previously noted that the cytoplasmic tail of E-cadherin does contain motifs with homology to known targeting signals (34, 35), which could potentially function to guide its trafficking. In this study we set out to test putative signals in the cytoplasmic tail of E-cadherin for possible basolateral targeting information. Our experiments also addressed the role of β-catenin in this targeting. We utilized a series of chimeras to express the E-cadherin cytoplasmic tail, or mutated versions thereof, using Tac (the α subunit of the IL-2 receptor) as an ectodomain marker. Tac is a 273-amino acid protein that has previously been used as a reporter protein for trafficking studies (36, 37).

These constructs were expressed in MDCK cells, in which the basolateral surface expression of E-cadherin is well established, and in another epithelial cell line LLC-PK1 cells. Using these model systems we have identified a positive targeting signal in E-cadherin that is responsible for basolateral delivery. Our results provide new insights into the trafficking of E-cadherin and its accessory proteins (catenins), and the findings are also significant to our understanding of cell polarity and sorting pathways in epithelia.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Madin-Darby canine kidney (MDCK) and pig kidney (LLC-PK1) cell lines were grown and passaged as described previously (38) in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal calf serum and 2 mM glutamine in 5% CO2 and 95% air.

**Antibodies**—Mouse monoclonal antibodies (Transduction Laboratories, Lexington, KY) raised against a conserved region of the cytoplasmic domain of human E-cadherin and against β-catenin were used; Tac was recognized using a mouse monoclonal antibody (B-10, BIO-SOURCE International, Camarillo, CA) and a rabbit polyclonal antibody directed against the green fluorescence protein (GFP) (Molecular Probes Inc., Eugene, OR) was also used for staining. Secondary antibodies included Cy3-conjugated sheep anti-mouse and goat anti-rabbit IgGs (Jackson ImmunoResearch Labs, West Grove, PA) and a horseradish peroxidase-conjugated sheep anti-mouse IgG (AMRAD, Victoria, Australia).

**cDNA Construction and Expression**—Chimeric fusions between human E-cadherin cDNAs and the cDNA encoding for Tac were generated in the pCDNA3 expression vector. Molecular cloning techniques were performed according to Sambrook et al. (39), using reagents from New England BioLabs (Beverly, MA). All constructs were confirmed by DNA sequencing. cDNAs encoding the transmembrane plus cytoplasmic domains (residues 554–728), the cytoplasmic tail (residues 578–728), or the amino-terminal half of the cytoplasmic tail (578–653) of human E-cadherin were amplified using PCR with specific oligonucleotide primers. E-cadherin residue numbers correspond to the mature protein as defined previously (6). The oligonucleotide primers contained restriction endonuclease sites allowing generation of in-frame fusions with the Tac cDNA. Cloning the respective PCR products into pCMV-IL2R (40) using the HindIII and XbaI sites generated the pCMV-Tac/Ecad(578–728) and pCMV-Tac/Ecad(578–653) plasmids. These were introduced at the carboxyl terminus of the full-length cDNA of E-cadherin. This was achieved by the PCR amplification of the entire E-cadherin cDNA using specific oligonucleotide primers using pCDNA3-hEcad (41) as a template. The 3′-primer included the SacII site and a XhoI site. The resulting PCR product was digested with SacI and XbaI and subcloned into pCDNA3-hEcad using the same SacII and XhoI sites. The resulting construct pCMV-Tac/Ecad was used as a template and mutagenized proteins, with reduced binding to β-catenin, were not efficiently delivered to the surface (34). It has also been previously noted that the cytoplasmic tail of E-cadherin does contain motifs with homology to known targeting signals (34, 35), which could potentially function to guide its trafficking. In this study we set out to test putative signals in the cytoplasmic tail of E-cadherin for possible basolateral targeting information. Our experiments also addressed the role of β-catenin in this targeting. We utilized a series of chimeras to express the E-cadherin cytoplasmic tail, or mutated versions thereof, using Tac (the α subunit of the IL-2 receptor) as an ectodomain marker. Tac is a 273-amino acid protein that has previously been used as a reporter protein for trafficking studies (36, 37).

