p120-Catenin Inhibits VE-Cadherin Internalization through a Rho-independent Mechanism

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p120-catenin is a cytoplasmic binding partner of cadherins and functions as a set point for cadherin expression by preventing cadherin endocytosis, and degradation. p120 is known to regulate cell motility and invasiveness by inhibiting RhoA activity. However, the relationship between these functions of p120 is not understood. Here, we provide evidence that p120 functions as part of a plasma membrane retention mechanism for VE-cadherin by preventing the recruitment of VE-cadherin into membrane domains enriched in components of the endocytic machinery, including clathrin and the adaptor complex AP-2. The mechanism by which p120 regulates VE-cadherin entry into endocytic compartments is dependent on p120's interaction with the cadherin juxtamembrane domain, but occurs independently of p120's prevention of Rho GTPase activity. These findings clarify the mechanism for p120's function in stabilizing VE-cadherin at the plasma membrane and demonstrate a novel role for p120 in modulating the availability of cadherins for entry into a clathrin-dependent endocytic pathway.

INTRODUCTION

VE-cadherin is a central component of endothelial adherens junctions and mediates homophilic interactions between neighboring endothelial cells. Adhesion and signaling events mediated by VE-cadherin play key roles in vascular barrier function and angiogenesis (Vestweber, 2008; Wallez and Huber, 2008). Likewise, several endothelial signaling pathways are known to regulate endothelial cell–cell interactions through regulation of VE-cadherin or other adherens junction molecules (Gavard and Gutkind, 2006; Lampugnani et al., 2006). The cytoplasmic domain of VE-cadherin interacts with members of the armadillo family of proteins, including β-catenin and p120-catenin (p120). These interactions regulate association of cadherins with the actin cytoskeleton and are important for strong cell–cell adhesion (Pokutta and Weis, 2007; Hartsock and Nelson, 2008). Furthermore, gene ablation studies have revealed key roles for VE-cadherin, β-catenin, and p120 in vascular development, underscoring the importance of adherens junction components in vascular biology and vertebrate development (Carmeliet et al., 1999; Gory-Faure et al., 1999; Cattelino et al., 2003; Xiao, Oas, and Kowalczyk, unpublished observations).

An important aspect of the ability of cells to dynamically modulate their adhesive state is the regulation of cadherin availability at the cell surface. One cellular mechanism that is commonly used to rapidly alter the expression of cell surface receptors is membrane trafficking. Cells utilize several endocytic pathways to retrieve receptors from the cell surface. The most common of these is clathrin-dependent, in which transmembrane cargos are sorted into clathrin-coated pits at the plasma membrane. Endocytic adaptor proteins, including the tetrameric adaptor complex AP-2, play a fundamental role in the recruitment and formation of clathrin-coated vesicles at the plasma membrane through the ability to bind to lipids, cargo, clathrin, and clathrin accessory proteins (Bonifacino and Traub, 2003; Maldonado-Báez and Wendland, 2006). Clathrin-independent pathways for receptor endocytosis are increasingly recognized as important portals of internalization (Mayor and Pagano, 2007). Cadherins can be internalized through both clathrin-dependent and -independent pathways, depending on the cadherin and the cellular context (Chiasson and Kowalczyk, 2008; Delva and Kowalczyk, 2008). Evidence suggests that E-cadherin and VE-cadherin are primarily internalized through clathrin-dependent pathways. However, a recent study demonstrated that the desmosomal cadherin desmoglein 3 is internalized through a clathrin- and dynamin-independent pathway (Delva et al., 2008). It remains unclear how the decision for cadherins to enter different endocytic compartments is regulated according to the needs of the cell.

Several years ago, a series of studies revealed a core function for p120 in regulating cadherin-mediated adhesion. p120 acts as a set point for cadherin expression by controlling the amount of cadherin available at cell junctions for adhesion (Davis et al., 2003; Xiao et al., 2003, 2007; Chiasson and Kowalczyk, 2008). In the absence of p120, cadherin-based junctions are destabilized, and the cadherin is targeted for degradation through an endosomal–lysosomal pathway. In addition to regulating cadherin endocytosis,

p120 also functions in a signaling capacity as a potent regulator of Rho-GTPase activity within the cell (Anastasiadis, 2007). Rho-GTPases make up a large family of proteins that regulate cytoskeletal organization. The first evidence for the involvement of p120 in regulating Rho-GTPase signaling was the observation of a dramatic branching phenotype in cells expressing high levels of exogenous p120 characterized by the formation of long dendritic processes extending from the cell body (Reynolds *et al.*, 1996; Anastasiadis *et al.*, 2000; Noren *et al.*, 2000). These and other studies have demonstrated that p120 acts as an inhibitor of RhoA, and through this activity p120 appears to regulate cell migration, proliferation, and inflammatory responses.

Although p120's functions in stabilizing cadherins and regulating RhoA signaling are well established, the relationship between these roles is unclear. Early studies suggested that p120-mediated inhibition of RhoA activity is spatially restricted to the cytoplasm and therefore mutually exclusive with the role of p120 at adherens junctions (Anastasiadis *et al.*, 2000; Fox and Peifer, 2007; Yanagisawa *et al.*, 2008). However, a recent study has proposed a potential mechanism linking these functions of p120, at least in certain cellular contexts. After PDGF signaling in fibroblasts, p120 was found to be required for the localized inhibition of RhoA activity through a mechanism that requires the recruitment of p190RhoGAP to cadherin-based junctions. Interestingly, p190RhoGAP not only inhibits RhoA, but also promotes adhesion (Wildenberg *et al.*, 2006). In the absence of p190RhoGAP, N-cadherin is mislocalized from cell junctions. These data provide the first evidence that localized inhibition of Rho activity by p120 at cell junctions may regulate cadherin localization and stability. These data raise the possibility that p120-mediated inhibition of RhoA may in turn regulate entry of cadherins into an endocytic pathway.

In the present article, we directly tested whether p120 inhibition of endocytosis and regulation of RhoA are mechanistically interdependent. Using a series of approaches, we demonstrate that p120 potently inhibits entry of the cadherin into clathrin- and AP-2–enriched membrane domains. VE-cadherin is internalized through a pathway that is dependent on clathrin, dynamin, and the clathrin adaptor complex AP-2. Lastly, we found that the ability of p120 to inhibit cadherin endocytosis can be experimentally uncoupled from p120 inhibition of RhoA, indicating that binding to the cadherin tail, but not the ability of p120 to inhibit Rho, is the crucial function of p120 in preventing endocytosis. These findings clarify the mechanism by which p120 regulates cadherin endocytosis and distinguish this activity from p120 regulation of RhoA.

MATERIALS AND METHODS

Cell Culture

Primary cultures of dermal microvascular endothelial cells (MECs) from human neonatal foreskin were isolated and cultured in MCDB 131 medium as described previously (Xiao *et al.*, 2005). The medium was supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), L-anyl-glutamine (Mediatech, Herndon, VA), cAMP (Sigma-Aldrich), hydrocortisone (Sigma-Aldrich), epidermal growth factor (Intergen, Purchase, NY), and antibiotic/antimyotic (Invitrogen, Carlsbad, CA). HMEC-1 cells were cultured as previously described (Venkiteswaran *et al.*, 2002). Cells were generally cultured overnight on 0.1% gelatin-coated plates and grown to ~80% confluency for experiments. For adenovirus production human embryonic kidney cell line QBI-293A (Qbiogene, Carlsbad, CA) were routinely cultured in DMEM supplemented with 10% FBS and antibiotic/antimyotic. Chloroquine was purchased from Sigma-Aldrich and used at 100 μM. Cell-permeable C3 Transferase was purchased from Cytoskeleton (Denver, CO) and used at 1.0 μg/ml for experiments. Y27632 was purchased from Calbiochem (San Diego, CA) and used at 10 μM.

