Increased Internalization of p120-uncoupled E-cadherin and a Requirement for a Dileucine Motif in the Cytoplasmic Domain for Endocytosis of the Protein*

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E-cadherin is a member of the cadherin family of Ca\(^{2+}\)-dependent cell-cell adhesion molecules. E-cadherin associates with \(\beta\)-catenin at the membrane-distal region of its cytosolic domain and with p120 at the membrane-proximal region of its cytoplasmic domain. It has been shown that a pool of cell surface E-cadherin is constitutively internalized and recycled back to the surface. Further, p120 knockdown by small interference RNA resulted in dose-dependent elimination of cell surface E-cadherin. Consistent with these observations, we found that selective uncoupling of p120 from E-cadherin by introduction of amino acid substitutions in the p120-binding site increased the level of E-cadherin endocytosis. The increased endocytosis was clathrin-dependent, because it was blocked by expression of a dominant-negative form of dynamin or by hypertonic shock. A dileucine motif in the juxtamembrane cytoplasmic domain is required for E-cadherin endocytosis, because substitution of these residues to alanine resulted in impaired internalization of the protein. The alanine substitutions in the p120-uncoupled construct reduced endocytosis of the protein, indicating that this motif was dominant to p120 binding in the control of E-cadherin endocytosis. Therefore, these results are consistent with the idea that p120 regulates E-cadherin endocytosis by masking the dileucine motif and preventing interactions with adaptor proteins required for internalization.

Cadherins are a family of structurally and functionally related molecules that mediate Ca\(^{2+}\)-dependent cell-cell adhesion in a homophilic manner (1, 2). Ca\(^{2+}\) protects the extracellular domains of cadherins from proteolytic degradation and is necessary for their function. Cadherin-based adhesion is a central determinant of cell patterning during development and is necessary for the preservation of established tissue architecture in mature organisms. E-cadherin is a prototypic member of the family and 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. Genetic studies have shown that E-cadherin is essential for epithelial integrity during development in Xenopus and mice (3–5). At the cellular level, E-cadherin facilitates assembly of specialized intercellular junctions (desmosomes, gap, and tight junctions) necessary to link epithelial cells into functional monolayers (6, 7).

The adhesive strength of cell-cell contacts depends on factors such as dimerization and lateral clustering of cadherin molecules (8, 9). Cadherins also form high affinity complexes with catenins and other molecules that participate in the overall function and stability of adherens junctions (10, 11). E-cadherin interacts with \(\beta\)-catenin and \(\alpha\)-catenin, which links the complex to the actin cytoskeleton (11, 12). The Rho family GTPases Rho, Rac, and Cdc42 produce different configurations of actin in cells (13) and have been implicated in remodeling actin for the regulation of E-cadherin-mediated adhesion (14, 15).

The trafficking of E-cadherin, its delivery to or removal from the cell surface, is one aspect of cadherin regulation that is not yet thoroughly understood. There is a growing body of evidence that shows that cell surface E-cadherin can be endocytosed and trafficked back into the cell. Ca\(^{2+}\) depletion induces internalization of cadherins and the subsequent disruption of junctions (16–18). It was shown that E-cadherin undergoes endocytosis and recycling in Madin-Darby canine kidney (MDCK)\(^{2}\) cell monolayers under physiological conditions (19). The adhesive strength at cell-cell contacts depends, in part, on the amount of E-cadherin at the cell surface (20). Therefore, endocytosis and recycling of E-cadherin provides one mechanism for dynamically regulating cadherin-based adhesion, by balancing the relative amounts of surface-exposed and sequestered intracellular E-cadherin. How the endocytosis and recycling of E-cadherin are regulated is therefore of prime importance to the control of cell-cell adhesion.