These constructs were expressed in MDCK cells, in which the basolateral surface expression of E-cadherin is well established, and in another epithelial cell line LLC-PK1 cells. Using these model systems we have identified a positive targeting signal in E-cadherin that is responsible for basolateral delivery. Our results provide new insights into the trafficking of E-cadherin and its accessory proteins (catenins), and the findings are also significant to our understanding of cell polarity and sorting pathways in epithelia.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Madin-Darby canine kidney (MDCK) and pig kidney (LLC-PK1) cell lines were grown and passaged as described previously (38) in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal calf serum and 2 mM glutamine in 5% CO2 and 95% air.

**Antibodies**—Mouse monoclonal antibodies (Transduction Laboratories, Lexington, KY) raised against a conserved region of the cytoplasmic domain of human E-cadherin and against β-catenin were used; Tac was recognized using a mouse monoclonal antibody (B-10, BIO-SOURCE International, Camarillo, CA) and a rabbit polyclonal antibody directed against the green fluorescence protein (GFP) (Molecular Probes Inc., Eugene, OR) was also used for staining. Secondary antibodies included Cy3-conjugated sheep anti-mouse and goat anti-rabbit IgGs (Jackson ImmunoResearch Labs, West Grove, PA) and a horseradish peroxidase-conjugated sheep anti-mouse IgG (AMRAD, Victoria, Australia).

**cDNA Construction and Expression**—Chimeric fusions between human E-cadherin cDNAs and the cDNA encoding for Tac were generated in the pCDNA3 expression vector. Molecular cloning techniques were performed according to Sambrook et al. (39), using reagents from New England BioLabs (Beverly, MA). All constructs were confirmed by DNA sequencing. cDNAs encoding the transmembrane plus cytoplasmic domains (residues 554–728), the cytoplasmic tail (residues 578–728), or the amino-terminal half of the cytoplasmic tail (578–653) of human E-cadherin were amplified using PCR with specific oligonucleotide primers. E-cadherin residue numbers correspond to the mature protein as defined previously (6). The oligonucleotide primers contained restriction endonuclease sites allowing generation of in-frame fusions with the Tac cDNA. Cloning the respective PCR products into pCMV-IL2R (40) using the HindIII and XbaI sites generated the pCMV-Tac/Ecad(578–728) and pCMV-Tac/Ecad(578–653) plasmids. These were introduced at the carboxyl terminus of the full-length cDNA of E-cadherin. This was achieved by the PCR amplification of the entire E-cadherin cDNA using specific oligonucleotide primers using pCDNA3-hEcad (41) as a template. The 3′-primer included the SacII site and a XhoI site. The resulting PCR product was digested with SacI and XbaI and subcloned into pCDNA3-hEcad using the same SacII and XhoI sites. The resulting construct pCMV-Tac/Ecad was used as a template and mutagenized proteins, with reduced binding to β-catenin, were not efficiently delivered to the surface (34). It has also been previously noted that the cytoplasmic tail of E-cadherin does contain motifs with homology to known targeting signals (34, 35), which could potentially function to guide its trafficking. In this study we set out to test putative signals in the cytoplasmic tail of E-cadherin for possible basolateral targeting information. Our experiments also addressed the role of β-catenin in this targeting. We utilized a series of chimeras to express the E-cadherin cytoplasmic tail, or mutated versions thereof, using Tac (the α subunit of the IL-2 receptor) as an ectodomain marker. Tac is a 273-amino acid protein that has previously been used as a reporter protein for trafficking studies (36, 37).

