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Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells

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The mechanisms by which catenins regulate cadherin function are not fully understood, and the precise function of p120 catenin (p120ctn) has remained particularly elusive. In microvascular endothelial cells, p120ctn colocalized extensively with cell surface VE-cadherin, but failed to colocalize with VE-cadherin that had entered intracellular degradative compartments. To test the possibility that p120ctn binding to VE-cadherin regulates VE-cadherin internalization, a series of approaches were undertaken to manipulate p120ctn availability to endogenous VE-cadherin. Expression of VE-cadherin mutants that competed for p120ctn binding triggered the degradation of endogenous VE-cadherin. Similarly, reducing levels of p120ctn using siRNA caused a dramatic and dose-related reduction in cellular levels of VE-cadherin. In contrast, overexpression of p120ctn increased VE-cadherin cell surface levels and inhibited entry of cell surface VE-cadherin into degradative compartments. These results demonstrate that cellular levels of p120ctn function as a set point mechanism that regulates cadherin expression levels, and that a major function of p120ctn is to control cadherin internalization and degradation.

Introduction

Cadherins are a family of cell–cell adhesion molecules that play crucial roles in tissue patterning, cellular growth control, and in the regulation of cell shape and migration (Angst et al., 2001; Perez-Moreno et al., 2003). Cadherins function as calcium-dependent adhesion molecules and interact homophilically with cadherins on adjacent cells (Leckband, 2002). Changes in cadherin function and expression levels are associated with numerous developmental events such as epithelial to mesenchymal transitions, and the loss of cadherin expression is associated with tumor cell invasion and metastasis (El-Bahrawy and Pignatelli, 1998; Conacci-Sorrell et al., 2002; Thiery, 2002). In vascular endothelial cells, the endothelial specific cadherin, VE-cadherin, is particularly important in the regulation of vascular barrier function (Stevens et al., 2000) and in the organization of endothelial cells during angiogenesis (Carmeliet and Collen, 2000; Bazzoni and Dejana, 2001; Dejana et al., 2001). Although a number of studies have demonstrated that cadherins are regulated at the level of gene expression (Hirohashi, 1998), very few studies have investigated post-transcriptional mechanisms that might also be used in the control of cadherin expression levels. Because of the important role for VE-cadherin in the control of vascular permeability and in the regulation of angiogenesis (Carmeliet and Collen, 2000), it is particularly important to identify cellular pathways involved in regulating VE-cadherin cell surface levels in microvascular endothelial cells (MEC).

Similar to other cadherins (Cowin, 1994; Pokutta and Weis, 2002), the VE-cadherin cytoplasmic domain interacts with several binding partners that couple the adhesion molecule to actin (Navarro et al., 1995) and vimentin cytoskeletal networks (Kowalczyk et al., 1998; Shasby et al., 2002; Calkins et al., 2003). Two distinct regions of the cytoplasmic domain of the classical cadherins have been identified, and these domains bind to different subfamilies of armadillo

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Key words: adhesion; cytoskeleton; endocytosis; cadherin; catenin

Abbreviations used in this paper: IL-2R, interleukin-2 receptor; MEC, microvascular endothelial cells; p120ctn, p120 catenin.

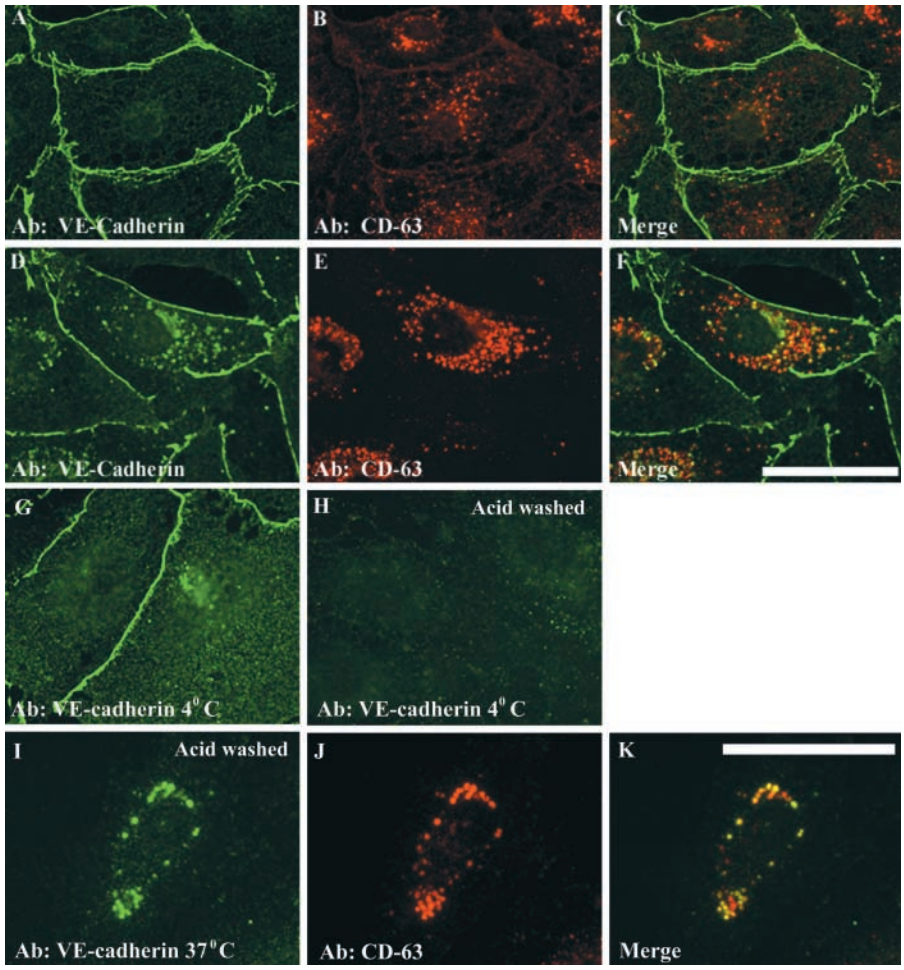

Figure 1. **VE-Cadherin accumulates in an endosomal–lysosomal compartment in chloroquine-treated MEC.** VE-Cadherin localization was examined by immuno–fluorescence microscopy and compared with CD63 localization in untreated MEC (A–C) and in MEC treated with the lysosomal inhibitor chloroquine for 4 h (D–F). Note the extensive accumulation of vesicular VE-cadherin in chloroquine-treated MEC (D) and the colocalization of this intracellular pool with CD63 (F), a marker for late endosomes and lysosomes. To determine if cell surface–derived VE-cadherin was internalized into CD63-positive vesicular compartments, cell surface VE-cadherin was labeled in living MEC at 4°C using an mAb (BV6) directed against the VE-cadherin extracellular domain. The cells were rinsed, fixed, and processed for immunofluorescence microscopy (G). Parallel cultures were labeled at 4°C and transferred to 37°C for 6 h in the presence of chloroquine to allow for cadherin internalization (I–K). After fixation, cells were incubated in an antibody directed against CD63, followed by processing for dual label immunofluorescence microscopy. A low pH wash (acid washed) was used to distinguish cell surface cadherin from internalized cadherin. Note that in H, cell surface VE-cadherin antibody was removed by the low pH wash, indicating that the vesicular VE-cadherin observed in I represents cadherin that was internalized from the cell surface. Bars, 50 μm.

proteins (Anastasiadis and Reynolds, 2000). The membrane distal region of the cadherins interacts with β-catenin and plakoglobin (Cowin and Burke, 1996; Angst et al., 2001; Hatsell and Cowin, 2001). β-Catenin links cadherins to the actin cytoskeleton through direct and indirect interactions with actin binding proteins, such as α-catenin and α-actinin (Knudsen and Wheelock, 1992; Aberle et al., 1994; Jou et al., 1995; Knudsen et al., 1995). Plakoglobin also links cadherins to the actin cytoskeleton, but in addition, plakoglobin interacts with desmoplakin (Kowalczyk et al., 1999; Green and Gaudry, 2000), an intermediate filament binding protein that is important for vascular organization during mammalian development (Gallicano et al., 2001).