Adenovirus Production

Adenoviral reagents for the interleukin (IL)-2R-VE-cad$_{cyto}$ chimera, IL-2R-Dsg3$_{cyto}$ chimera, and p120ctn 1A were generated as described previously (Xiao *et al.*, 2005). To generate the p120-4A K622,628A adenovirus, PCR was performed using a p120-1A K622,628A construct (provided by A. Reynolds, Vanderbilt University, Nashville, TN) as a template and the forward primers consisting of the start site of p120-4A isoform with a NotI restriction site and Kozak (5'-GCGGCCGCGCCACCATGATTGGTGAGGAGGTGCCA-3') along with the reverse primer containing a SwaI site and the C-terminus of p120 (5'-ATTTAAATGAATCTTCTGCATCAAGGGTGC-3'). The recombinant fragment was placed in pShuttle in front of enhanced green fluorescent protein (EGFP). Adenovirus was then produced using the AdEasy packaging system described previously (Xiao *et al.*, 2005). The dominant negative DynII (K44A) adenovirus (Altschuler *et al.*, 1998) was provided by S. Schmid (The Scripps Institute, La Jolla, CA), and the dominant negative RhoA (N19) adenovirus (Kalman *et al.*, 1999) was provided by D. Kalman (Emory University, Atlanta, GA).

Immunofluorescence

Immunofluorescence was carried out as described previously (Xiao *et al.*, 2005). Antibodies used were as follows: rabbit anti VE-cadherin (Alexis Biochemicals, San Diego, CA), anti-IL-2R IgG from 7G7B6 mouse hybridoma (American Type Culture Collection, Manassas, VA), chicken anti-myc epitope tag (Bethyl Laboratories, Montgomery, TX), a mouse mAb (BD Biosciences Pharmigen, San Diego, CA) or a polyclonal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) against p120, mouse mAb against dynamin (BD Biosciences Pharmigen), mouse mAb against RhoA (Santa Cruz Biotechnology), mouse mAbs against clathrin (BD Transduction Laboratories, Lexington, KY) or AP-2 (Santa Cruz Biochemicals), a rabbit antibody against caveolin-1 (BD Biosciences Pharmigen). The localization of actin was determined with an Alexa Fluor 488–conjugated phalloidin (Molecular Probes, Eugene, OR). Secondary antibodies conjugated to Alexa Fluors (Molecular Probes) were used for double- and triple-label experiments. Microscopy was performed using either a wide-field fluorescence microscope (model DMR-E; Leica, Wetzlar, Germany) equipped with narrow bandpass filters and a digital camera (model Orca; Hamamatsu, Bridgewater, NJ) or an inverted Leica DMI-6000B microscope equipped with an Infinity II confocal scanning module, 561- and 491-nm lasers, and a Hamamatsu CCD camera (C9100-12). Images were captured, pseudocolored, and processed using Simple PCI software (Compix, Cranberry Township, PA) or Metamorph Software (Universal Imaging, West Chester, PA). For colocalization experiments, a nearest neighbors deconvolution algorithm was performed on successive 0.20-μm focal planes.

Internalization Assay

Assays to follow internalization of transferrin and the IL-2R-VE-cadherin polypeptides were carried out as previously described (Xiao *et al.*, 2005). For colocalization experiments with clathrin and AP-2, cells were labeled at 4°C with IL-2R antibodies and then incubated for 5 min at 37°C to allow for internalization. Cells were then fixed and processed for immunofluorescence without being acid-washed. Potassium depletion was performed as described previously (Xiao *et al.*, 2005), and cells were treated with 5 μg/ml chlorpromazine for 30 min at 37°C. To measure internalization of endogenous VE-cadherin, cells were treated with 100 μM chloroquine for 3 h. Cells were then rinsed, fixed, and processed for immunofluorescence as described above. Internalization was quantified using Simple PCI software to measure either total intracellular fluorescence or object number. Statistical analysis was performed using two-tailed *t* test. Error bars represent the SEM.

Cross-linking and Immunoprecipitation

To analyze low-affinity interactions between VE-cadherin and AP-2, we performed cross-linking in MECs with DSP [dithiobis(succinimidylpropionate)] as described (Craige *et al.*, 2008; Salazar *et al.*, 2008). Briefly, MECs were grown to confluency in 10-cm dishes and infected with Il-2R-VE-cadherin constructs. On the day of the experiment, cells were placed on ice, rinsed twice with PBS, and incubated with 1 mM DSP (Pierce, Rockford, IL) or vehicle for 2 h on ice. The reaction was quenched by adding 25 mM Tris. The cells were then lysed in buffer A (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, and 0.1 mM MgCl$_2$, pH 7.4) + 0.5% TX-100, scraped from the dish, and incubated on ice for 30 min. Cell homogenates were centrifuged at 16,100 $\times$ *g* for 10 min, and supernatants were diluted to 1 mg/ml in 0.5 ml of buffer A + 0.5% TX-100. The supernatants were incubated overnight at 4°C with Dynal magnetic beads (Invitrogen) conjugated to mAbs against AP-2 (α subunit, BD Transduction Laboratories) or clathrin (Calbiochem, San Diego, CA). The beads were then washed with buffer A + 0.1% TX-100 and eluted with SDS-PAGE sample buffer at 75°C for 5 min. Immunoblotting under reducing conditions reverses the DSP cross-linking and allows for the detection of immunoprecipitated material.

Small Interfering RNA Knockdown of AP-2

Small Interfering RNA (siRNA) siRNA oligonucleotides to the AP-2 μ subunit and control, nontargeting siRNA were purchased from Dharmacon (Lafayette, CO) and have been previously described (Urs *et al.*, 2008; Motley *et al.*, 2003). HMEC-1 cells were seeded in 60-mm dishes at 50% confluency. Cells were transfected on consecutive days with 200 pM siRNA using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Twenty-four hours after the second transfection, cells were trypsinized and plated onto coverslips or 35-mm dishes to assay internalization or efficiency of knockdown. Cells were infected with adenovirus expressing IL-2R-VE-cad$_{cyto}$ at the time of plating. Internalization assays were performed as described above.

RESULTS

VE-Cadherin Is Internalized via a Clathrin-, AP-2–, and Dynamin-dependent Pathway

The cytoplasmic domain of VE-cadherin mediates internalization of the cadherin from the cell surface, and p120 is a potent inhibitor of this process (Xiao *et al.*, 2005). However, the molecular machinery utilized for VE-cadherin internalization and the mechanism of p120 inhibition are not fully understood. In previous studies, we demonstrated that a chimeric molecule consisting of the cytoplasmic domain of VE-cadherin fused to the extracellular domain of the IL-2 receptor (IL-2R-VE-cad$_{cyto}$) is rapidly internalized from the cell surface (Xiao *et al.*, 2005). To further elucidate the endocytic machinery used by VE-cadherin, the IL-2R-VE-cad$_{cyto}$ chimera was expressed in primary cultures of human MECs, and a series of pharmacological and genetic approaches were utilized to inhibit endocytosis. Cells were subjected to K$^+$ depletion (Salazar and Gonzalez, 2002; Xiao *et al.*, 2005) or treatment with chlorpromazine (Wang *et al.*, 1993) to inhibit clathrin-dependent endocytosis. To measure internalization of IL-2R-VE-cad$_{cyto}$ chimera, MECs were incubated at 4°C with antibodies directed against IL-2R to label the cell surface. After incubation at 37°C for 5 min to allow for endocytosis, cells were washed with a low pH acid wash buffer to remove antibody remaining at the cell surface, making it possible to distinguish internalized IL-2R-VE-cad$_{cyto}$ from that at the cell surface. An myc epitope at the carboxy-terminal tail of the VE-cadherin cytoplasmic domain was used to identify cells expressing the IL-2R-VE-cad$_{cyto}$. As shown in Figure 1, untreated cells expressing the IL-2R-VE-cad$_{cyto}$ chimera were found to display high levels of internalized IL-2R-VE-cad$_{cyto}$ after 5 min of internalization at 37°C (Figure 1, A, a′ and b′, and B). As expected from previous data (Xiao *et al.*, 2005) K$^+$ depletion dramatically reduced the extent of internalization of the IL-2R-VE-cad$_{cyto}$ chimera (Figure 1, A, c′ and d′, and B). Treatment with chlorpromazine, which disrupts localization of the clathrin adaptor complex AP-2 to membranes and prevents clathrin assembly at the plasma membrane (Wang *et al.*, 1993), also strongly inhibited internalization of the IL-2R-VE-cad$_{cyto}$ chimera (Figure 1, A, e′ and f′, and B).