These constructs were expressed in MDCK cells, in which the basolateral surface expression of E-cadherin is well established, and in another epithelial cell line LLC-PK1 cells. Using these model systems we have identified a positive targeting signal in E-cadherin that is responsible for basolateral delivery. Our results provide new insights into the trafficking of E-cadherin and its accessory proteins (catenins), and the findings are also significant to our understanding of cell polarity and sorting pathways in epithelia.
and 20 mM Tris-HCl (pH 7.4) prior to solubilization in concentrated SDS-PAGE sample buffer. Proteins in cell extracts and immunoprecipitates were separated on 8% SDS-PAGE reducing gels and then transferred to polyvinylidene difluoride Immunobilon-P membranes (Millipore, Bedford, MA) and stained with 0.1% Coomassie Brilliant Blue to ensure even protein transfer and protein loading. Membranes were immunoblotted by sequential incubations in primary antibody, horseradish peroxidase-conjugated secondary antibody followed by chemiluminescence detection with Supersignal West Pico (Pierce Chemical Co., Rockford, IL). Different luminescence exposures were collected and exposures in the linear range were used.

Surface Biotinylation—Confluent monolayers of LLC-PK, cells stably expressing Tac/Ecad-(578–728) or Tac/Ecad-(578–653) grown on filters were incubated in media containing 1.5 mg/ml Sulfo-NHS-SS-biotin (Pierce), applied to either the apical or basal side of the filter, for 60 min on ice. Filters were then washed several times in cold PBS before cells were scraped off and lysed in cold RIPA buffer. Soluble cell fractions were incubated with streptavidin beads (Sigma) in RIPA buffer, pH 7.4, for 2 h with rotation. Beads were then washed in several rounds of RIPA buffer and 20 mM Tris-HCl (pH 7.4). Biotinylated proteins bound to the streptavidin beads and unlabeled proteins in the supernatants were analyzed by SDS-PAGE, immunoblotting, and densitometry to quantitate the relative amounts of biotinylated Tac/Ecad proteins.

RESULTS

Basolateral Targeting of E-cadherin—E-cadherin is delivered to the basolateral surface of polarized MDCK cells, where it gives a typical and widely documented surface staining pattern using specific antibodies (Fig. 1a). The same antibody did not stain E-cadherin in paraformaldehyde-fixed LLC-PK, cells (Fig. 1c), but it did produce cell surface staining of E-cadherin in methanol-fixed LLC-PK, cells (31 and Fig. 1c). Hence E-cadherin is expressed endogenously in both cell lines and is found in a polarized distribution. A tagged construct of human GFP-E-cadherin was expressed in MDCK cells, generating a clear basolateral surface staining pattern with GFP antibodies, showing that GFP-E-cadherin is targeted in a manner analogous to the endogenous protein (Fig. 1d). GFP-E-cadherin was also expressed in epithelial LLC-PK, cells, where it was also targeted in a polarized fashion to the basolateral membrane (Fig. 1e).

Targeting of Tac/E-cadherin Chimeras—For targeting studies we utilized chimeras consisting of the ectodomain of Tac fused to the cytoplasmic tail of E-cadherin (Fig. 2A). Chimeric cDNAs expressed in epithelial cells all produced proteins of the expected molecular masses (Fig. 2B). Tac typically localizes to the apical membrane in polarized cells (Ref. 36 and Fig. 3a and b), therefore, any basolateral signal in the E-cadherin cytoplasmic domain is predicted to redirect Tac from apical to basolateral membranes. cDNAs for Tac alone or chimeric proteins were transfected into MDCK and LLC-PK, cells, and clonal, stably transfected cell lines were selected. Antibodies against Tac were used to detect the chimeric proteins and determine their localization by indirect immunofluorescence and confocal microscopy.

Chimeras containing the full cytoplasmic tail of E-cadherin were expressed and found to redirect Tac to the basolateral domain in both MDCK and LLC-PK, cells. Both Tac/Ecad-(578–728) and Tac/Ecad-(554–728), which additionally encodes the transmembrane domain of E-cadherin, were localized by epifluorescence and by confocal imaging to the basolateral membranes of MDCK and LLC-PK, cells (Fig. 3). The presence or absence of the E-cadherin transmembrane domain had no effect on targeting. These results indicate that the Tac/E-cadherin chimeras are efficiently synthesized and transported to the cell surface and that the cytoplasmic tail of E-cadherin contains positive sorting information, capable of rerouting Tac to a basolateral trafficking pathway. MDCK cells expressing Tac/Ecad-(578–728) showed no concomitant loss of endogenous E-cadherin staining on the basolateral surface (not shown), suggesting that the sorting and targeting machinery in
proximal tails are trafficked similarly and have the same polarized surface distribution.