In addition to β-catenin and plakoglobin, cadherins interact with a second subfamily of armadillo proteins through a highly conserved domain on the cytoplasmic side of the cadherin membrane spanning domain (Anastasiadis and Reynolds, 2000). This juxtamembrane domain is thought to play an important role in cadherin clustering and in strengthening of cadherin adhesive interactions (Yap et al., 1998). The major binding partner for the cadherin juxtamembrane domain is an armadillo protein termed p120 catenin (p120ctn; Thoreson et al., 2000). p120ctn is part of a group of related armadillo proteins that includes ARVCF, δ-catenin, and p0071 (Hatzfeld, 1999). The juxtamembrane domain of VE-cadherin is known to bind directly to both p120ctn and p0071 (Calkins et al., 2003). Several studies indicate that p120ctn functions to promote cadherin clustering and to strengthen adhesion (Thoreson et al., 2000; Pettitt et al., 2003), but other studies have suggested that p120ctn may function as a negative regulator of cadherin function (Aono et al., 1999; Ohkubo and Ozawa, 1999). Recently, the absence of p120ctn in a colon carcinoma cell line was found to cause a corresponding loss of E-cadherin metabolic stability, indicating an important role for p120ctn in the maintenance of E-cadherin expression in differentiated epithelial cells (Ireton et al., 2002). However, recent studies in *Drosophila melanogaster* indicate that p120ctn is not an essential component of adherens junctions in these organisms (Myster et al., 2003; Pacquelet et al., 2003), underscoring the elusive nature of p120ctn contributions to cadherin function.

Here, we examined whether VE-cadherin internalization and degradation are regulated by armadillo family proteins that bind to the cadherin cytoplasmic tail. Morphological analysis indicated that neither p120ctn nor β-catenin colocalized with VE-cadherin that had entered an endocytic pathway, suggesting that the disruption of catenin binding to the cadherin cytoplasmic tail might be associated with cadherin endocytosis. Consistent with this possibility, expression of cadherin mutants that compete for catenin binding caused the disruption of intercellular junctions and a dramatic down-regulation of endogenous VE-cadherin. Interestingly, competition for p120ctn binding, but not

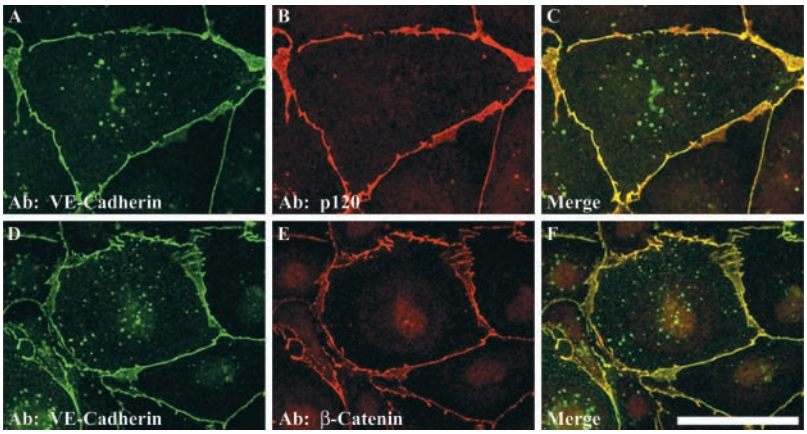

Figure 2. **The internalized pool of VE-cadherin does not colocalize with β-catenin or p120ctn.** Dual label immunofluorescence analysis was performed on MEC treated with chloroquine for 4 h. The internalized pool of VE-cadherin exhibited limited colocalization with (A–C) p120ctn and (D–F) β-catenin. Bar, 50 μm.

β-catenin binding, was found to be critical for the induction of VE-cadherin degradation. Similarly, siRNA knockdown experiments revealed that the loss of p120ctn resulted in a corresponding loss of VE-cadherin. Finally, overexpression of p120ctn inhibited VE-cadherin entry into endocytic compartments, and caused a corresponding increase in cell surface levels of VE-cadherin. These findings indicate that p120ctn levels function as a set point for cadherin expression levels, and demonstrate for the first time that p120ctn regulates cadherin cell surface presentation by preventing cadherin degradation via an endosomal–lysosomal pathway.

Results

VE-Cadherin constitutively enters a degradative pathway in MEC

In previous studies, VE-cadherin was found to enter degradative compartments in response to a VE-cadherin dominant negative mutant (Xiao et al., 2003). To further investigate the regulation of VE-cadherin turnover, the localization of VE-cadherin was examined in the presence or absence of chloroquine. This drug treatment prevents endosome–lysosome acidification, and thereby allows VE-cadherin internalization but prevents lysosomal degradation. In untreated MEC, very little vesicular VE-cadherin was observed (Fig. 1 A). In contrast, in MEC treated with chloroquine for 4 h, extensive accumulation of a vesicular pool of VE-cadherin was evident (Fig. 1 D). To determine if VE-cadherin was entering a degradative compartment, vesicular VE-cadherin in chloroquine-treated cells was examined for colocalization with CD63, a marker for late endosomal and lysosomal compartments (Metzelaar et al., 1991). VE-Cadherin exhibited extensive colocalization with CD63 in chloroquine-treated cells (Fig. 1, D–F), suggesting that VE-cadherin was constitutively internalized and degraded by an endosomal–lysosomal pathway.

To demonstrate directly that the vesicular VE-cadherin observed in chloroquine-treated cells (Fig. 1 D) represented a pool of VE-cadherin that was internalized from the cell surface, mAbs were used to label cell surface VE-cadherin in living MEC cultures. MEC were incubated at 4°C with an mAb directed against the VE-cadherin extracellular domain. The cells were rinsed to remove unbound antibody, fixed, and processed for immunofluorescence microscopy (Fig. 1 G). Binding of the VE-cadherin antibody was removed when the cells were washed in a low pH buffer (acid washed), indicating that the labeled cadherin was present on the cell surface (Fig. 1 H). After incubation with the mAb at 4°C, parallel cultures were returned to 37°C in the presence of chloroquine to allow for cadherin internalization. The cells were acid washed to remove antibody bound to cell surface VE-cadherin, and subsequently fixed and processed for immunofluorescence microscopy to reveal the localization of the internalized cadherin. This approach demonstrated that a cell surface pool of VE-cadherin was internalized and targeted to CD63-positive compartments (Fig. 1, I–K). In nonchloroquine-treated cells, internalized VE-cadherin also could be detected (unpublished data). However, the amount of intracellular VE-cadherin detected was dramatically enhanced by treating the cells with chloroquine, indicating that VE-cadherin is constitutively metabolized by an endosomal–lysosomal pathway. To determine whether VE-cadherin was cointernalized with its catenin binding partners, the localization of β-catenin and p120ctn was determined in endothelial cells treated with chloroquine for 4 h. As shown in Fig. 2, extensive vesicular VE-cadherin accumulation was observed in chloroquine-treated MEC. However, p120ctn (Fig. 2, A–C) and β-catenin (Fig. 2, D–F) exhibited limited colocalization with this vesicular pool of VE-cadherin. Similar results were obtained when VE-cadherin was surface labeled; neither β-catenin nor p120ctn exhibited significant colocalization with the internalized pool of VE-cadherin (see Fig. 7). These results suggest that the cadherin-catenin complex dissociates during VE-cadherin internalization.