To determine if dynamin, which is required for scission of clathrin-coated vesicles from the plasma membrane (Ungewickell and Hinrichsen, 2007), is involved in VE-cadherin endocytosis, a dominant negative dynamin II (DN DynII) mutant was utilized (Altschuler *et al.*, 1998). Transferrin internalization was entirely blocked by expression of DN DynII (Figure 1, C and D). Intracellular accumulation of endogenous VE-cadherin after treatment with chloroquine to inhibit lysosomal degradation was used as a measure of VE-cadherin endocytosis (Xiao *et al.*, 2003). Chloroquine-treated cells expressing DN DynII (Figure 1, E, e′ and f′, and F) had limited vesicular staining of VE-cadherin compared with cells expressing empty vector (Figure 1, E, c′ and ′, and F), suggesting a role for dynamin in VE-cadherin endocytosis.

Inhibition of VE-cadherin endocytosis by inhibitors of clathrin-mediated endocytosis suggests a role for adaptor molecules involved in the formation and transport of clathrin-coated vesicles. To determine if the adaptor complex AP-2 is required for VE-cadherin endocytosis, we used siRNA to deplete expression of AP-2. For these experiments, HMEC-1 cells, an immortalized line of dermal microvascular endothelial cells, were used to overcome transfection inefficiencies in primary cells. Treatment of cells with siAP-2, but not siControl, reduced expression of AP-2 by 75% as measured by Western blot (Figure 1G). Treatment with siAP-2 also inhibited internalization of transferrin, a well-characterized AP-2 cargo, by 80% (Figure 1, H and I). Internalization of IL-2R-VE-cad$_{cyto}$ was also reduced by 80% in siAP-2–treated cells compared with siControl-treated cells (Figure 1, J and K). A dominant negative AP-2 mutant also reduced internalization of endogenous VE-cadherin in chloroquine-treated cells (data not shown). Together, these results demonstrate that VE-cadherin endocytosis is dependent on components of the clathrin endocytic machinery, including AP-2 and dynamin.

To test the hypothesis that the VE-cadherin cytoplasmic tail forms a complex with clathrin, we immunoprecipitated clathrin from MEC lysates expressing either IL-2R or IL-2R-VE-cad$_{cyto}$ chimera. Reducing SDS-PAGE and immunoblotting with IL-2R antibodies revealed that the IL-2R-VE-cad$_{cyto}$ chimera coprecipitated with clathrin but the IL-2R lacking the VE-cadherin tail did not (Figure 2, cf. lanes 13 and 14), demonstrating that clathrin specifically associates with the VE-cadherin cytoplasmic domain. To analyze interactions between VE-cadherin and adaptor complexes, we used limited whole cell cross-linking followed by immunoprecipitation. DSP, a homobifunctional cell-permeable cross-linker, was used to stabilize cargo-adaptor interactions (Craige *et al.*, 2008). In cells treated with DSP, IL-2R-VE-cad$_{cyto}$, but not IL-2R, was consistently immunoprecipitated with AP-2 (Figure 2, lanes 11 and 12). IL2-R and IL-2R-VE-cad$_{cyto}$ did not coprecipitate with beads without antibody (Figure 2, lanes 5–8), demonstrating that clathrin and AP-2 specifically interact with the VE-cadherin cytoplasmic tail.

To determine if the association between clathrin, AP-2, and the VE-cadherin tail could be observed in cells, the localization of these proteins was examined by deconvolution immunofluorescence microscopy. Transferrin was used as a positive control for colocalization with clathrin. A chimeric molecule consisting of the extracellular domain of the IL-2 receptor fused to the cytoplasmic tail of the desmosomal cadherin desmoglein 3 (IL-2R-Dsg3$_{cyto}$), which has been shown to be internalized through a clathrin-independent pathway (Delva *et al.*, 2008), was used as a negative control. To measure colocalization, cells expressing IL-2R, IL-2R-VE-cad$_{cyto}$, or IL-2R-Dsg3$_{cyto}$ were labeled with IL-2R antibodies at 4°C and transferred to 37°C for 5 min to allow for internalization. For transferrin, uninfected cells were labeled with fluorescently conjugated transferrin at 4°C and transferred to 37°C for 5 min. In contrast to internalization assays, cells were not acid-washed, allowing for the visualization of clusters of the chimeric molecules at early endocytic steps. As expected, extensive colocalization was observed between transferrin and clathrin (Figure 3, A, a′–d′, and B). We also observed significant levels of colocalization between IL-2R-VE-cad$_{cyto}$ and clathrin (Figure 3, A, e′–h′, and B). This colocalization was dependent on the VE-cadherin cytoplasmic tail, because only minimal colocalization was observed between IL-2R or IL-2R-Dsg3$_{cyto}$ and clathrin (Figure 3, A, i′–p′, and B). Furthermore, the IL-2R-VE-cad$_{cyto}$ did not colocalize with caveolin-1 (Figure 3, A, q′–t′, and B),
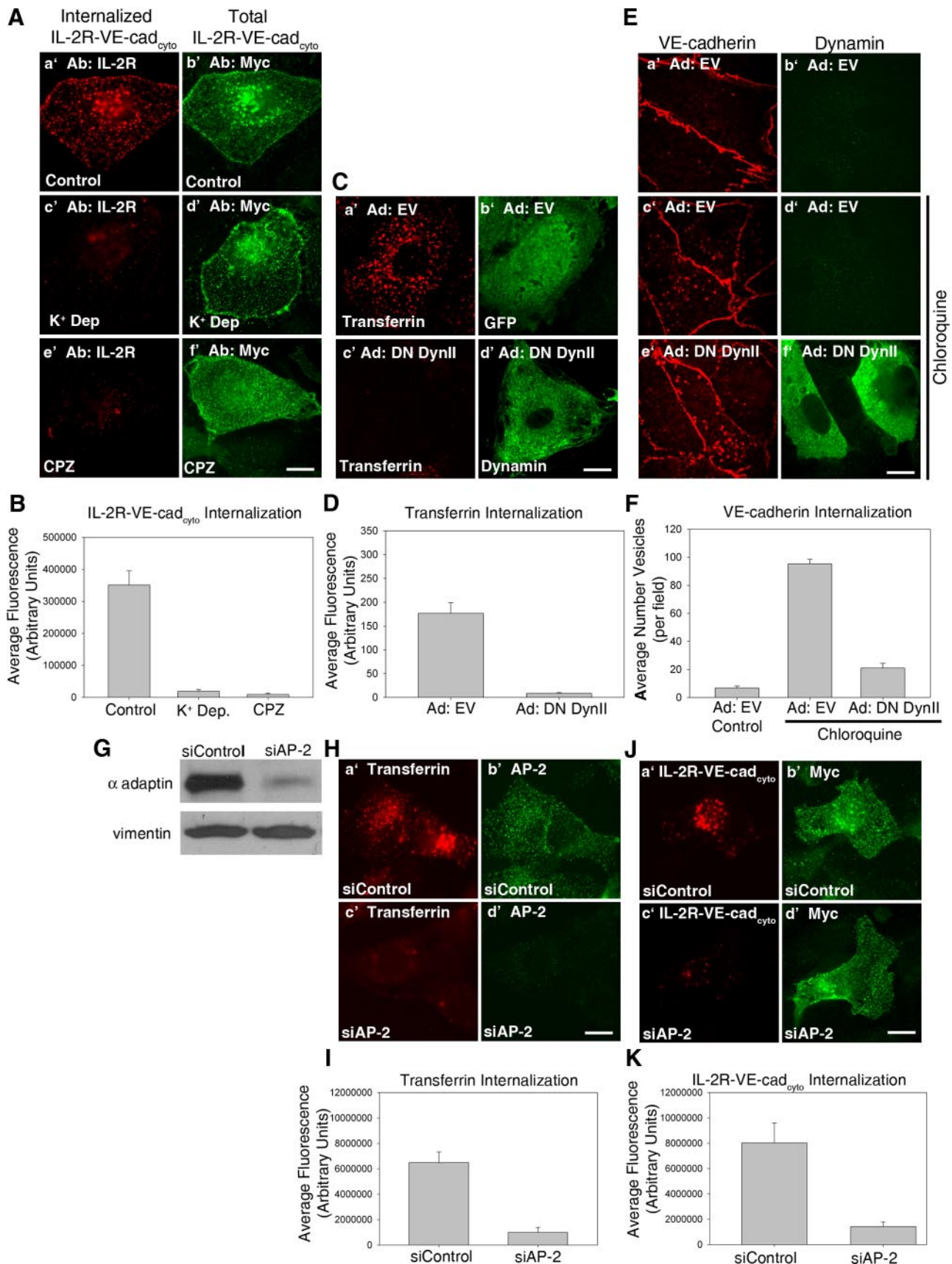
Figure 1. VE-cadherin endocytosis is mediated through a clathrin-, AP-2–, and dynamin-dependent pathway. (A and B) Cell surface IL-2R-VE-cad_cyto was labeled with IL-2R antibodies at 4°C. Cells were incubated at 37°C for 5 min, washed in a low pH buffer at 4°C to
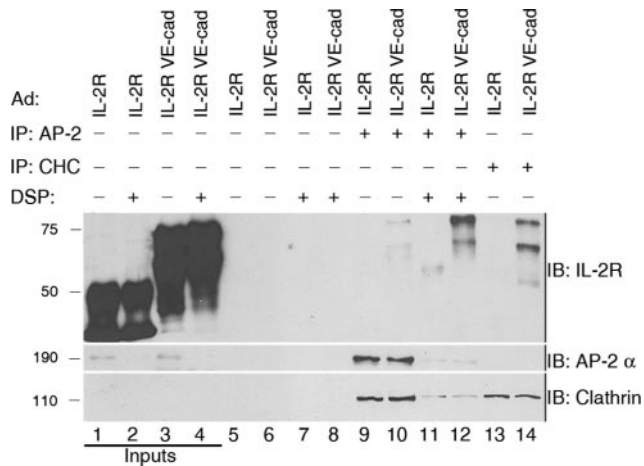
Figure 2. The VE-cadherin cytoplasmic tail specifically associates with clathrin and AP-2. MECs expressing IL-2R or IL-2R-VE-cad$_{cyto}$ were treated with DSP (lanes 2, 4, 7–8, and 11–12) or DMSO vehicle (lanes 1, 3, 5–6, 9–10, and 13–14), and extracted in detergent, and proteins were immunoprecipitated with beads alone (lanes 5–8), beads coated with AP-2 α antibodies (lanes 8–12), or beads coated with clathrin antibodies (CHC; lanes 13–14). Western blot analysis using antibodies against IL-2R to detect IL-2R or IL-2R-VE-cad$_{cyto}$ demonstrates that the IL2R-VE-cad$_{cyto}$ specifically interacts with AP-2 (lane 12) and clathrin (lane 14). Inputs represent 5% of sample.