In addition to \(\beta\)-catenin, which binds to the distal portion of the E-cadherin cytoplasmic domain, another member of the armadillo family, p120, interacts with cadherins at the membrane-proximal region of the cytosolic domain (21–23). Although not directly involved in coupling cadherins to the

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\(2\) The abbreviations used are: MDCK, Madin-Darby canine kidney cell; EA substitution, amino acid substitutions in which two conserved glutamic acid residues and a conserved aspartic acid residue in the p120-binding site were substituted with alanine residues; GFP, green fluorescent protein; HA, hemagglutinin; LA substitution, amino-acid substitutions in which a dileucine motif was changed to a dialanine motif; mAb, monoclonal antibody; siRNA, small interference RNA; Ecad, E-cadherin; EA substitution; LAEcad, Ecad with LA substitution; LEAEcad, Ecad with EA substitution; EALAEcad, Ecad with EA and LA substitutions.

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cytoskeleton, p120 has emerged as a critical regulator of cadherin adhesive activity and cytoskeletal organization (22, 23). In addition to regulating adhesion and actin cytoskeletal organization, recent studies have demonstrated a core function for p120 in regulating cadherin expression levels. Separate studies have shown that p120 regulates steady-state levels of E-cadherin in epithelial cells (24) and VE-cadherin in vascular endothelial cells (25). In the absence of p120, cadherins become destabilized and targeted for degradation. The precise mechanism by which p120 regulates cadherin levels is not fully understood. However, several lines of evidence suggest that p120 regulates the entry of cell surface cadherins into a lysosomal degradation pathway (24, 25). In the absence of p120, E-cadherin is efficiently delivered to the cell surface but is then rapidly turned over (24). Similarly, cell surface labeling experiments indicate that p120 regulates the lysosomal degradation of cell surface pools of VE-cadherin (25). These data suggest that p120 likely functions at the plasma membrane to regulate cadherin internalization.

In this study, we aimed to identify the amino acid residues in E-cadherin that are required for internalization of the protein. A recent study demonstrated that the signal responsible for the endocytosis of VE-cadherin resides in the cytoplasmic domain (26). A candidate dileucine motif that was able to mediate association with clathrin-containing endocytic vesicles was identified at positions 587–588 in the juxtamembrane region of the E-cadherin cytoplasmic domain. These two leucine residues were substituted with alanine residues. Endocytosis of the substituted E-cadherin was impaired, demonstrating that this motif was required for efficient internalization of the protein. Disrupting the binding of E-cadherin to p120 increased the rate at which E-cadherin was endocytosed, whereas introduction of the substitution in the p120-uncoupled construct decreased its endocytosis. Thus, the dileucine motif is dominant to p120 in the control of E-cadherin endocytosis. Based on these observations, we propose a model wherein p120 regulates E-cadherin endocytosis by masking the motif to prevent interactions with adaptors proteins required for internalization.

**EXPERIMENTAL PROCEDURES**

**cDNA Construction**—To distinguish exogenously expressed protein from endogenous canine E-cadherin, all constructs expressing proteins fused to a hemagglutinin (HA) epitope at their carboxyl termini. Expression vectors containing the cDNAs encoding the following constructs were described previously (27, 28): HA-tagged full-length E-cadherin (Ecad), and a full-length E-cadherin construct in which two conserved glutamic acid residues and a conserved aspartic acid residue in the p120-binding site were substituted with alanine residues (EAEcad). The EA substitution results in uncoupling of p120 binding (28).

To mutate the two leucine residues at positions 587 and 588 to alanine residues (LA mutation) (Fig. 1B), the primer pairs 5′-GGCGGGGTCTTACAGACCGTTCT and 5′-GCGCCTCTTACAGACCGTTCT were used. EALAEcad, a full-length E-cadherin construct carrying the EA and LA substitutions, was generated by replacing a 1938-bp Smal-Smal fragment from the EAEcad construct with the same fragment from the LAEcad construct. All PCR products were sequenced and subcloned into expression vectors.

Expression vectors for green fluorescent protein (GFP)-tagged wild-type and dominant-negative dynamin IIaa, pEGFP-N2Ya and pEGFP-N2Ya(K44A), respectively (29), were kindly provided by Dr. Kazuhisa Nakayama (Kyoto University, Japan).