Disruption of p120ctn binding to the VE-cadherin cytoplasmic tail causes loss of VE-cadherin expression

The observation that the catenins do not colocalize with VE-cadherin in endocytic compartments raised the possibility that the loss of catenin binding to the VE-cadherin cytoplasmic domain might be causally related to VE-cadherin internalization and degradation. Therefore, we used a series of approaches to manipulate the availability of the catenins for binding to VE-cadherin, and determined the consequences of those manipulations on endogenous VE-cadherin distribution and expression levels.

The first approach undertaken was to express various cadherin mutants to compete with endogenous VE-cadherin for

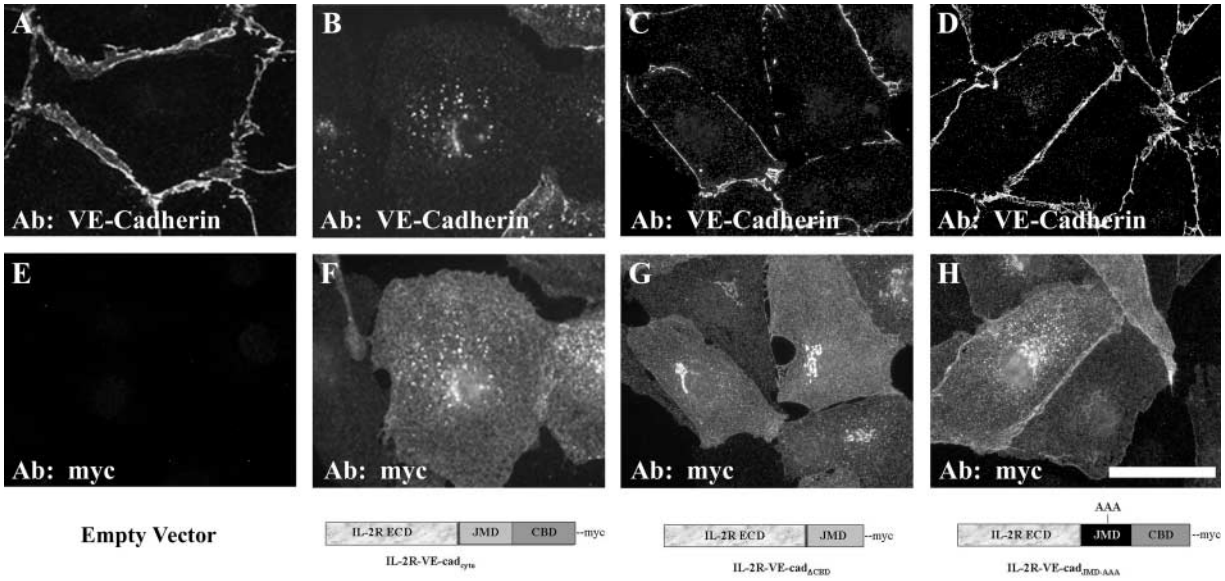

Figure 3. **Cadherin mutants that bind to p120ctn disrupt MEC intercellular junctions.** MEC were infected by (A and E) empty adenoviral vector, (B and F) the IL-2R-VE-cad$_{cyto}$, (C and G) IL-2R-VE-cad$_{\Delta CBD}$, or (D and H) IL-2R-VE-cad$_{JMD-AAA}$ mutants, respectively, for 18 h. The IL-2R-VE-cad$_{\Delta CBD}$ mutant lacks the β-catenin binding site, whereas the IL-2R-VE-cad$_{JMD-AAA}$ mutant contains the β-catenin binding domain, but harbors a triple alanine substitution that abrogates p120ctn binding. The cells were fixed in methanol and processed for dual label immunofluorescence using antibodies directed against (A–D) endogenous VE-cadherin (cad-5 antibody) and (E–H) the myc epitope tag. Bar, 50 μM.

binding to p120ctn and/or β-catenin. In previous studies, the expression of an IL-2 receptor-VE-cadherin chimera comprising the interleukin-2 receptor (IL-2R) extracellular domain and the VE-cadherin cytoplasmic tail (IL-2R-VE-cad$_{cyto}$) was shown to cause the down-regulation of endogenous VE-cadherin (Xiao et al., 2003). Two additional mutants were constructed for the present work (Fig. 3). The IL-2R-VE-cad$_{\Delta CBD}$ mutant lacks the β-catenin binding site and the IL-2R-VE-cad$_{JMD-AAA}$ mutant contains a triple alanine substitution at amino acids 562–564 (EMD-AAA), which abrogates p120ctn and p0071 binding to classical cadherins (Thoreson et al., 2000; Calkins et al., 2003). To determine the localization of endogenous VE-cadherin in MEC expressing the various VE-cadherin mutants, immunofluorescence analysis was performed after infection with adenovirus carrying the empty virus or the various mutants (Fig. 3). An antibody directed against the VE-cadherin extracellular domain was used to specifically identify endogenous VE-cadherin, and antibodies directed against the myc epitope were used to detect the VE-cadherin mutants. In control cells expressing empty virus, extensive VE-cadherin staining was observed at MEC cell borders (Fig. 3 A). In striking contrast, in MEC cultures expressing the IL-2R-VE-cad$_{cyto}$ mutant, endogenous VE-cadherin was distributed in a punctate cytoplasmic distribution (Fig. 3 B). MEC expressing the IL-2R-VE-cad$_{\Delta CBD}$ mutant also exhibited disrupted junctions, as evidenced by the thinning of VE-cadherin staining at intercellular junctions (Fig. 3 C). However, overall, the IL-2R-VE-cad$_{\Delta CBD}$ mutant had somewhat less dramatic effects than the IL-2R-VE-cad$_{cyto}$. Interestingly, the IL-2R-VE-cad$_{JMD-AAA}$ mutant had only minor effects on MEC intercellular junctions (Fig. 3 D), suggesting that competition for

Figure 4. **Cadherin mutants cause down-regulation of endogenous VE-cadherin by competing for p120ctn.** (A) MEC were transduced with adenoviruses carrying the various IL-2R-VE-cad mutants and endogenous VE-cadherin levels were monitored by Western blot analysis. The mutant lacking the p120ctn binding site (IL-2R-VE-cad$_{JMD-AAA}$) failed to cause down-regulation of endogenous VE-cadherin. (B) p120ctn and β-catenin were coexpressed with the IL-2R-VE-cad$_{cyto}$ mutant to determine which armadillo family proteins could prevent the down-regulation of endogenous VE-cadherin in response to the cadherin mutant. p120ctn was able to prevent the down-regulation of endogenous VE-cadherin by the IL-2R-VE-cad$_{cyto}$ mutant, but β-catenin expression did not rescue endogenous VE-cadherin.