providing additional evidence that VE-cadherin is internalized through a clathrin-dependent pathway. Significant co-localization was also observed between IL-2R-VE-cad$_{cyto}$ and AP-2 (Figure 3, C, e'–h', and D), but not between IL-2R and AP-2 (Figure 3, C, a'–d', and D) or IL-2R-Dsg3$_{cyto}$ and AP-2 (data not shown). These data indicate that VE-cadherin associates with clathrin and AP-2 and localizes to membrane domains enriched in both proteins during internalization.

Figure 1 (cont). remove surface bound antibody, and then processed for immunofluorescence to visualize intracellular IL-2R-VE-cad$_{cyto}$ (A, a', c', and e'). Antibodies against the myc epitope tag at the carboxyl terminal domain of the IL-2R-VE-cad$_{cyto}$ were used to verify expression of the polypeptide (A, b', d', and f'). Note that untreated cells exhibited high levels of internalized IL-2R-VE-cad$_{cyto}$, whereas treatment with K$^+$ depletion or chlorpromazine completely inhibited internalization. (C–F) MECs were infected with adenoviruses expressing either GFP (C, a' and b') or dominant negative DynII (C, c' and d'). The DynII completely blocked internalization of Alexa Fluor 555–conjugated transferrin (C and D). To monitor internalization of VE-cadherin, MECs were either untreated (E, a' and b') or treated with 100 μM chloroquine for 3 h (E, c'–f'). Note that intracellular vesicular accumulation of VE-cadherin is reduced in cells expressing DynII (E, e' and f', and F). (G–K) HMEC-1 cells were transfected with an siRNA oligo targeted to the AP-2 μ subunit or a nontargeting control siRNA. Lysates prepared from cells treated with control siRNA or AP-2 siRNA were separated by SDS-PAGE and immunoblotted for AP-2 to confirm AP-2 knockdown (G). Western blot and immunofluorescence reveals ~75% reduction in AP-2 levels in siRNA-treated cells compared with control, as well as an 85% decrease in transferrin internalization (G–I). IL-2R-VE-cad$_{cyto}$ internalization assays were performed and AP-2 siRNA-treated cells (J, c' and d') exhibited an 80% decrease in internalization of IL-2R-VE-cad$_{cyto}$ compared with control (J, a' and b'), indicating a requirement for AP-2 in VE-cadherin internalization (K). Error bars, SEM; n > 20 cells. Scale bar, 20 μm.

p120 Prevents VE-Cadherin from Entering a Clathrin- and AP-2–enriched Membrane Domain

To test the hypothesis that p120 inhibits the entry of VE-cadherin into membrane domains enriched in clathrin and AP-2, we monitored the localization of the IL-2R-VE-cad$_{cyto}$ chimera in cells expressing exogenous p120. Interestingly, in cells expressing exogenous p120 the IL-2R-VE-cad$_{cyto}$ chimera did not form punctate clusters as seen in control cells (Figure 4, A and C, a'–j'). Additionally, colocalization between the IL-2R-VE-cad$_{cyto}$ chimera and clathrin or AP-2 was dramatically reduced in these cells (Figure 4, B and D). To test whether this function of p120 requires its interaction with the juxtamembrane domain (JMD) of VE-cadherin, we expressed a mutant IL-2R-VE-cad$_{cyto}$ construct that contains a triple alanine substitution in the p120-binding site of VE-cadherin (IL-2R-VE-cad$_{JMD-AAA}$; Xiao *et al.*, 2005). This mutation completely abrogates the binding of p120 to VE-cadherin (Calkins *et al.*, 2003). The IL-2R-VE-cad$_{JMD-AAA}$ displays a punctate localization and colocalizes with clathrin after incubation at 37°C in cells containing endogenous levels of p120 (Figure 4A, k'–o'). However, unlike the wild-type IL-2R-VE-cad$_{cyto}$ chimera, the distribution and colocalization of the IL-2R-VE-cad$_{JMD-AAA}$ chimera was not affected by exogenous expression of p120 (Figure 4, A, p'–t', and B). These results indicate that p120 prevents VE-cadherin from localizing to clathrin- and AP-2–enriched membrane domains in a manner that requires its binding to the VE-cadherin JMD.