**Cells and Transfections**—MDCK cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells (5 × 10^6^) were transfected with the appropriate expression vectors (10 μg) using the calcium phosphate method (10). To determine the effects of dynamin expression on the distribution of EAEcad, MDCK cell clones stably expressing EAEcad were transiently transfected with expression vectors for either wild-type or mutant dynamin using Lipofectamine 2000 (Invitrogen) as described (29). siRNAs against canine p120 were obtained from Dharmacon (Lafayette, CO), and 100 nm of four pooled siRNAs was transfected using Lipofectamine 2000.

**Antibodies**—A mouse mAb (12CA5) directed against HA was kindly provided by Dr. Akihiko Yoshimura (Kyushu University, Japan) and was used for immunoblotting. Rat mAb against HA (3F10) and fluorescein isothiocyanate-labeled 3F10 antibody were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Rabbit anti-E-cadherin antibody was kindly provided by Dr. Rolf Klemper (Max-Planck Institut für Immunologie, Germany). Rabbit anti-N-cadherin antibody (H-63) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse mAbs against p120 and early endosomal antigen 1 were purchased from BD Transduction Laboratories (Lexington, KY). LysoTracker Red DND-99 and Alexa Fluor 594-conjugated transferrin were obtained from Molecular Probes (Eugene, OR). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Immunoblotting and Immunoprecipitation**—Immunoprecipitation and immunoblot analyses were carried out as described previously (27). In brief, cells (2 × 10^6^) were lysed in lysis buffer (25 mM Tris–HCl buffer, pH 7.4, containing 1% Triton X-100, 2 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 25 μg/ml aprotinin). Immunoprecipitations were carried out with anti-HA mAb pre-absorbed to protein G-Sepharose.

**Fluorescence Microscopy**—Immunofluorescence labeling of cells was performed as described previously (22). In brief, cells were fixed with 3% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 and incubated with primary and secondary antibodies. Cells were analyzed using a conventional Olympus fluorescence microscope (Tokyo, Japan) equipped with a CoolSNAP charge-coupled device camera (Nippon Roper).

**Quantification of Cell Surface E-cadherin**—Cells were incubated in HEPEFS-buffered saline with 0.01% trypsin for 10 min at 37 °C in the presence of 2 mM Ca^{2+} (TC treatment) or 1 mM EGTA (TE treatment). Proteins were detected by anti-HA mAb. Cell surface E-cadherins are resistant to trypsin digestion.
in the presence of Ca\(^{2+}\) but can be degraded in the absence of Ca\(^{2+}\). Thus, the E-cadherin remaining after TC treatment represents the total pool of E-cadherin, whereas intracellular E-cadherin remains undigested after TE treatment.

**Apical and Basolateral Biotinylation**—Cells (5 \times 10^5 cells) were seeded on Transwell filters, and tight cell monolayers were incubated twice with 0.5 mg/ml sulfo-N-hydroxysuccinimide-biotin (Pierce) in either the apical or basolateral chamber at 4 °C. The cells were washed with 50 mM NH_4Cl in phosphate-buffered saline at 4 °C. Cells were then stripped with 20 mM Tris-HCl (pH 8.0) containing 1% SDS, boiled for 3 min, and passed four or five times through a 23-gauge needle. 9 volumes of 2% Triton X-100 was added, and biotinylated proteins were collected using streptavidin beads.

**Biotinylation Assay for Endocytosis and Recycling**—The assay was performed as described with minor modifications (19). Cells were incubated with 0.5 mg/ml sulfo-N-hydroxysuccinimide-sbstibin at 4 °C. After washing, cells were incubated in medium containing 20 mM HEPES at 18 °C for the indicated periods of time. Cell surface biotin was removed by incubation with 60 mM glutathione or with 50 mM 2-mercaptoethanesulfonic acid at 4 °C. Cells were then lysed in lysis buffer as described above. Biotinylated proteins were collected with streptavidin beads.