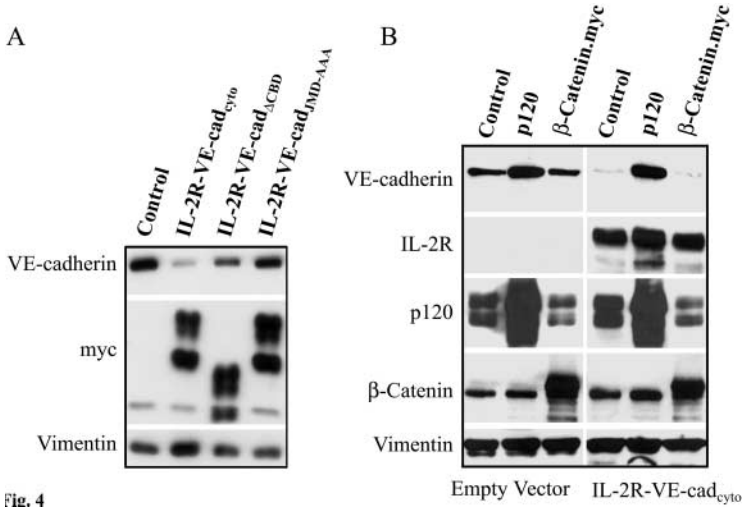

Fig. 4

p120ctn was required for the rapid disruption of MEC junctions in cells expressing mutant cadherins.

To determine the effects of these IL-2R-VE-cad mutants on endogenous VE-cadherin protein levels, the mutants were expressed for 18 h and the level of endogenous VE-cadherin was monitored (Fig. 4 A). Both the IL-2R-VE-cad$_{cyto}$ and the IL-2R-VE-cad$_{\Delta CBD}$ mutant decreased the level of endogenous VE-cadherin compared with empty virus. Interestingly, the IL-2R-VEcad$_{JMD\text{-}AAA}$ mutant had only a modest effect on the level of endogenous VE-cadherin (Fig. 4 A), suggesting that competition for p120ctn but not β-catenin was causing the rapid down-regulation of endogenous VE-cadherin. To test this possibility further, the IL-2R-VE-cad$_{cyto}$ mutant was coexpressed with either β-catenin or p120ctn (Fig. 4 B). Overexpression of p120ctn completely abrogated the ability of the IL-2R-VE-cad$_{cyto}$ mutant to cause down-regulation of endogenous VE-cadherin, whereas β-catenin overexpression had no effect (Fig. 4 B). These results demonstrate that the rapid down-regulation of VE-cadherin in response to cadherin mutants is caused by competition for p120ctn binding to endogenous VE-cadherin.

p120ctn levels function as a set point for cadherin expression levels in MEC

The aforementioned results above demonstrated that competition for p120ctn by mutant cadherins causes the down-regulation of endogenous VE-cadherin. These findings suggested that cellular levels of VE-cadherin might be regulated by the availability of p120ctn. To test this possibility directly, dose-response experiments were performed in which various levels of p120ctn were expressed in MEC using an adenoviral delivery system (Fig. 5). An increase in p120ctn levels resulted in a dose dependent increase in VE-cadherin accumulation, whereas up-regulation of β-catenin levels had little or no effect on VE-cadherin expression levels (Fig. 5 A). Control experiments indicated that overexpression of p120ctn or β-catenin had no effect on the expression of PECAM-1, a member of the Ig family of cell–cell adhesion molecules (Newman et al., 1990; Albelda et al., 1991; Fig. 5 B). Furthermore, expression of exogenous p120ctn increased VE-cadherin cell surface levels as determined by ELISA (Fig. 5 C). Finally, siRNA was used to knock down p120ctn expression in MEC, and the resulting effects on VE-cadherin levels were monitored by Western blot analysis. Transfection with irrelevant negative control siRNA had no effect on p120ctn or VE-cadherin levels (Fig. 5 D). However, Western blot analysis revealed that the loss of p120ctn expression resulted in a dramatic and dose-related down-regulation of VE-cadherin levels (Fig. 5 D). Collectively, these data indicate that cellular levels of VE-cadherin are tightly and specifically coupled to p120ctn expression levels.

p120ctn regulates the internalization and degradation of cell surface VE-cadherin

As shown in Fig. 1, the vesicular pool of VE-cadherin in MEC treated with chloroquine represents cadherin that is localized to degradative endocytic compartments. To directly test if the cytoplasmic availability of p120ctn regulates internalization of cell surface cadherin, live cell labeling and immunofluorescence analysis was performed to monitor VE-cadherin internalization in MEC expressing various cadherin mutants. Cell surface VE-cadherin was labeled at 4°C using an mAb directed against the VE-cadherin extracellular domain. The cells were switched to 37°C for 3 h in the presence of chloroquine to allow for VE-cadherin internalization. Antibody bound to cell surface VE-cadherin was removed using a low pH wash, and the internalized pool of VE-cadherin was detected by fixing and processing the cells for immunofluorescence microscopy. Antibodies directed against the myc epitope tag were applied after fixation to verify expression of the mutant cadherins. Similar to the results shown in Fig. 1, an internalized vesicular pool of VE-cadherin could be detected in control MEC expressing empty adenoviral vector

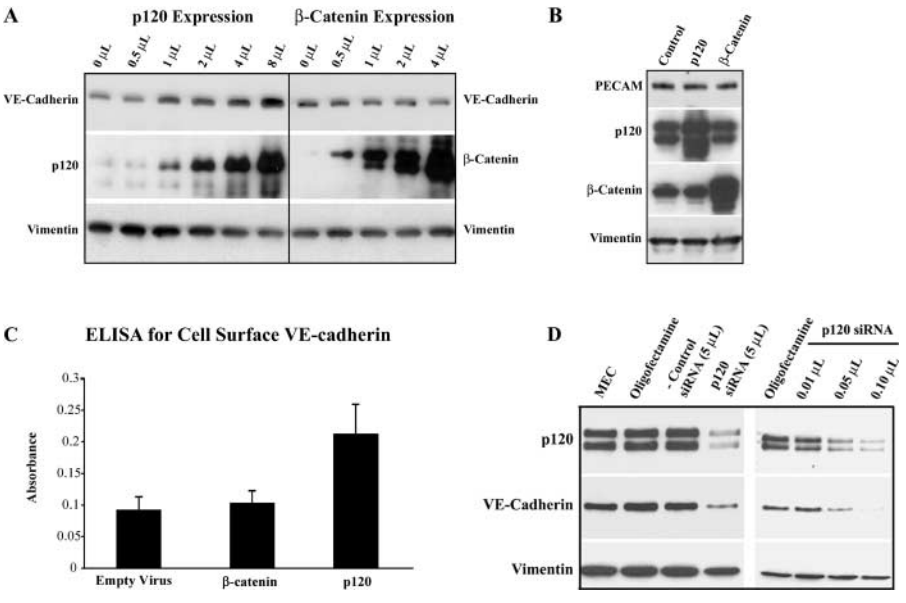

Figure 5. **Cellular levels of VE-cadherin are tightly coupled to p120ctn expression levels.** (A) p120ctn or β-catenin were overexpressed in MEC using an adenoviral delivery system. Expression of exogenous p120ctn leads to a dose-dependent increase in VE-cadherin accumulation, whereas β-catenin has no affect on VE-cadherin levels. (B) Expression levels of PECAM-1 were unaltered in MEC overexpressing p120ctn or β-catenin. (C) Relative cell surface levels of VE-cadherin were monitored using an ELISA on unpermeabilized MEC. Exogenous expression of p120ctn increased cell surface levels of VE-cadherin. Error bars represent the SD. (D) siRNA was used to knock down p120ctn levels in MEC. Western blot analysis was performed to determine p120ctn, VE-cadherin, and vimentin levels in untreated and siRNA-treated MEC. The loss of p120ctn results in a corresponding and dose-related loss of VE-cadherin.