We noted that, in cells from several different clones stably expressing Tac/Ecad-(578–653), there was intracellular staining of Tac/Ecad-(578–653) in a perinuclear, Golgi-like pattern in addition to basolateral surface staining (Fig. 4c). This pattern was not regularly seen in cell lines expressing chimeras with full-length tails (Tac/Ecad-(554–728) or Tac/Ecad-(578–728)). LLC-PK1 cells expressing Tac/Ecad-(578–653) were treated with cycloheximide to stop protein synthesis and fixed and stained at various times after addition of the drug. There was a sequential loss of intracellular staining followed at longer times by a diminution of cell surface staining. Fig. 4 shows that after 2 h of treatment, all of the intracellular staining had disappeared, leaving only staining of Tac/Ecad-(578–653) at the basolateral surface. From this it was concluded that intracellular staining in these cells represents a transient accumulation of newly synthesized Tac/Ecad-(578–653) in the biosynthetic pathway and that the membrane-proximal chimera is transported to the basolateral membrane at a slower rate than Tac/Ecad-(578–728).

The targeting of Tac/Ecad-(578–653) was finally tested using a surface biotinylation assay to measure and quantify its appearance on the plasma membrane domains of confluent, polarized cells. Cell surface biotinylation was performed on cell lines stably expressing Tac/Ecad-(578–728) and Tac/Ecad-(578–653). Addition of biotin reagents to the basolateral surface of the monolayers labeled most of the Tac/Ecad-(578–728) and Tac/Ecad-(578–653) proteins, whereas from the apical side almost no labeling occurred in either case (see Table 1). Together these results show that a chimeric protein, containing only the membrane-proximal half of the E-cadherin cytoplasmic tail, has sufficient information to direct efficient sorting and targeting to the basolateral cell surface, albeit perhaps at a slower rate than constructs with the full-length cytoplasmic tail.

A Dileucine Motif Is Responsible for Basolateral Targeting of E-cadherin—Sequence analysis of the membrane-proximal E-cadherin tail encoded by the region in Tac/Ecad-(578–653) revealed the presence of two putative targeting motifs. Sequence alignment of members of the type I cadherins, of which E-cadherin is the prototype, and type II cadherins (42), revealed that a dileucine motif at position 587 is highly conserved across species and present in almost all members of the family (Fig. 5A). To test this dileucine motif for targeting information, the leucines at positions 587 and 588 were changed to alanines in the chimeras encoding the full-length tail and membrane-proximal tail, using oligonucleotide cloning. The resulting mutated chimeras, termed Tac/Ecad-(578–728)Δ587588 and Tac/Ecad-(578–653)Δ588, were transfected into LLC-PK1 and MDCK cells and then localized by immunofluorescence (Fig. 5B). Tac/Ecad-(578–728)Δ587 and Tac/Ecad-(578–653)Δ588 were localized at the apical surfaces of transfected LLC-PK1 and MDCK cells, in patterns similar to the apical Tac (Fig. 3, a and 3b) and distinct from the basolateral non-mutated chimeras. Thus removal of the dileucine motif at 587 from the E-cadherin cytoplasmic domain resulted in a loss of basolateral targeting information. We conclude that this motif is a positive sorting signal for the basolateral membrane localization of E-cadherin and that it is necessary to direct sorting. The high level of conservation of this dileucine motif throughout the cadherins family further suggests that it has a key functional role. A second potential targeting signal of the type NPXY is present in the sequence of Tac/Ecad-(578–653). This tyrosine-based signal at position 600 was not tested here and is not considered a likely candidate for targeting, based on experimental data from another study (34) and our observation that these cells has not been saturated or subverted by the overexpressed protein.