To measure recycling of endocytosed proteins accumulated at 18 °C, cells were stripped using glutathione or 2-mercaptoethanesulfonic acid at 4 °C and then returned to 37 °C for various times in medium containing anti-E-cadherin antibody. Cells were then washed to remove unbound antibody and lysed in lysis buffer. E-cadherin bound to anti-E-cadherin antibody during the incubation was collected using protein G-Sepharose. After washing, E-cadherin was released from the beads by boiling in 20 mM Tris-HCl (pH 8.0) containing 1% SDS. After addition of 9 volumes of 2% Triton X-100, biotinylated E-cadherin was recovered by incubation with streptavidin beads. The materials were analyzed by SDS-PAGE and immunoblotting with anti-HA antibodies.

**RESULTS**

**Selective Uncoupling of p120 from E-cadherin Results in Intracellular Accumulation of the Protein**—Previous studies have shown that, in MDCK cells, a pool of cell surface E-cadherin is constitutively internalized and recycled back to the surface (19). A construct expressing wild-type E-cadherin tagged with an HA epitope (Ecad) (Fig. 1B) was introduced into MDCK cells, and stable transfectants were isolated. In confluent MDCK cells, Ecad was localized mostly on the lateral plasma membrane (Fig. 2). Substitution of the two conserved glutamic acid residues and a conserved aspartic acid residue (Glu-Glu-Asp) in the p120-binding site of E-cadherin with alanine residues (the EA substitution) (Fig. 1B). EAEcad expressed from this construct in MDCK cells did not co-precipitate p120 (Fig. 3A). In contrast to wild-type E-cadherin, a significant fraction of EAEcad was detected intracellularly (Fig. 2). To determine the origin of the intracellular EAEcad, cells were pretreated with 10 \mu M cycloheximide to inhibit translation and stained for the HA tag. Although some labeling in the perinuclear region, which represents the Golgi apparatus, disappeared upon treatment (data not shown), the overall localization of EAEcad was not altered significantly, indicating that the intracellular labeling did not represent a newly synthesized pool of EAEcad. Thus, although a portion of EAEcad was present on the lateral plasma membrane, the intracellular staining suggests that a significant pool of EAEcad was internalized from the cell surface. Similar changes in the cellular distribution of N-cadherin were observed after uncoupling of p120 binding (data not shown). These results are consistent with observations that p120 knockdown by siRNA expression resulted in dose-dependent elimination of cell surface E-cadherin (24) and that overexpression of p120 selectively inhibited VE-cadherin internalization from the plasma membrane by inhibiting clathrin-dependent endocytosis of the protein (26).
Next, we biochemically quantified the amount of EAEcad on the surface of stably transfected MDCK cells. It is well established that Ca\(^{2+}\) protects cell surface cadherins from proteolytic degradation. Cells were trypsinized in the presence of Ca\(^{2+}\) (TC treatment) or EGTA (TE treatment) (see “Experimental Procedures”). Immunoblot analysis of cells following TC or TE treatment using anti-HA antibodies demonstrated that 90% of wild-type E-cadherin was digested following TE treatment (Fig. 3, B and C). In contrast, <40% of the p120-uncoupled construct was digested after TE treatment (Fig. 3B). Thus, <40% of EAEcad resided on the cell surface.

Cell surface biotinylation of the apical and basolateral membranes of cells cultured on Transwell filters showed that EAEcad was, like wild-type E-cadherin, exclusively transported to the basolateral membrane (Fig. 3D). Therefore, intracellular accumulation of EAEcad was not caused by mis-sorting of the protein.