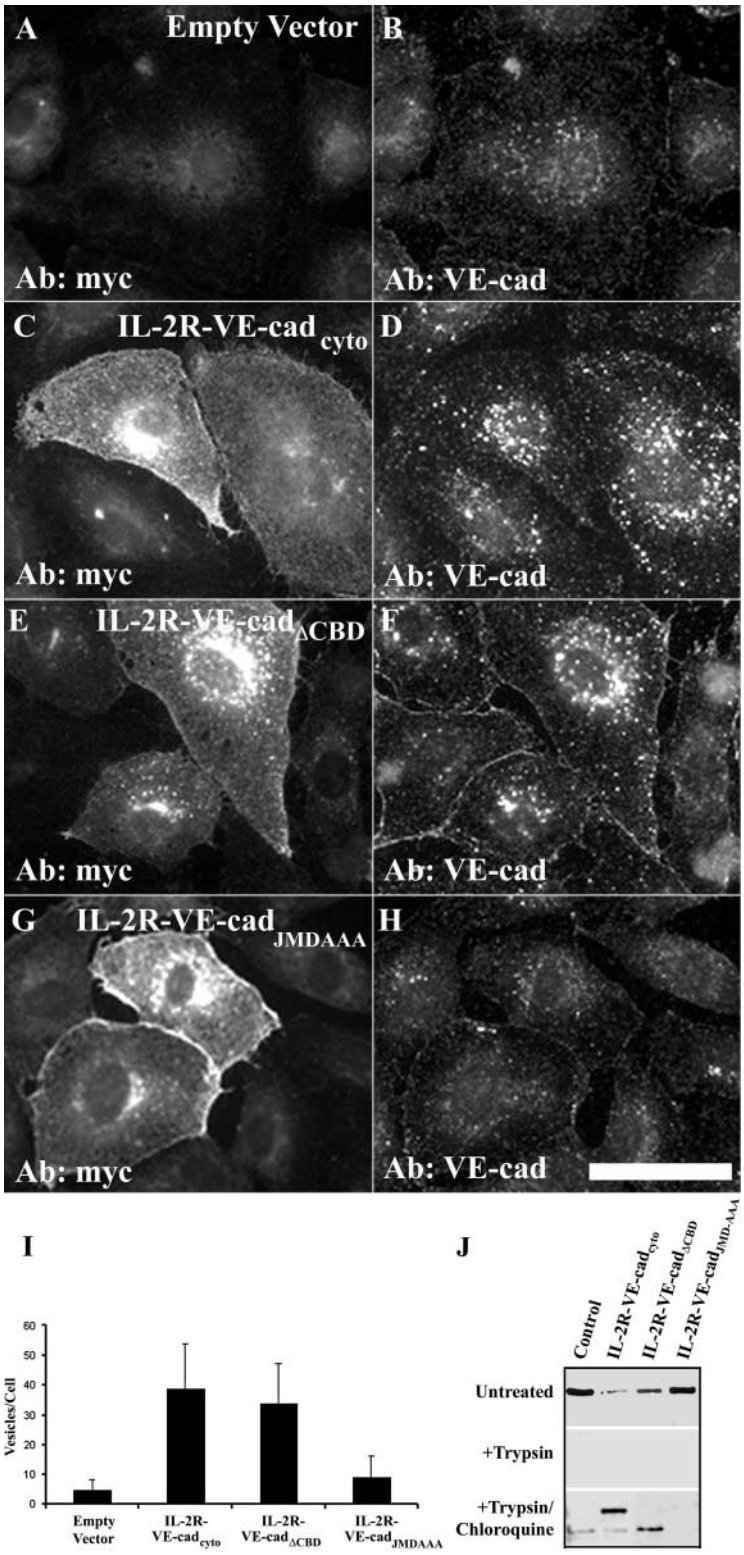

Figure 6. **Sequestration of cytoplasmic p120ctn by exogenously expressed cadherin mutants causes internalization of cell surface VE-cadherin.** The internalization of cell surface VE-cadherin was monitored in MEC expressing various IL-2R-VE-cadherin mutants. MEC were incubated with the BV6 antibody at 4°C and transferred to 37°C for 3 h in the presence of chloroquine. The cells were acid washed to remove surface bound VE-cadherin antibody, and fixed and processed for dual label immunofluorescence microscopy to detect both the internalized cadherin and the IL-2R-VE-cadherin mutants. Internalized VE-cadherin was detected in control MEC expressing empty adenoviral vector (A and B). VE-Cadherin internalization was dramatically increased in MEC expressing either the IL-2R-VE-cad$_{cyto}$ (C and D) or the IL-2R-VE-cad$_{\Delta CBD}$ mutant (E and F). However, the IL-2R-VE-cad$_{JMDAAA}$ mutant (G and H), which does not bind and compete for cytoplasmic p120ctn, failed to cause increased VE-cadherin internalization relative to cells expressing empty vector. (I) Quantitative representation of vesicular VE-cadherin detected in cells expressing the various cadherin mutants (results representative of greater than three independently conducted experiments. Error bars indicate the SD; $n >$ 10 cells). (J) MEC were infected with empty virus, IL-2R-VE-cad$_{cyto,}$ IL-2R-VE-cad$_{\Delta CBD}$, or the IL-2R-VE-cad$_{JMD-AAA}$ mutant and Western blot analysis was performed using an antibody against the extracellular domain of VE-cadherin to monitor endogenous VE-cadherin levels. Expression of the mutants was verified using the c-myc epitope tag (not depicted). Mutant cadherins that bind p120ctn cause down-regulation of endogenous VE-cadherin (top). In untreated cells, VE-cadherin was completely removed by trypsinization (middle). In MEC treated with chloroquine for 8 h, VE-cadherin is detected in trypsinized cells (bottom), indicating that this pool of VE-cadherin is intracellular. The results indicate that the IL-2R-VE-cad$_{cyto}$ and the IL-2R-VE-cad$_{\Delta CBD}$ mutant cause internalization of endogenous VE-cadherin, whereas the IL-2R-VE-cad$_{JMD-AAA}$ mutant does not trigger VE-cadherin internalization. Bar, 50 μm.

(Fig. 6 B). However, VE-cadherin internalization was dramatically increased in MEC expressing either the IL-2R-VE-cad$_{cyto}$ (Fig. 6 D) or the IL-2R-VE-cad$_{\Delta CBD}$ mutant (Fig. 6 F). In contrast, the IL-2R-VE-cad$_{JMDAAA}$ mutant, which does not bind to p120ctn, failed to increase VE-cadherin internalization over background levels (Fig. 6 H). These results are shown quantitatively in Figure 6 I.

To determine if the increase in VE-cadherin internalization observed using this live cell labeling approach correlated with the accumulation of an intracellular pool of VE-cadherin, MEC expressing the cadherin mutants were treated with chloroquine to prevent lysosomal degradation and trypsinized to remove cell surface cadherin (Fig. 6 J). In untreated cells, the IL-2R-VE-cad$_{cyto}$ mutant and the IL-2R-