p120 Inhibits VE-Cadherin Endocytosis Independently of RhoA

In addition to its central function in regulating cadherin expression at adherens junctions, an important role has also been established for p120 in regulating Rho GTPases, particularly as an inhibitor of RhoA activity. To determine if this function of p120 is integral to its ability to stabilize VE-cadherin at the plasma membrane by preventing VE-cadherin endocytosis, we utilized several methods to inhibit Rho activity in endothelial cells and measured the effect on internalization of endogenous VE-cadherin. As expected, expression of exogenous p120 inhibited the accumulation of intracellular VE-cadherin after chloroquine treatment (Figure 5A, e' and f'). Expression of a dominant negative RhoA mutant (N19; Figure 5A, g'–i'), treatment with the selective Rho inhibitor C3 toxin (Figure 5A, j' and k') or treatment with Y27632 (Figure 5A, l' and m'), an inhibitor of ROCK, a downstream Rho effector, were effective in reducing RhoA activity within endothelial cells, as indicated by the dramatic loss of actin stress fibers. However, none of these treatments had any discernable impact on internalization of VE-cadherin in chloroquine-treated cells (Figure 5, B and C), suggesting that inhibition of RhoA does not phenocopy the activity of p120.

To look specifically at the role of local p120-dependent regulation of RhoA on VE-cadherin stability, we tested the effects of a p120 mutant that is unable to inhibit RhoA activity. The p120 mutant lacks the N-terminal regulatory domain and also contains mutations within the Rho-binding domain (p120 4A K622,628A). Deletion of these domains eliminates p120's ability to inhibit RhoA activity (Castano *et al.*, 2007; Yanagisawa *et al.*, 2008). In MECs expressing high levels of wild-type p120, cells exhibit a dramatic branching phenotype characterized by dendritic outgrowths, which have been shown to result from loss of RhoA activity (Figure 6Aa'). No cells with this branching phenotype could be found in cells expressing the p120 Rho-uncoupled mutant,
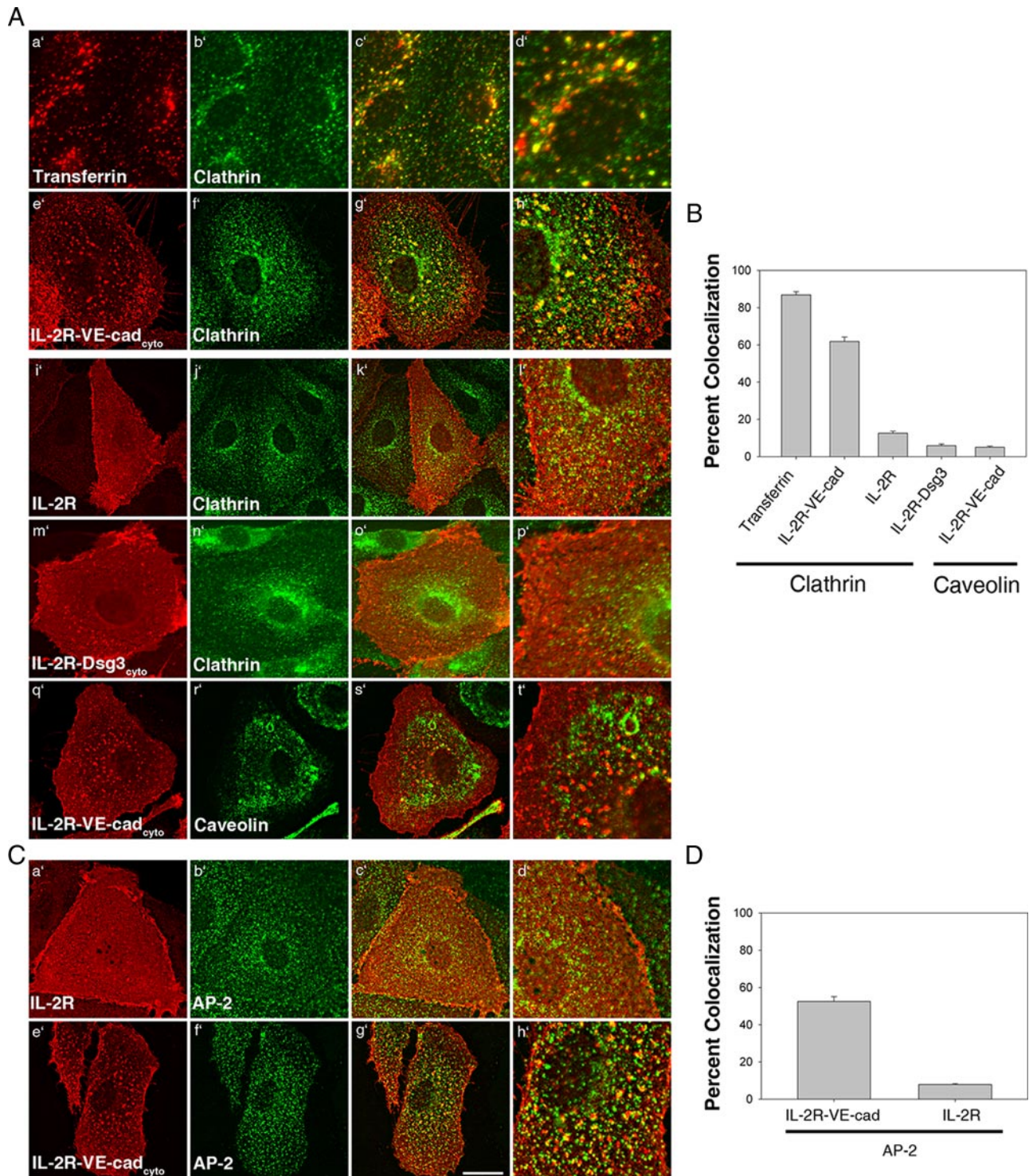
Figure 3. The VE-cadherin cytoplasmic tail specifically colocalizes with clathrin and AP-2. MECs expressing IL-2R, IL-2R-VE-cad$_{cyto}$, or IL-2R-Dsg3$_{cyto}$ were labeled for 30 min at 4°C with IL-2R antibodies, transferred to 37°C for 5 min, and then processed for immunofluorescence microscopy. As a positive control, internalization of transferrin receptor was monitored by labeling MECs with fluorescently conjugated transferrin at 4°C, transferring cells to 37°C for 5 min, and then processing for immunofluorescence microscopy. (A and B) Colocalization of transferrin, IL-2R, IL-2R-VE-cad$_{cyto}$, or IL-2R-Dsg3$_{cyto}$ with clathrin or caveolin was monitored. Colocalization was quantified as the percentage of transferrin, IL-2R, IL-2R-VE-cad$_{cyto}$, or IL-2R-Dsg3$_{cyto}$ that colocalize with clathrin or caveolin, using Metamorph software. (C and D) Colocalization of IL-2R and IL-2R-VE-cad$_{cyto}$ with AP-2 was also measured. Colocalization was quantified as the percentage of transferrin, IL-2R, or IL-2R-VE-cad$_{cyto}$ that colocalize with AP-2. Error bars, SEM; n = 25 cells. Scale bar, 20 μm.