The p120-uncoupled E-cadherin Co-localized with an Early Endosomal Antigen—To define the site of EAEcad accumulation, transfected cells were incubated with medium containing LysoTracker, a fluorescently labeled marker for lysosomes. EAEcad did not co-localize well with the lysosome marker (Fig. 4, upper panel), but a significant fraction of the protein co-localized with an early endosomal marker (30), early endosomal antigen 1 (Fig. 4, middle panel). EAEcad also co-localized with fluorescently tagged transferrin internalized from the cell surface (lower panel). Cells were incubated with Alexa Fluor 594-conjugated transferrin at 4°C and then transferred to 37°C for 30 min. After washing the cells with acid, cells were fixed and processed for immunofluorescence microscopy to detect internalized transferrin.
**E-cadherin Endocytosis**

early endosomes. Treatment of cells with chloroquine to inhibit lysosomal hydrolases did not change the expression of EAEcad (Fig. 3E).

Enhanced Internalization of the p120-uncoupled E-cadherin—Accumulation of EAEcad in early endosomes suggested that interfering with p120 binding facilitated the endocytosis of the protein. To compare the extent of endocytosis of different E-cadherin proteins expressed in MDCK cells, we used a biotinylation assay (19) that measures the internalization of cell surface E-cadherin. Following biotinylation of cell surface proteins at 4 °C, cells were incubated for various periods of time at 18 °C, a temperature that causes the accumulation of internalized proteins in early or sorting endosomes by preventing further progression into the endocytic or recycling pathways (19). Next, biotin was stripped from the cell surface, and the remaining sequestered biotin was measured. Under these conditions, the amount of internalized wild-type Ecad increased over the first 120 min (Fig. 5, A and B). After 2 h at 18 °C, the internalized pool represented <10% of the total E-cadherin biotinylated at the beginning of the experiment. In contrast, the pool of internalized EAEcad increased more rapidly over time (Fig. 5, A and B); after 60 min, the intracellular pool of EAEcad was more than twice that of wild-type E-cadherin. Therefore, uncoupling of p120 binding to E-cadherin increases internalization of the protein.

Enhanced Internalization of the p120-uncoupled E-cadherin Is Clathrin-mediated—E-cadherin endocytosis occurs via a clathrin-dependent pathway and can be inhibited by hypertonic shock (31) or dominant-negative dynamin, a GTPase that is a key regulator of clathrin-mediated endocytosis (32). To study whether the enhanced internalization of the p120-uncoupled E-cadherin is mediated by a clathrin-dependent mechanism, cells expressing EAEcad were transfected with expression vectors for wild-type or dominant negative (K44A) dynamin II tagged with GFP (29), and the effects on EAEcad localization were determined. Cells overexpressing wild-type dynamin II showed efficient internalization of EAEcad (Fig. 6A, upper panel), similar to untransfected cells (data not shown). In contrast, cells overexpressing dominant-negative dynamin II (marked by green fluorescence) failed to internalize EAEcad and showed no intracellular accumulation of the protein (Fig. 6A, lower panel). Thus, p120-uncoupled E-cadherin is internalized by clathrin-mediated endocytosis requiring dynamin.

To quantitatively analyze clathrin dependence of endocytosis, we used hypertonic shock, a method that has been shown to specifically inhibit clathrin-coated pit uptake of the low density lipoprotein receptor (33) and other receptors. As shown in Fig. 6B, treatment of cells with hypertonic medium blocked endocytosis of wild-type E-cadherin. Under the same conditions, the internalization of EAEcad was also blocked. From these data, we conclude that the p120-uncoupled E-cadherin is internalized by clathrin-dependent endocytosis.