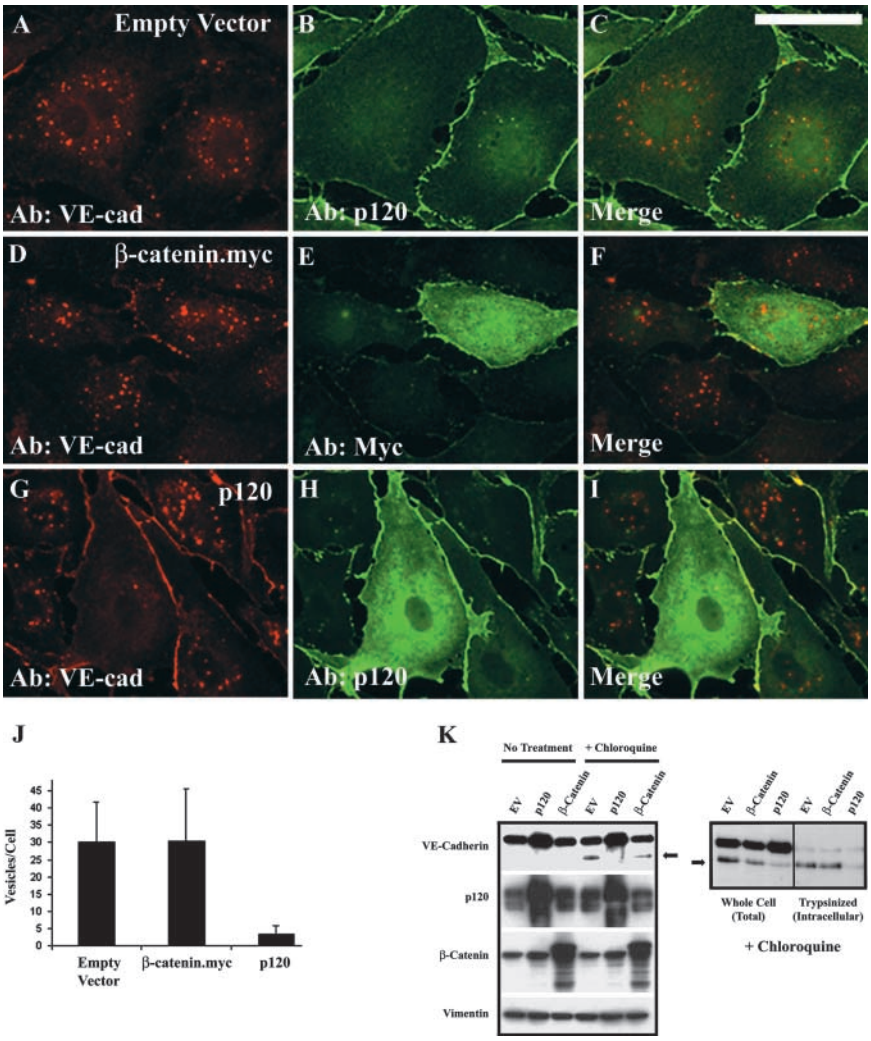

Figure 7. **Expression of exogenous p120ctn decreases internalization of cell surface VE-cadherin.** MEC were transduced with adenovirus carrying β-catenin or p120ctn overnight. The cells were surfaced labeled with the BV6 VE-cadherin mAB and transferred to 37°C for 6 h in the presences of chloroquine to allow for the accumulation of internalized VE-cadherin (Fig. 1). The cells were acid washed to remove cell surface cadherin and processed for immunofluorescence microscopy to detect both internalized VE-cadherin and the exogenously expressed catenins. Extensive vesicular accumulation of internalized VE-cadherin was apparent in chloroquine-treated MEC expressing (A–C) empty virus or (D–F) β-catenin. In contrast, expression of exogenous p120ctn dramatically inhibited (G–I) VE-cadherin internalization. (J) Quantitative representation of the results shown in A–I (results representative of three independent experiments. $n > 10$ cells. Error bars represent the SD. (K) MEC were transduced with adenovirus carrying p120ctn or β-catenin. The cells were chloroquine treated overnight and harvested for Western blot analysis. As reported previously (Xiao et al., 2003), chloroquine treatment results in the accumulation of an intracellular truncated form of VE-cadherin (arrows). To distinguish intracellular from cell surface cadherin, cells were trypsinized before harvesting the cells for Western blot analysis to remove cell surface pools of cadherin (K, right). p120ctn, but not β-catenin, inhibited the accumulation of this intracellular-processed form of VE-cadherin. Bar, 50 μm.

VE-cad$_{\Delta CBD}$ caused down-regulation of endogenous VE-cadherin, whereas the IL-2R-VE-cad$_{JMD\text{-}AAA}$ did not significantly alter endogenous VE-cadherin levels (Fig. 6 J, top). Trypsinization of untreated cells quantitatively removed cell surface VE-cadherin (Fig. 6 J, middle). However, in MEC treated with chloroquine for 8 h before trypsinization (Fig. 6 J, bottom), significant levels of intracellular VE-cadherin were detected in trypsinized MEC expressing either the IL-2R-VE-cad$_{cyto}$ and the IL-2R-VE-cad$_{\Delta CBD}$ mutants, but not the IL-2R-VE-cad$_{JMD\text{-}AAA}$ mutant (Fig. 6 J). As described previously (Xiao et al., 2003), chloroquine treatment results in the intracellular accumulation of a 100-kD processed form of VE-cadherin lacking the β-catenin binding domain (Fig. 6 J, bottom band). Interestingly, in cells expressing the IL-2R-VE-cad$_{cyto}$ mutant, the intracellular VE-cadherin remains intact, and is not processed to the 100-kD fragment. The precise reason for this difference is not known, but this result is highly reproducible and may reflect competition between the IL-2R-VE-cad$_{cyto}$ mutant and endogenous VE-cadherin for a processing event. These results indicate that mutant cadherins that compete for p120ctn cause internalization of cell surface cadherin (Fig. 6, A–H) and lead to the accumulation of intracellular pools of cadherin in chloroquine-treated cells (Fig. 6 J). These data suggest that mutant cadherins trigger the internalization and degradation of endogenous cadherins through an endosomal–lysosomal pathway, and that competition for p120ctn causes VE-cadherin internalization.

If the depletion of cytoplasmic p120ctn by cadherin mutants increases VE-cadherin internalization, then increased levels of p120ctn in the cytosol should inhibit cadherin internalization. To test this possibility, exogenous p120ctn was expressed in MEC and the amount of VE-cadherin internalization was monitored. For these experiments, MEC were surface labeled at 4°C with VE-cadherin antibody and switched to 37°C for 6 h in the presence of chloroquine to allow for extensive VE-cadherin internalization. Under these conditions, numerous cytoplasmic vesicles containing cell surface-derived VE-cadherin were visualized (Fig. 7 A). Very little p120ctn (Fig. 7 B) or β-catenin (not depicted) colocalized with the internalized cadherin. Expression of exogenous β-catenin had no discernible impact on the amount of VE-cadherin internalization (Fig. 7, D–F). In contrast, expression of exogenous p120ctn dramatically inhibited entry of cell surface VE-cadherin into cytoplasmic vesicles (Fig. 7, G–I). These results suggested that p120ctn expression was inhibiting constitutive internalization and degradation of VE-cadherin. Therefore, the accumulation of an intracellu-

lar pool of VE-cadherin in chloroquine-treated cells was monitored by Western blot analysis in MEC expressing p120ctn or β-catenin. As discussed above (Fig. 6 J), chloroquine treatment results in the accumulation of an intracellular 100-kD processed form of VE-cadherin. As shown in Fig. 7 K, the accumulation of this intracellular pool of VE-cadherin was prevented by the expression of exogenous p120ctn but not β-catenin. These data are consistent with the inhibition of VE-cadherin internalization observed in MEC expressing exogenous p120ctn (Fig. 7, G–I). Collectively, these findings indicate that cytoplasmic availability of p120ctn regulates the delivery of cell surface VE-cadherin into endocytic compartments for endosomal–lysosomal degradation.

Discussion

The results of the present work indicate that VE-cadherin is constitutively internalized and degraded in MEC, and that the armadillo family protein p120ctn plays a central role in regulating this process. p120ctn was found to regulate cadherin expression by controlling entry of the cadherin into an endosomal–lysosomal pathway. These findings reveal a new mechanism by which armadillo family proteins contribute to the regulation of cadherin-mediated cell adhesion, and clearly establish a role for p120ctn as a modulator of the adhesive properties of the plasma membrane in MEC.