Basolateral Targeting Mediated by the Membrane-proximal E-cadherin Tail—As the first step in a more detailed analysis of the cytoplasmic tail of E-cadherin, a truncation mutant was created to effectively bisect the cytoplasmic tail, leaving only the membrane-proximal portion of the tail fused to Tac. The resulting Tac/Ecad-(578–653) construct was expressed in MDCK and LLC-PK1 cells (Fig. 4). Immunofluorescence staining and confocal analysis showed that it was distributed in a polarized fashion. There was no staining of apical membranes when antibody was applied to either unpermeabilized cells (not shown) or permeabilized cells, however, there was staining of the basolateral membranes in LLC-PK1 and MDCK cells expressing Tac/Ecad-(578–653) (Fig. 4, a and c). Thus, chimeras containing either the full-length tails or only the membrane-proximal tails are trafficked similarly and have the same polarized surface distribution.
the motif is not conserved in cadherins across different species.

Binding of β-Catenin—β-Catenin is known to bind to the cytoplasmic tail of E-cadherin early in the biosynthetic pathway and has previously been implicated in trafficking to the cell surface (34). Therefore, Tac/E-cadherin chimeras were tested for their ability to bind to β-catenin. The interaction of endogenous E-cadherin in MDCK cells with β-catenin was demonstrated by co-immunoprecipitation of the two proteins using an E-cadherin antibody (Fig. 6A). Tac/Ecad-(578–728) was immunoprecipitated with the Tac antibody from extracts of transfected MDCK and LLC-PK1 cells, and β-catenin co-precipitating in the complex was detected by immunoblotting.

The membrane-proximal Tac/Ecad-(578–653) construct is missing the carboxyl-terminal β-catenin binding domain, and co-immunoprecipitation experiments confirm that, as expected, β-catenin was not co-precipitated with this truncated chimeric protein (Fig. 6B). Endogenous E-cadherin and chimeras with full-length E-cadherin tails were able to efficiently bind and co-immunoprecipitate β-catenin (Fig. 6, A and B). Deletion of the 587 dileucine targeting signal had no effect on binding of β-catenin, which was efficiently co-precipitated with Tac/Ecad-(578–728)ΔS1 (Fig. 6B). The correct basolateral targeting of Tac/Ecad-(578–653) in the absence of bound β-catenin now demonstrates that β-catenin is not essential for basolateral sorting and targeting in either MDCK or LLC-PK1 cells. The complexing of β-catenin with newly synthesized E-cadherin may be required for other roles in biosynthetic processing or trafficking, for instance, the loss of β-catenin binding may account for the increased...
intracellular accumulation and apparently slower trafficking of the Tac/Ecad-(578–653) mutants.

**DISCUSSION**

E-cadherin is one of the proto-typical polarized membrane proteins in epithelia. It is delivered to the basolateral membrane and is concentrated in adherens junctions where it participates in cell-cell adhesion. To address how E-cadherin is trafficked and targeted in polarized cells, we made use of Tac/Ecad-(578–728) and Tac/Ecad-(554–728), which contains similar tail regions to some of the mutants in a previous study in which a series of chimeras representing truncation mutants of the E-cadherin tail fused to a GP-2 ectodomain, were found to be very poorly trafficked (34). Only about 10% of the truncated GP-2 chimeric proteins in that study reached the surface of MDCK cells and were randomly sorted, whereas the majority of the proteins were blocked or degraded early in the secretory pathway (34). On the basis of the correct targeting and delivery of Tac/Ecad-(578–653), which contains similar tail regions to some of the mutants in the previous study, we hypothesize that the poor processing and trafficking of those chimeras may have been due to the influence of the GP-2 ectodomain rather than being a function of E-cadherin trafficking or accessory proteins (see below).