Dileucine Motif Leu<sup>587</sup>-Leu<sup>588</sup> Is Required for Internalization of E-cadherin—Dileucine motifs are known to mediate endocytosis into endosomal compartments (34) and usually correspond to a (DE)XXLX(LI) or DXXLL consensus. Despite the fact that E-cadherin lacks the acidic residue(s) preceding the dileucine motif, we nevertheless tested the hypothesis that the strongly conserved dileucine repeat Leu<sup>587</sup>-Leu<sup>588</sup> is required for E-cadherin endocytosis. Substitution of the leucine residues to alanine had a less striking effect on the distribution of E-cadherin (LAEcad) at the steady state, with no endocytic vesicles observed inside the cells (Fig. 2). Interestingly, substitution of the leucine residues to alanine in the p120-uncoupled E-cadherin (EALAEcad) resulted in an almost total loss of intracellular accumulation of the protein (Figs. 2,3B, and 3C). Although this dileucine motif has been reported to be critical for the targeting of human E-cadherin to the basolateral membrane of MDCK and LLC-PK1 epithelial cells (35, 36), we observed expression of LAEcad and EALAEcad on the basolateral membrane of MDCK cells under these conditions (Fig. 3D).

![FIGURE 5. The dileucine motif is required for endocytosis of E-cadherin.](http://example.com/figure5.png)
Biotinylation assays showed that the percentage of internalized LAEcad was significantly reduced (Fig. 5, A and B), consistent with the idea that the dileucine motif is involved in internalization. Mutation of the dileucine motif in the p120-uncoupled E-cadherin (EALAEcad) also resulted in reduced internalization of protein from the cell surface (Fig. 5B). Thus, the dileucine motif is required for the internalization of E-cadherin.

To examine the effects of p120 knockdown, cells expressing wild-type E-cadherin and LAEcad were characterized by immunoblotting and by immunofluorescence. Transfection with specific siRNAs against p120 reduced p120 protein to ~30% of the control (Fig. 7, A and B). Consistent with previous reports (24, 25), the p120 knockdown induced reduction (~40% of control) of wild-type E-cadherin levels (Fig. 7, A and B). Remarkably, levels of LAEcad were not affected (Fig. 7, A and B). Levels of vimentin, which is an intermediate filament component, were unaffected.

For immunofluorescence analysis, cells were incubated for 2 h at 18 °C to accumulate internalized proteins in early or sorting endosomes by preventing them from progressing further into the endocytic pathways. Because the 18 °C temperature block also prevent exit of newly synthesized proteins from the trans-Golgi network, cycloheximide (10 μM) was added 1 h before and included during the incubation. As shown in Fig. 7C, the p120 knockdown cells exhibit a decreased cell surface and increased intracellular vesicular labeling of wild-type E-cadherin (Fig. 7C). Contrary to wild-type E-cadherin, LAEcad was exclusively detected on the cell surface at cell-cell contacts of the p120 knockdown cells.

Endocytosis of wild-type E-cadherin and LAEcad was measured using biotinylation assay described above. Endocytosis of wild-type E-cadherin was enhanced by p120 depletion, but that of LAEcad was not (Fig. 7D). Although the amount of E-cadherin internalized under the conditions used is small, the enhanced internalization is consistent with the accumulation of intracellular vesicles of E-cadherin in the p120 knockdown cells.

Reduced Recycling of p120-uncoupled E-cadherin—Using an approach similar to that used to study the surface expression of lysosomal glycoproteins in MDCK cells (37), we next developed an assay to identify the recycling of internalized biotinylated E-cadherin back to the cell surface. Surface proteins were biotinylated at 4 °C, and cells were incubated for 2 h at 18 °C to allow the internalization and accumulation of E-cadherin. After treatment with reducing reagents to strip remaining biotinyl groups from cell surface proteins, the cells were released at 37 °C to resume trafficking. Cells were incubated with anti-E-cadherin antibody, washed, and lysed. E-cadherin bound to anti-E-cadherin antibodies was collected by immunoprecipitation with protein G-Sepharose. The immunocomplexes were then precipitated with streptavidin-beads to collect biotinylated E-cadherin. The resulting precipitate was separated by SDS-PAGE and immunoblotted with anti-HA antibodies. Almost no biotinylated E-cadherin was detected on the surface of cells that were not incubated at 37 °C (incubation time 0). However, E-cadherin was detected on the surface after a 10-min incubation at 37 °C (Fig. 8A). Recycled E-cadherin increased rapidly for the first 20 min at 37 °C, after which the rate slowed (Fig. 8, A and B). At the end of analysis, >90% of internalized E-cadherin recycled back to the cell surface. The amount of recycled EAEcad increased at a slower rate than wild-type E-cadherin for 40 min at 37 °C. After 40 min at 37 °C, ~17% of the internalized EAEcad recycled back. This indicates that internalized EAEcad can also be recycled to the cell surface but at a significantly slower rate than wild-type E-cadherin.