The findings presented here reveal a remarkable reciprocity in the regulation of cadherin and catenin expression levels. Previous studies demonstrated that cytoplasmic pools of β-catenin and plakoglobin are metabolically unstable, and that cadherin binding to β-catenin or plakoglobin rescues these armadillo family proteins from degradation (Kowalczyk et al., 1994; Aberle et al., 1997). Because of the important functions of β-catenin in the regulation of gene expression, the rapid turnover of cytosolic β-catenin is central to the regulation of β-catenin entry into the nucleus and in the modulation of cell proliferation and migration (Willert and Nusse, 1998; Polakis, 1999; Gottardi and Gumbiner, 2001). As shown here, p120ctn rescues VE-cadherin from entry into a degradative pathway. VE-Cadherin is degraded in an endosomal–lysosomal compartment when p120ctn is removed from the cadherin tail, either by competition with cadherin mutants or by siRNA knock down approaches. Because cadherins antagonize β-catenin signaling (Heasman et al., 1994; Fagotto et al., 1996; Sadot et al., 1998), the regulation of cadherin expression by p120ctn may control β-catenin availability to signal transduction pathways. Thus, interactions between p120ctn and the cadherin cytoplasmic domain may function as a global regulator of cadherin and catenin signaling.

The role of p120ctn in junction assembly and cadherin function has been difficult to establish and the results obtained through the use of various model systems have been difficult to reconcile (Aono et al., 1999; Ohkubo and Ozawa, 1999; Thoreson et al., 2000). The Reynolds laboratory recently demonstrated in a colon carcinoma cell line that the loss of p120ctn expression due to gene mutation resulted in a corresponding loss of E-cadherin (Ireton et al., 2002). This was an important finding because it provided a potential explanation for the widespread down-regulation of E-cadherin expression that is observed in tumor cells, even when mutations in the E-cadherin gene are not apparent (Thoreson and Reynolds, 2002). However, recent studies in *Drosophila* indicate that p120ctn is not required for adherens junction assembly or for DE-cadherin expression (Myster et al., 2003; Pacquelet et al., 2003). It is possible that certain tumor cell lines might harbor additional genetic anomalies that would render E-cadherin vulnerable to the loss of p120ctn. However, our current results using siRNA knock down approaches in primary cultures of MEC indicate clearly that p120ctn is required for cadherin expression in normal human cells. The reason for this apparent discrepancy between flies and mammalian systems is not clear. However, it is interesting that vertebrates express not only p120ctn, but also several other related armadillo family members, including ARVCF, δ-catenin, and p0071 (Hatzfeld, 1999; Anastasiadis and Reynolds, 2000). Thus, the appearance of multiple p120ctn family members in vertebrates may reflect the evolution of distinct cadherin regulatory mechanisms that are required for tissue patterning or integrity in higher organisms.

The present work, as well as our previous work (Xiao et al., 2003), indicates that cadherin mutants trigger endocytosis and degradation of endogenous cadherins. Elimination of the p120ctn binding site on the mutant cadherin severely compromised the ability of the mutant to trigger VE-cadherin internalization (Fig. 6). In contrast, deletion of the β-catenin binding domain had very little effect on this process, at least over the relatively short time courses that were examined. Thus, we conclude that VE-cadherin mutants trigger degradation of endogenous VE-cadherin by competing for p120ctn binding. This interpretation is based on the fact that overexpression of p120ctn could prevent the down-regulation of endogenous VE-cadherin, whereas β-catenin overexpression could not. Furthermore, knock down of p120ctn levels using siRNA resulted in a corresponding decrease in VE-cadherin expression. Interestingly, overexpression of N-cadherin also caused the down-regulation of VE-cadherin in this model system (unpublished data). These data are consistent with previous studies suggesting that cells possess mechanisms that function as sensors for cadherin levels (Troxell et al., 1999). The results presented here indicate that p120ctn is the central component of this sensing mechanism.

Recently, endocytosis has emerged as a regulatory mechanism that modulates cadherin cell surface levels in epithelial cells (Le et al., 1999; Akhtar and Hotchin, 2001; Palacios et al., 2002). E-Cadherin is internalized and recycled back to the plasma membrane (Le et al., 1999), and this process is modulated by PKC (Le et al., 2002). It is formally possible that p120ctn does not regulate the initial cadherin internalization event, but rather that p120ctn regulates subsequent sorting decisions. The juxtamembrane domain of E-cadherin binds to Hakai, an E3 ubiquitin ligase that targets E-cadherin for internalization and degradation in epithelial cells (Fujita et al., 2002). Although Hakai binds to sequences unique to E-cadherin, these findings suggest that the cadherin juxtamembrane domain, and proteins that bind this region of cadherins, are critical in the control of cadherin expression levels. Consistent with this possibility,

we found that p0071, which also binds to the VE-cadherin juxtamembrane domain (Calkins et al., 2003), also regulates VE-cadherin expression levels (unpublished data). Reynolds and colleagues (Ireton et al., 2002) found that p120ctn mutants that are unable to bind to E-cadherin are also unable to rescue E-cadherin expression in a p120ctn null background. These data suggest that p120ctn family proteins bind to the cadherin juxtamembrane domain and function as a "cap" that prevents cadherin internalization and degradation. Such a model is consistent with the competitive association of Hakai and p120ctn for the cadherin juxtamembrane domain (Fujita et al., 2002) and with the apparent dissociation of p120ctn from the cadherin tail (Fig. 2) during endocytosis. Regardless of the precise mechanism, this work reveals a key role for p120ctn in the regulation of cadherin cell surface levels by modulating cadherin delivery to degradative endocytic pathways.

Materials and methods

Cell culture

Primary cultures of human dermal MEC were obtained from the Emory Skin Diseases Research Center (Core B) and cultured as described previously (Venkiteswaran et al., 2002; Xiao et al., 2003). In brief, the cells were grown in MCDB131 medium (Invitrogen) supplemented with 10% FBS (HyClone), L-glutamine (Mediatech, Inc.), cAMP (Sigma-Aldrich), hydrocortisone (Sigma-Aldrich), EGF (Intergen), and antibiotic/antimycotic (Invitrogen). Cells were typically cultured overnight and grown to 80% confluence for most experiments. For adenovirus production, a human embryonic kidney cell line QBI-293A (Qbiogene) was routinely cultured in DME supplemented with 10% FBS and penicillin/streptomycin/amphotericin. Chloroquine was purchased from Sigma-Aldrich and used at 100 μM.