The correct basolateral targeting of the Tac/Ecad-(578–653) construct first indicated that the membrane-proximal tail region alone is sufficient for membrane targeting and that distal regions of the tail are not required for targeting or surface delivery. In our hands, mutants with the truncated tail of E-cadherin, Tac/Ecad-(578–653), were processed, targeted, and delivered to the basolateral cell surface, albeit at a slower rate than constructs with the full cytoplasmic tail. Transient accumulation of Tac/Ecad-(578–653) in the biosynthetic pathway, seen as intracellular staining in LLC-PK1 cells, indicated that it was trafficked less efficiently than the full-length tail. Partial accumulation of Tac/Ecad-(578–653) was similarly noted in MDCK cells, although the truncated tail mutants did eventually reach the cell surface. Our findings are in contrast to those of a previous study in which a series of chimeras representing truncation mutants of the E-cadherin tail fused to a GP-2 ectodomain, were found to be very poorly trafficked (34). Only about 10% of the truncated GP-2 chimeric proteins in that study reached the surface of MDCK cells and were randomly sorted, whereas the majority of the proteins were blocked or degraded early in the secretory pathway (34). On the basis of the correct targeting and delivery of Tac/Ecad-(578–653), which contains similar tail regions to some of the mutants in the previous study, we hypothesize that the poor processing and trafficking of those chimeras may have been due to the influence of the GP-2 ectodomain rather than being a function of E-cadherin trafficking or accessory proteins (see below).

The membrane-proximal tail region of E-cadherin has been implicated in a number of functions. Cadherin clustering and adhesive strength were shown to be affected by mutations in the membrane-proximal region of the C-cadherin tail (45). Binding of the p120ctn protein has also been shown to occur in this region (45, 46), and some of the amino acid sequences mapped by two-hybrid analysis, as being specifically involved in the binding of p120ctn (47), overlap with the dileucine targeting signal identified in this study. It is therefore likely that this membrane-proximal region of the tail is involved in multiple, temporally regulated protein interactions that are important, initially for E-cadherin transport, and then for adhesive function at the cell surface. Positive sorting mediated by the Tac/Ecad-(578–653) membrane-proximal tail allowed us to focus on putative sorting signals in this region. The dileucine motif at 587 was chosen here as a candidate targeting signal. Despite being in the non-conserved region of the tail, sequence alignments revealed that the dileucine consensus motif is highly conserved throughout cadherins of type I or II cadherin family members, including E-cadherin (cadherin 1), N-cadherin (cadherin 2), and VE-cadherin (cadherin 5). A dileucine motif at position 587 (underlined) in the membrane-proximal region of the tail of E-cadherin (cadherin 1) is highly conserved across members of the family and is relatively close to the transmembrane domain (42). Sequence alignment of cadherins with conserved cytoplasmic tails (type I and II families as defined by Nollet et al. (42)) in the membrane-proximal region of the tail. Three main conclusions emerged from these experiments: (i) that a single dileucine motif at 587 (shaded) in E-cadherin (cadherin 1) is highly conserved across members of the family and is relatively close to the transmembrane domain (42). Sequence alignment of cadherins with conserved cytoplasmic tails (type I and II families as defined by Nollet et al. (42)), targeting B, targeting of dileucine deletion mutants. MDCK and LLC-PK1 cells expressing Tac/Ecad(578–728)ΔS1 or Tac/Ecad-(578–653)ΔS1 were stained with the Tac antibody. Both constructs with deleted dileucine motifs, now show apical targeting. Prominent apical staining only, is now seen in both cell lines expressing Tac/Ecad-(578–728)ΔS1 or Tac/Ecad-(578–653)ΔS1. Bars, 2.5 μm.