DISCUSSION

In this study, we show that wild-type E-cadherin and E-cadherin uncoupled for p120 binding undergo endocytosis by a clathrin-dependent mechanism. We found that the p120-uncoupled E-cadherin is internalized from the cell surface at a rate four times faster than that of wild-type E-cadherin. Enhanced internalization of p120-uncoupled E-cadherin allowed us to focus on putative sorting signals in this region. A conserved dileucine motif at 587–588 in the juxtamembrane cytoplasmic
domain was chosen as a candidate targeting signal and shown to be necessary for E-cadherin endocytosis. Replacement of the dileucine motif with two alanine residues resulted in reduced internalization.

**FIGURE 7.** RNA interference of p120 in MDCK cells enhances internalization of wild-type E-cadherin but not that of the mutant E-cadherin with the dileucine to dialanine substitution. A, immunoblot of extracts of MDCK cells expressing either wild-type (Ecad) or p120-uncoupled E-cadherin (EAECad) that were transfected with either p120 siRNAs or controls for 48 h. Expression of the p120 isoforms is reduced by ~70% in cells that express the p120 siRNA compared with control cells as assessed by densitometry. B, quantification of the p120 (left) and E-cadherin (right). The relative amounts of the proteins in the p120 siRNA cells were quantified using NIH Image and expressed as a percentage of control RNA cells. These data are the averaged results of the three independent experiments. Error bars represent ± S.D.

**FIGURE 8.** Reduced recycling of p120-uncoupled E-cadherin. A, surface appearance of biotinylated-internalized E-cadherin. Subconfluent monolayers of MDCK cells expressing either wild-type (Ecad) or p120-uncoupled E-cadherin (EAECad) were surface-biotinylated and incubated for 2 h at 18 °C to allow the internalization and accumulation of E-cadherin. After treatment with glutathione to strip the remaining biotinyl groups from cell surface proteins, the cells were released at 37 °C to resume trafficking for 10, 20, or 40 min in the presence of E-cadherin antibodies added to the culture medium. The recycled E-cadherin was collected as described under "Experimental Procedures." B, quantification of the recycled E-cadherin. The relative amounts of the recycled proteins shown in A were quantified using NIH Image and expressed as a percentage of the total, initial internalized pool of protein. These data are the averaged results of the three independent experiments. Error bars represent ± S.D.
endocytosis of both wild-type and p120-uncoupled E-cadherin. This indicates that the dileucine motif is likely to be a signal for the E-cadherin endocytic pathway.

The dileucine motif of E-cadherin is somewhat unusual, as it lacks the acidic amino acids present in the consensus (DE)XX-\(\text{LI}L\) or DXLL sequences (34). However, it is located very close to the transmembrane domain like dileucine motifs in other proteins (34). Although dileucine motifs have been shown to be crucial to mediate rapid endocytosis and generally target receptors to late endosomes or lysosomes, acidic residues prior to the motif appear to be important for targeting to late endosomes or lysosomes but not for internalization (34). The absence of acidic residues prior to the dileucine repeat, therefore, may prevent the efficient lysosomal targeting of E-cadherin. Consistent with this idea, E-cadherin uncoupled for p120 binding is internalized more efficiently and is localized in early endosomes but not lysosomes. Treatment of cells with chloroquine to inhibit lysosomal hydrolases did not change the expression of p120-uncoupled E-cadherin, indicating that the protein was not extensively processed for lysosomal degradation. A recent report showed that ubiquitination is essential for the shuttling of E-cadherin to lysosomes (38). Therefore, it is possible that inhibiting p120 binding to E-cadherin did not result in decreased steady-state levels, because another process, such as ubiquitination, is the limiting step; uncoupling p120 binding alone was not sufficient for lysosomal targeting and subsequent degradation. Although inhibiting p120 binding to E-cadherin does not decrease steady-state level of the protein, depletion of p120 reduces wild-type E-cadherin levels. At present we do not know the reason for this discrepancy. Intracellular vesicular labeling of E-cadherin was detected by incubating the p120 knockdown cells at 18 °C, which is a condition that prevents progression of endocytotic pathways. Therefore, it is possible that other binding interactions involved in post internalization events, including ubiquitination, are perturbed by the mutation.