cDNA constructs

A cDNA clone encoding full-length human VE-cadherin was provided by E. Dejana (FIRC Institute of Molecular Oncology, Milan, Italy; Navarro et al., 1995), and an expression construct encoding the extracellular and transmembrane domains of the IL-2R was provided by S. LaFlamme (Albany Medical College, Albany, NY; LaFlamme et al., 1994). This IL-2R construct was used to generate a chimeric cDNA with the IL-2R extracellular domain, the entire VE-cadherin cytoplasmic domain, and a carboxyl-terminal c-myc epitope tag, as described previously (Venkiteswaran et al., 2002). A deletion mutant of the VE-cadherin cytoplasmic tail lacking the catenin binding domain of VE-cadherin was constructed based on a previous report in which the catenin binding domain of VE-cadherin was mapped (Navarro et al., 1995). This catenin binding domain deletion construct encodes VE-cadherin amino acid positions 621–702 followed by a carboxyl-terminal c-myc epitope tag. To generate this construct, a VE-cadherin cDNA was used as a template for PCR using the 5′ primer 5′ATGGAAGCTTCGGCGGCGGCTCCGGAAGCAGGCC3′, which includes a HindIII site and the 3′ primer 5′ACGTCTCGAGCTACAAGTCCTCTTCAGAAATGAGCTTTTGCTCCACGGGCCCTCCGTGTGC3′, which includes a c-myc tag, stop codon, and a XhoI site. The resulting PCR product was ligated in frame to the IL-2R extracellular domain using the HindIII and XhoI restriction sites. An additional VE-cadherin cytoplasmic mutant lacking the p120ctn binding domain was generated as described elsewhere (Calkins et al., 2003). This mutant encodes the VE-cadherin cytoplasmic tail with mutations altering the sequence EMD-AAA at amino acid positions 562–564, which abrogates binding of the VE-cadherin cytoplasmic tail to p120ctn as determined by yeast two hybrid analysis (Calkins et al., 2003). This mutant lacking the p120ctn binding site was ligated to the IL-2R extracellular domain as described above to generate the IL-2R-VE-cad$_{JMD\text{-}AAA}$ mutant. p120ctn 1A cDNA was provided by A.B. Reynolds (Vanderbilt University School of Medicine, Nashville, TN) and a human, myc tagged β-catenin cDNA was obtained from P. McCrea (University of Texas M.D. Anderson Cancer Center, Houston, TX).

Adenovirus production

The VE-cadherin mutants, β-catenin, and p120ctn 1A were subcloned into the pAd-Track vector, which coexpresses GFP with the cDNA of interest (He et al., 1998). Adenoviruses carrying the VE-cadherin constructs, p120ctn, and β-catenin were produced using the pAdeasy adenovirus-packaging system as described previously (Xiao et al., 2003). For most experiments, infection rates of 80–90% were used as monitored by GFP expression.

Immunofluorescence

MEC cultured on gelatin-coated glass coverslips were fixed in methanol or 3.7% PFA followed by extraction in 0.5% Triton X-100. Endogenous VE-cadherin was detected using mouse mAbs cad-5 (Transduction Laboratories), BV6 (Research Diagnostics Inc.), or a goat polyclonal VE-cadherin antibody (Santa Cruz Biotechnology, Inc.). VE-Cadherin mutants were followed using a rabbit antibody directed against the c-myc epitope tag (Bethyl Laboratories, Inc.). The localization of p120ctn and β-catenin was determined using rabbit polyclonal antibodies against p120ctn (Santa Cruz Biotechnology, Inc.) or β-catenin (Neo Markers), respectively. An mAb H5C6 directed against CD63 (Developmental Studies Hybridoma Bank at the University of Iowa) was used to detect late endosomes/lysosomes. Secondary antibodies conjugated to various Alexa Fluors (Molecular Probes) were used for dual label immunofluorescence. The mouse BV6 and CD63 antibodies were distinguished using secondary antibodies specific for IgG2a and IgG1 subtypes. Microscopy was performed using a fluorescence microscope (model DMR-E; Leica) equipped with narrow band pass filters and a camera (model Orca; Hamamatsu). Images were captured, pseudo colored, and processed using Open Lab software (Improvision Inc.).

VE-Cadherin internalization assay

VE-cadherin internalization assays were performed using procedures adapted from Paterson et al. (2003). An mAb directed against the VE-cadherin extracellular domain (BV6) was dialyzed into MCDB 131 medium containing 20 mM Hepes and 3% BSA. The dialyzed antibody was incubated with MEC cultures at 4°C for 1 h. Unbound antibody was removed by rinsing cells in ice-cold MCDB 131. Cells were incubated at 4°C or transferred to 37°C for various amounts of time (3–6 h) in the presence of 100–150 μM chloroquine. To remove cell surface bound antibody while retaining internalized antibody, cells were washed for 15 min in PBS, pH 2.7, containing 25 mM glycine and 3% BSA. The cells were rinsed, fixed, and processed for dual label immunofluorescence as described in the previous paragraph. For experiments to monitor internalization in response to the IL-2R-VE-cadherin mutants, MEC were infected with adenovirus for 6 h to allow time for infection and expression of the mutants. The cells were surface labeled at 4°C and transferred to 37°C for 3 h. To determine if p120ctn inhibits internalization, cells were infected with p120ctn or β-catenin overnight to allow expression of the proteins. Cells were labeled at 4°C and transferred to 37°C for 6 h to allow time for significant levels of internalization in control cells. In each case, the amount of vesicular VE-cadherin present was quantified by a blinded observer by counting VE-cadherin vesicles/cell.

Western blot analysis

MEC were harvested in Laemmli gel sample buffer (Bio-Rad laboratories) and analyzed by SDS-PAGE and immunoblot using antibodies directed against the extracellular domain of VE-cadherin (cad-5; Transduction Laboratories), the myc epitope tag (Bethyl Laboratories), p120ctn (Santa Cruz Biotechnology, Inc.), β-catenin (Transduction Laboratories or Neo Marker), PECAM-1 (Santa Cruz Biotechnology, Inc.), or vimentin (V9; Sigma-Aldrich). HRP-conjugated secondary antibodies (Bio-Rad Laboratories) were used at 1:3,000 dilution and detected using ECL (Amersham Biosciences). In some experiments, MEC were rinsed and incubated in trypsin/EDTA at 37°C for 2 min to proteolytically remove cell surface VE-cadherin before Western blot analysis (Xiao et al., 2003). Trypsin was inactivated using normal growth medium and cells recovered by centrifugation. Cell pellets were dissolved in SDS-PAGE sample buffer for Western blot analysis. Control cells were harvested in SDS-PAGE sample buffer without trypsinization.

siRNA

Inhibition of p120ctn expression in MEC was performed using p120ctn-directed siRNA reagents. A human p120ctn-specific 21-nt siRNA (5′-AACGAGGTTATCGCTGAGAAC-3′) was constructed using the Silencer™ siRNA Construction Kit (Ambion). MEC were transfected with the 21-nt duplexes using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Ambion's Silencer™ negative control siRNAs were purchased and used as controls. Cells were rinsed and harvested for Western blot analysis 48–72 h after transfection.

ELISA

ELISA was performed to measure cell surface VE-cadherin levels. MEC were seeded into a 96-well plate overnight and infected by empty virus or adenoviruses carrying p120ctn or β-catenin for 18 h. Cells were rinsed and fixed in 1% formaldehyde at room temperature for 10 min. Cells were blocked in HBSS supplemented with 1 mM Ca^{2+} and 10% FBS for 45 min, and incubated in a mouse VE-cadherin antibody directed against the cadherin extracellular domain (BD Biosciences). Antibodies directed against vimentin were used as negative controls to verify that only cell surface antigens were being detected in the assay. HRP-conjugated goat anti–mouse secondary antibodies (Bio-Rad Laboratories) were used at 1:500 and detected using TMB One Step Substrate System (DakoCytomation). The reaction was stopped using 8 N sulfuric acid and the results were determined by Microplate Autoreader.

We acknowledge Dr. S. LaFlamme for providing cDNA reagents, and Drs. M. Powers and K.J. Green for helpful comments and suggestions. We gratefully acknowledge Dr. A. Reynolds for providing cDNA reagents. Special thanks to S. Summers for assistance with adenoviral reagents.

This work was supported by the American Cancer Society (RPG CSM-100348), American Heart Association (AHA) (0355293B), and by National Institutes of Health grants (1R01AR048266 and P30AR042687). K. Xiao was supported by an AHA fellowship.

Submitted: 2 June 2003
Accepted: 19 September 2003

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