**FIG. 5.** A, sequence alignment of cadherins with conserved cytoplasmic tails (type I and II families as defined by Nollet et al. (42)). Sequence alignment of the membrane-proximal tail regions of cadherin family members, including E-cadherin (cadherin 1), N-cadherin (cadherin 2), and VE-cadherin (cadherin 5). A dileucine motif at position 587 (shaded) in E-cadherin (cadherin 1) is highly conserved across members of the family and is relatively close to the transmembrane domain (underlined) in the membrane-proximal region of the tail. B, targeting of dileucine deletion mutants. MDCK and LLC-PK1 cells expressing Tac/Ecad-(578–728)ΔS1 or Tac/Ecad-(578–653)ΔS1 were stained with the Tac antibody. Both constructs with deleted dileucine motifs, now show apical targeting. Prominent apical staining only, is now seen in both cell lines expressing Tac/Ecad-(578–728)ΔS1 or Tac/Ecad-(578–653)ΔS1. Bars, 2.5 μm.
The dileucine signal in E-cadherin has an acidic nature of the adaptor complexes or that required for post-Golgi trafficking of E-cadherin and Tac/Ecad constructs in LLC-PK₁ cells acts as further evidence that this targeting does, in fact, rely on dileucine rather than tyrosine motifs. The full nature of the adaptor complexes or that required for post-Golgi trafficking of E-cadherin in either LLC-PK₁ or MDCK epithelial cells have yet to be characterized.

Finally, the current study also provides new insights into the role of β-catenin in E-cadherin trafficking. β-Catenin is a cytoplasmic protein with affinity for the cytoplasmic tail of E-cadherin, it binds to E-cadherin early in the biosynthetic pathway, forming a stable complex that is transported to the cell surface (33). Chen and colleagues (34) concluded that β-catenin is required for the biosynthetic processing and trafficking of E-cadherin, based on a correlation between deletion of residues within or near the β-catenin binding domain and loss of surface delivery in GP-2-E-cadherin chimeras and other constructs. However, our current results suggest a different scenario. We showed that full-length tail chimeras co-precipitated β-catenin in similar proportion to that seen in endogenous E-cadherin-β-catenin complexes. Upon expressing the Tac/Ecad-(578-653) chimera, which clearly did not bind β-catenin, we found it was correctly targeted and transported to the cell surface, suggesting that β-catenin is not required for sorting or delivery under these conditions. β-Catenin may have a role in facilitating or optimizing the transport, processing, or folding of newly synthesized E-cadherin. All of these factors might well contribute to the slower processing and transient accumulation of Tac/Ecad-(578-653) that we noted in the absence of β-catenin.

Recent biophysical and biochemical analyses further suggest that β-catenin binding might also serve to protect the E-cad-

---

**Fig. 6. Co-immunoprecipitation of β-catenin.** A, proteins immunoprecipitated from untransfected MDCK cells with a monoclonal antibody to E-cadherin were probed by immunoblotting with the E-cadherin antibody (top) or with a β-catenin antibody (bottom). Proteins at 120 and 92 kDa, respectively, were detected. B, the Tac antibody was used to immunoprecipitate chimeras from transfected LLC-PK₁ cells. The β-catenin antibody was then used for immunoblotting supernatants (SN lanes 1, 3, and 5) and immunoprecipitates (IP lanes 2, 4, and 6). β-Catenin was co-precipitated with Tac/Ecad-(578–728) (lane 2) but not with the truncated Tac/Ecad-(578–653) chimera (lane 4). Deletion of the dileucine targeting motif (Tac/Ecad-(578–728)ΔS1) did not affect co-precipitation of β-catenin (lane 6).
herin tail from degradation (57). Our finding, that β-catenin is not required for basolateral targeting or surface delivery, sheds new light on β-catenin as having a role in facilitating, but not directing, cadherin trafficking.

The polarized targeting of E-cadherin is of seminal importance to the maintenance of epithelial polarity and function. Targeting signals and mechanisms must ensure the accurate basolateral delivery of newly synthesized E-cadherin and of internalized and recycled E-cadherin for its incorporation into adherens junctions. In this study we demonstrate one such mechanism, basolateral sorting directed via a dileucine signal. Future studies will address specific roles for additional signals and perhaps for some of the accessory proteins in cadherin/ catenin complexes.

Acknowledgments—We thank Susan La Flamme for providing materials and acknowledge Darren Brown, Marita Goodwin, and Juliana Venturato for their technical support.