even at very high levels (Figure 6Ab′). Next, internalization of endogenous VE-cadherin in cells expressing either wild-type p120 (Figure 6B, c′ and d′) or the Rho-uncoupled p120 mutant (Figure 6B, e′ and f′) was assessed. Both wild-type
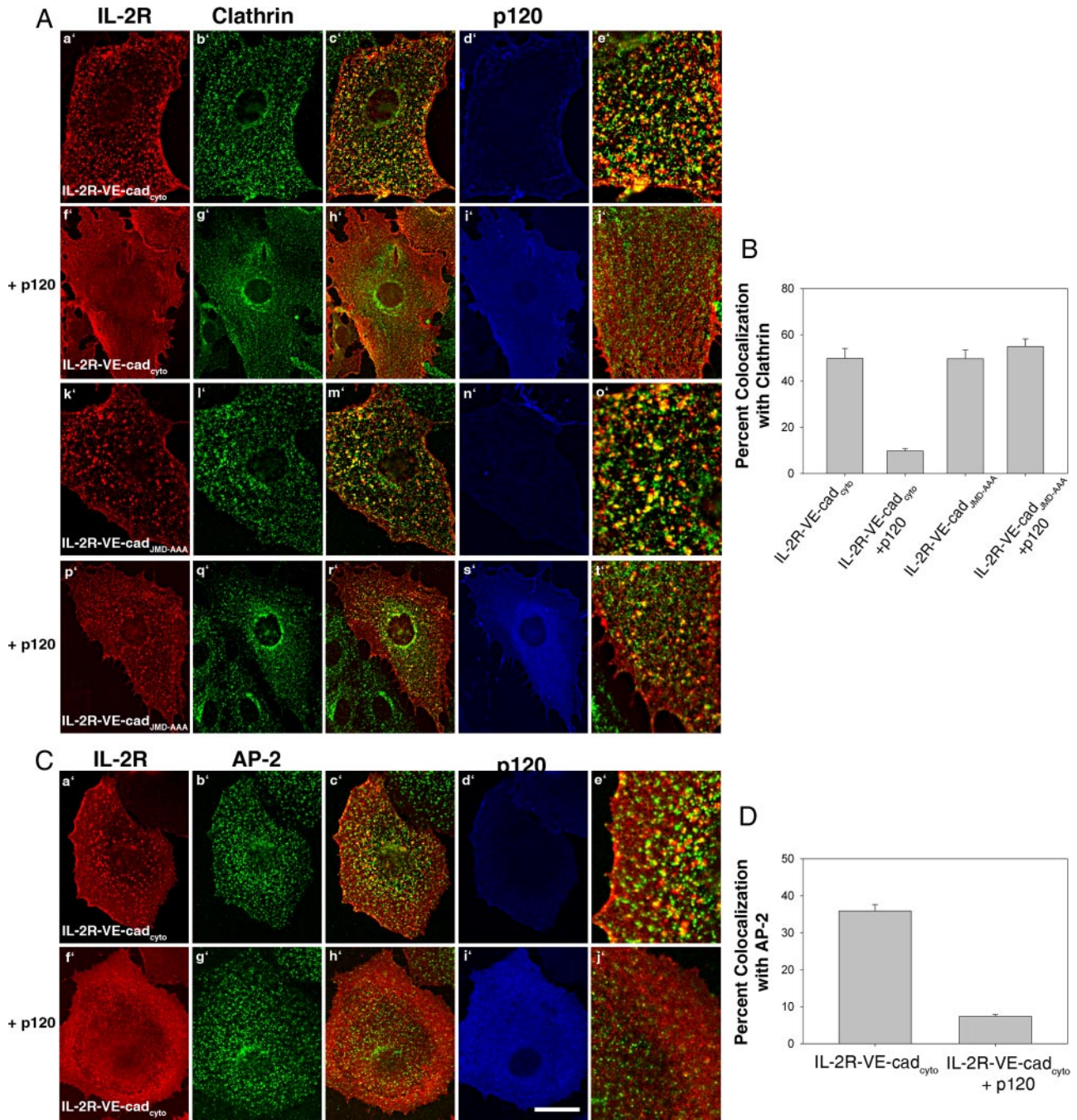
Figure 4. Exogenous expression of p120 prevents IL-2R-VE-cad$_{cyto}$ from colocalizing with clathrin and AP-2 in a manner dependent on the interaction of p120 with the VE-cadherin JMD. (A) p120 was coexpressed in MECs with either IL-2R-VE-cad$_{cyto}$ or IL-2R-VE-cad$_{JMD-AAA}$, which is unable to bind to p120, and internalization assays were conducted as described in Figure 3. Cells were fixed and processed for triple-label immunofluorescence to monitor the IL-2R-VE-cadherin polypeptides, p120, and clathrin. (B) Colocalization was quantified as the percentage of IL-2R-VE-cad$_{cyto}$ or IL-2R-VE-cad$_{JMD-AAA}$ that colocalize with clathrin. In the presence of p120, colocalization between IL-2R-VE-cad$_{cyto}$ and clathrin is greatly reduced. High levels of colocalization between IL-2R-VE-cad$_{JMD-AAA}$ and clathrin in cells expressing exogenous p120 demonstrate that p120 must bind to the VE-cadherin JMD to prevent VE-cadherin recruitment into clathrin-enriched membrane domains. (C) Colocalization between IL-2R-VE-cad$_{cyto}$ and AP-2 was also monitored. In the presence of p120, IL-2R-VE-cad$_{cyto}$ colocalization with AP-2 is dramatically reduced (D). Error bars, SEM; n = 25 cells. Scale bar, 20 μm.

p120 and the Rho-uncoupled p120 mutant significantly reduced the amount of internalized VE-cadherin (Figure 6C). To determine if p120 Rho-uncoupled mutant can block the association of VE-cadherin with clathrin and the AP-2 adaptor, we measured colocalization between the IL-2R-VE-cad$_{cyto}$ and clathrin (Figure 7A) or AP-2 (Figure 7C) in cells expressing

either wild-type (Figure 7, A and C, f'–j') or mutant p120 (Figure 7, A and C, k'–o'). The p120 Rho-uncoupled mutant was able to prevent colocalization between the IL-2R-VE-cad$_{cyto}$ and clathrin (Figure 7B) and AP-2 (Figure 7D) similarly to wild-type p120. Together, these findings suggest that p120 stabilizes VE-cadherin at the plasma membrane by
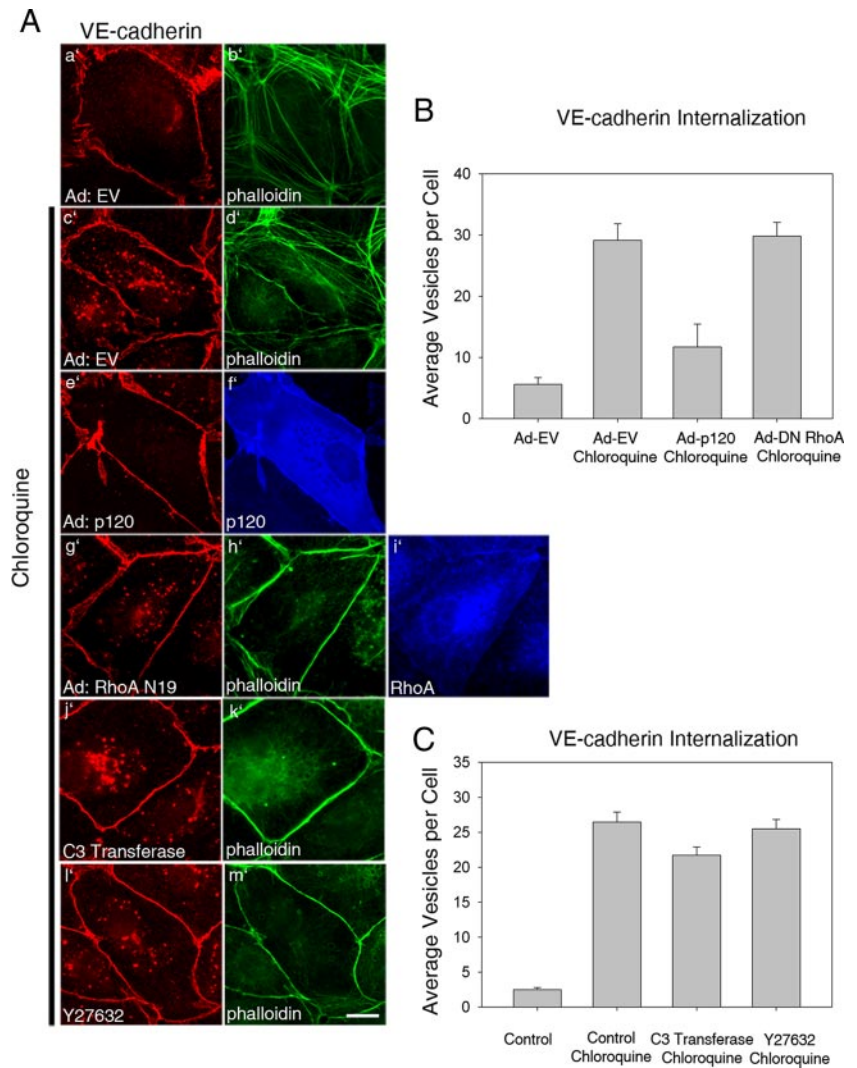
Figure 5. Inhibition of RhoA activity does not inhibit VE-cadherin endocytosis. (A) MECs were infected with empty adenovirus (a'–d'), virus expressing p120 (1A; e' and f'), or a RhoA dominant negative mutant (N19; g'-i'). RhoA activity was also inhibited by treating cells with a cell-permeable C3 transferase (j'–k') or Y27632 (l' and m'), an inhibitor of Rho kinase. Accumulation of vesicular VE-cadherin was measured in untreated or chloroquine-treated cells as described in Figure 1, and inhibition of Rho activity was verified by loss of stress fibers as detected by Alexa Fluor 488—conjugated phalloidin (b', d', h', k', and m'). The number of VE-cadherin–containing vesicles was quantified as shown in B and C. Error bar, SEM; n = 50 cells.