The dileucine motif of E-cadherin may mediate binding to adaptor complexes, such as AP-2, and thereby couple the protein to clathrin-coated pits (34). The dileucine motif was separated by 13 amino acids from the most amino-terminal residues necessary for p120 binding (Fig. 1B). This study suggests that p120 competes for these interactions and thereby functions to prevent the entry of cadherins into the endocytic pathway. It has been shown that overexpression of p120 reduced the internalization of a chimeric molecule composed of the interleukin-2 receptor extracellular domain and the VE-cadherin cytoplasmic domain, but not a chimera with amino acid substitutions in the p120-binding site that uncouple p120 binding (26). Thus, p120 must bind to the cadherin to function as a plasma membrane retention signal. The p120 effect is selective for VE-cadherin and not the endocytosis pathway globally, because p120 had no discernible impact on transferrin internalization (26). Based on these data, we propose a model in which p120 binding functions to mask the dileucine motif of cadherins, thereby preventing interactions with adaptor proteins and subsequent endocytosis. A similar model has been proposed by the Kowalczyk group (26). Consistent with this model, we found that p120 knockdown enhanced endocytosis of wild-type E-cadherin but not that of the mutant E-cadherin lacking the dileucine motif.

We found that the uncoupling of p120 binding not only increased the uptake of surface-biotinylated E-cadherin, but also decreased its recycling back to the cell surface, suggesting that p120 binding affects at least two transport steps in trafficking. These changes in trafficking of the mutant protein result in more of the protein in endosomes and less on the cell surface at steady state. Although our studies do not provide specific evidence to implicate p120 as a regulator of E-cadherin recycling, p120 has been implicated in other trafficking pathways, such as the movement of vesicular pools of N-cadherin toward intercellular junctions (39). Disappearance of some labeling in the Golgi region upon treatment of cells with cycloheximide suggests the presence of an intracellular newly synthesized pool of the p120-uncoupled E-cadherin. Our results that the p120-uncoupled E-cadherin recycled back to the cell surface at a rate slower than that of wild-type E-cadherin seem to be consistent with this observation. Previous studies showed that endocytic trafficking of E-cadherin is regulated by cell-cell contact (19) or by cellular proteins that include Rho family GTPases, Rac/Cdc42 (18). At present we do not know whether internalization and subsequent recycling of the p120-uncoupled E-cadherin occurs constitutively or regulated. Uncoupling of p120 binding has been shown to inhibit the ability of E-cadherin to activate Rac (40). Recently, it has been shown that p120 is involved in local inhibition of Rho by recruiting p190RhoGAP to membrane domains containing cadherins (41). Therefore it is interesting to determine the molecular mechanisms that regulate endocytosis of this mutant protein.

The results of this study support the conclusion that p120 regulates E-cadherin endocytosis by masking the dileucine motif to prevent interactions with adaptor proteins required for internalization. Although sorting adaptor proteins known to bind to dileucine motifs are candidate receptor proteins that may be responsible for the internalization of E-cadherin (34), the one or more molecules responsible for the recognition of the dileucine motif have yet to be identified. An attempt to identify the binding partner(s) of the dileucine motif is in progress in our laboratory.

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