REFERENCES

1. Takeichi, M. (1995) Curr. Opin. Cell Biol. 7, 619–627
2. Yap, A. S., Bricher, W. M., and Gumbiner, B. M. (1995) Annu. Rev. Cell Dev. Biol. 11, 119–146
3. Gumbiner, B. M. (1996) Cell 84, 345–357
4. Gumbiner, B. M. (2000) Curr. Opin. Cell Biol. 12, 599–604
5. Yap, A. S. (1996) Cancer Invest. 14, 252–261
6. Rimm, D. L., and Moreau, J. S. (1994) Annu. Rev. Cell Dev. Biol. 10, 119–146
7. Sheikh, H., and Isacke, C. M. (1996) J. Biol. Chem. 271, 12185–12190
8. Roux, D. L., Gattard, C. J., Naim, H. Y., Roth, M. G., and Caplan, M. J. (1998) J. Biol. Chem. 273, 26862–26869
9. Ohno, H., Tomomori, T., Nakagawa, F., Okazaki, Y., Aguiar, R. C., Fuchs, H., Mellman, I., Saito, T., Shirasawa, T., and Bonifacino, J. S. (1999) FEBS Lett. 449, 215–220
10. Folsch, U., Ohno, H., Bonifacino, J. S., and Mellman, I. (1999) Cell 99, 189–198
11. Lechner, J., Krali, M., Netzer, A., Radmayr, C., Ryan, M. P., and Pfaller, W. (1999) Kidney Int. 55, 2178–2191
12. Tanentzapf, G., Smith, C., Mcglade, J., and Tepass, U. (2000) J. Cell Biol. 151, 901–904
13. Hinck, L., Nathke, I. S., Papkoff, J., and Nelson, W. J. (1994) J. Cell Biol. 125, 1327–1340
14. Chen, Y. T., Stewart, D. B., and Nelson, W. J. (1999) J. Cell Biol. 144, 687–699
15. Le, T. L., Yap, A. S., and Stow, J. L. (1999) J. Cell Biol. 146, 239–252
16. Rajasekaran, A. K., Humphrey, J. S., Wagner, M., Miesenbock, G., Le Bivic, A., Bonifacino, J. S., and Rodriguez-Boulan, E. (1994) Mol. Biol. Cell 5, 1093–1103
17. Mallette, W. G., and Maxfield, F. R. (1999) J. Cell Biol. 146, 345–359
18. Narula, N., McMorrow, I., Plepper, G., Dobert, J., Matlin, K. S., Burke, B., and Stow, J. L. (1992) J. Cell Biol. 117, 27–38
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. LaFlamme, S. E., Akiyama, S. K., and Yamada, K. M. (1992) J. Cell Biol. 117, 497–517
21. Pond, L., Kuhn, L. A., Teyton, L., Schutze, M. P., Tainer, J. A., Jackson, M. R., and Cossart, P. (1999) EMBO J. 18, 3956–3963
22. Lin, S., Naim, H. Y., and Roth, M. G. (1997) J. Biol. Chem. 272, 26860–26865
23. Tanentzapf, G., Smith, C., McGlade, J., and Tepass, U. (2000) J. Cell Biol. 151, 901–904
24. Distel, B., Bauer, U., Le Borgne, R., and Hoflack, B. (1998) J. Biol. Chem. 273, 186–193
25. Hubert, M. E., Kil, S. J., Medof, M. E., and Carlin, C. R. (1997) J. Biol. Chem. 272, 32901–32909
26. El Nemer, W., Colon, Y., Bauvy, C., Codogno, P., Fraser, R. H., Carton, J. P., and Le Van Kim, C. L. (1999) J. Biol. Chem. 274, 31903–31908
27. Sheikhi, H., and Isacke, C. M. (1996) J. Biol. Chem. 271, 12185–12190
28. Roux, D. L., Gattard, C. J., Naim, H. Y., Roth, M. G., and Caplan, M. J. (1998) J. Biol. Chem. 273, 26862–26869