preventing the cadherin from entering a clathrin- and AP-2–dependent endocytic pathway. The mechanism by which p120 functions in this manner requires its interaction with the VE-cadherin cytoplasmic tail, but occurs independently of RhoA activity.

DISCUSSION

The results presented in this study demonstrate that p120 stabilizes VE-cadherin at the plasma membrane by preventing its entry into a clathrin-, AP-2–, and dynamin-dependent endocytic pathway through a mechanism that does not involve RhoA. These studies identify the endocytic machinery involved in VE-cadherin endocytosis and help to clarify the mechanism by which p120 functions as a set point to regulate cadherin expression levels. Additionally, they establish a novel role for p120 as part of a plasma membrane retention mechanism that modulates the availability of a cargo protein for recruitment into membrane-trafficking pathways.

In previous studies, we demonstrated that the cytoplasmic domain of VE-cadherin mediates rapid endocytosis of the protein and that p120 prevents VE-cadherin from being internalized (Xiao *et al.,* 2005). However, these previous studies did not fully explain how p120 functions to stabilize

the cadherin at the plasma membrane. In the present study, we found that internalization of endogenous VE-cadherin or an IL-2R-VE-cad$_{cyto}$ chimera was reduced by inhibition of clathrin-, AP-2–, or dynamin-dependent endocytosis (Figure 1). After a short period of internalization, a significant fraction of the IL-2R-VE-cad$_{cyto}$ chimera colocalizes with clathrin and AP-2 (Figure 3), providing further evidence that VE-cadherin is internalized through a clathrin-mediated pathway. We were also able to specifically immunoprecipitate the IL-2R-VE-cad$_{cyto}$ with antibodies against clathrin and the clathrin adaptor AP-2 (Figure 2). Together, these results provide the first evidence for a role for the AP-2 adaptor complex in the trafficking of VE-cadherin through a clathrin-mediated pathway.

Expression of exogenous p120 dramatically inhibits internalization of VE-cadherin during the earliest stages of endocytosis. The IL-2R-VE-cad$_{cyto}$ colocalizes extensively with clathrin and AP-2. However, in cells expressing exogenous p120, the IL-2R-VE-cad$_{cyto}$ fails to colocalize with clathrin or AP-2. Importantly, the interaction of p120 with the VE-cadherin JMD is crucial for p120's ability to prevent VE-cadherin from clustering with components of the clathrin machinery (Figure 4). These data support previous findings that p120 prevents VE-cadherin endocytosis and indicate
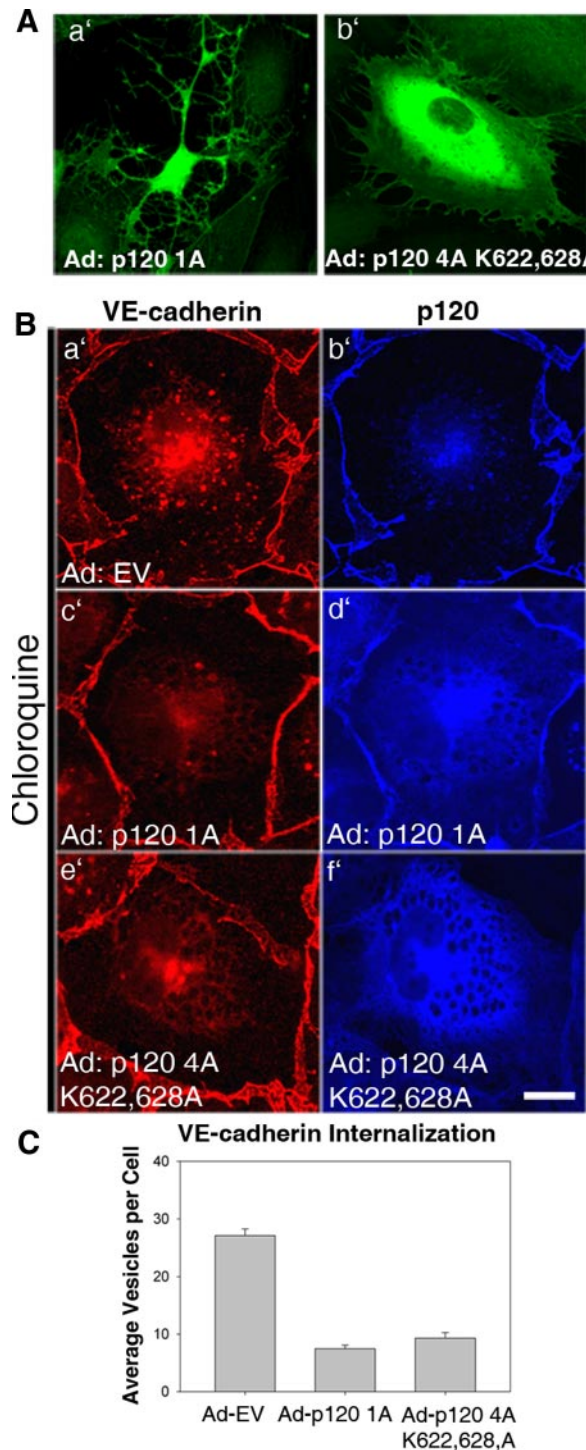
Figure 6. A p120 Rho-uncoupled mutant is effective at preventing VE-cadherin endocytosis. (A) Wild-type p120 1A or a RhoA-uncoupled mutant (p120 4A K622,628A) were expressed in MECs. Note dendritic appearance of cells expressing wild-type but not mutant p120. (B) Internalization of VE-cadherin was measured in MECs expressing EV (a' and b'), p120 1A (c' and d'), or the RhoA-uncoupled p120 4A K622,628A mutant (e' and f') by treating cells with chloroquine for 3 h. Total intracellular levels of VE-cadherin were quantified as shown in C. VE-cadherin internalization was dramatically reduced in cells expressing either exogenous wild-type p120 or the RhoA-uncoupled p120 mutant. Error bars, SEM; n = 100 cells. Scale bar, 20 μm.

that p120 acts to stabilize VE-cadherin at the plasma membrane at an early step in endocytosis, most likely by preventing adaptor binding and entry into a clathrin-enriched membrane domain.

In addition to functioning as a critical regulator of adherens junction stability, p120 also plays a key signaling role in regulating activity of Rho-GTPases (Anastasiadis, 2007). The relationship between the function of p120 in regulating RhoA activity and its role in stabilizing cadherins at cell junctions is not understood. The data presented in this study indicate that p120 regulates cadherin stability at the plasma membrane independently of Rho activity. Internalization of VE-cadherin was not impacted by inhibition of Rho activity by either genetic or pharmacological manipulation (Figure 5). Similarly, a p120 mutant that is unable to inhibit Rho activity retains its ability to prevent VE-cadherin endocytosis (Figure 6) and also prevents the IL-2R-VE-cad$_{cyto}$ from colocalizing with clathrin and AP-2 (Figure 7). Importantly, these data indicate that VE-cadherin endocytosis occurs independently of Rho activity and that p120 stabilizes VE-cadherin at the cell surface through a mechanism that does not require its Rho inhibitory function.

On the basis of our findings that the function of p120 in preventing VE-cadherin endocytosis can be uncoupled from the ability of p120 to inhibit RhoA, we hypothesize that p120 prevents the VE-cadherin cytoplasmic tail from interacting with components of the endocytic machinery that selectively recruit transmembrane receptors into a clathrin-mediated pathway. The identification of a complex containing VE-cadherin, clathrin, and AP-2, together with the inhibitory effect of silencing AP-2 expression on the internalization of VE-cadherin, demonstrates an important role for the AP-2 adaptor complex in the regulation of VE-cadherin endocytosis. In general, clathrin interacts with transmembrane cargo through multiple adaptor and accessory proteins (Bonifacino and Traub, 2003; Robinson, 2004; Sorkin, 2004). Recently, vascular endothelial growth factor (VEGF)-induced internalization of VE-cadherin was found to involve β-arrestin (Gavard and Gutkind, 2006), a clathrin adaptor that often works cooperatively with AP-2 to regulate clathrin-dependent endocytosis. Studies are currently underway to further characterize the interaction between VE-cadherin and AP-2, to identify additional adaptor proteins that may be required for VE-cadherin endocytosis and to determine if p120 prevents them from interacting with the VE-cadherin cytoplasmic tail.

The ability of p120 to prevent VE-cadherin from entering domains enriched in clathrin and AP-2 in a manner that requires p120 binding to the VE-cadherin juxtamembrane domain further supports a model in which p120 functions as a cap on the VE-cadherin cytoplasmic tail to prevent adaptor interactions. An alternative model is that p120 stabilizes cadherins at the plasma membrane by inhibiting the activity of another component of the endocytic machinery. On the basis of this hypothesis, one would predict that p120 may function as a general inhibitor of clathrin-dependent endocytosis. Thus far, we have found no evidence that this is the case. p120 has no effect on internalization of transferrin receptor or epidermal growth factor receptor (EGFR; Xiao *et al.*, 2005, our unpublished observation). Additionally, the requirement for p120 binding to the cadherin tail for the inhibitory effect suggests that p120's function is cadherin-specific. Therefore, we currently favor a model in which the association of p120 with the VE-cadherin JMD prevents the clathrin adaptor AP-2 from recruiting VE-cadherin into clathrin-coated pits.
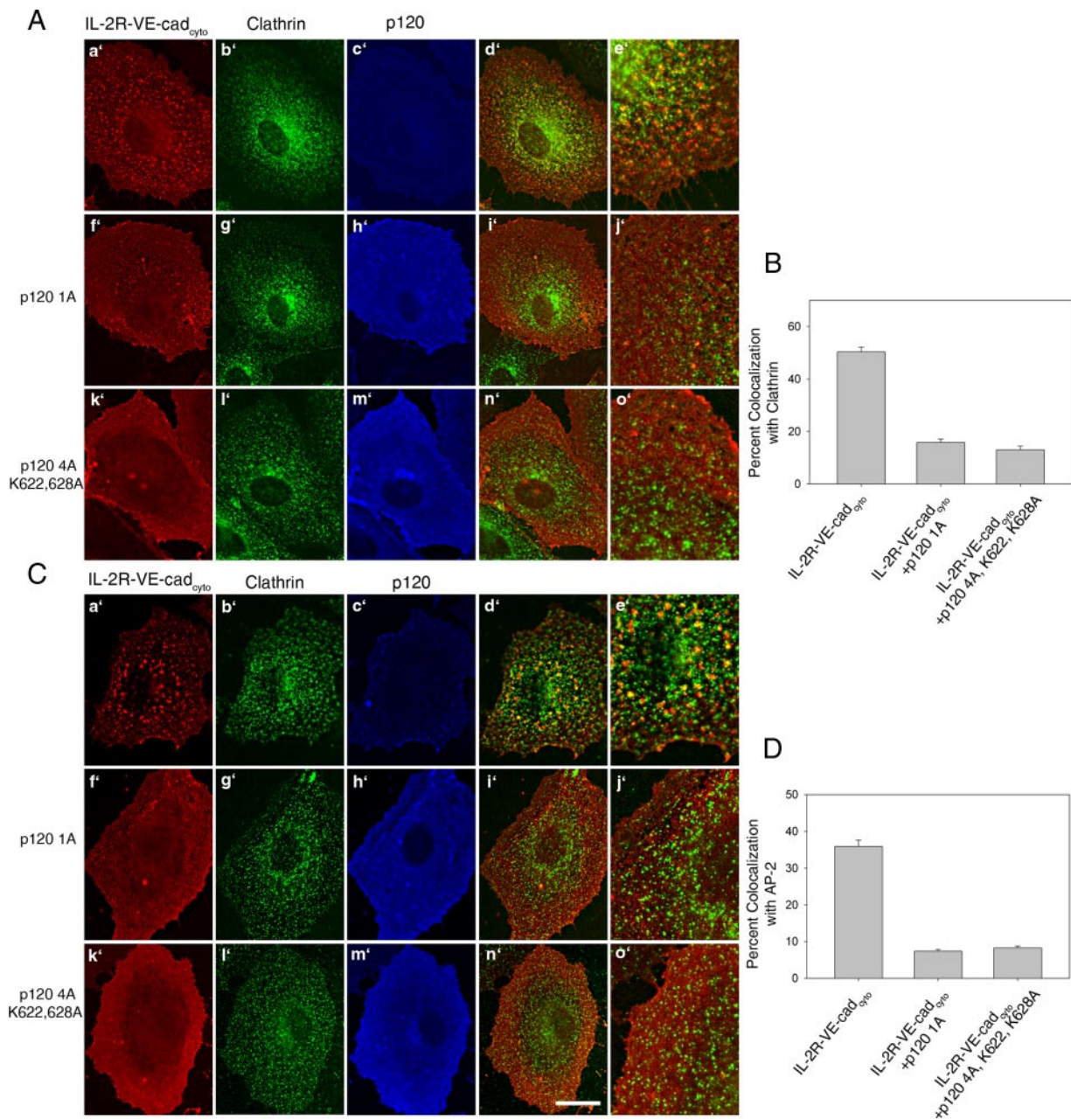
Figure 7. A p120 Rho-uncoupled mutant prevents entry of VE-cadherin into membrane compartments containing clathrin and AP-2. (A and C) MECs were infected with adenovirus carrying IL-2R-VE-cad$_{cyto}$ and either p120 1A or p120 4A K622,628A. Cells were then processed for immunofluorescence to determine the extent of colocalization between IL-2R-VE-cad$_{cyto}$ and clathrin (A) or AP-2 (C) in cells expressing either wild-type or Rho-uncoupled p120 (p120 4A K622,628A). Colocalization was quantified as the percentage of IL-2R-VE-cad$_{cyto}$ that colocalize with clathrin or AP-2. The RhoA-uncoupled p120 mutant was as effective at preventing colocalization of IL-2R-VE-cad$_{cyto}$ as wild-type p120 (B and D). Error bars, SEM; n = 25 cells. Scale bar, 20 μm.

Endocytic adaptor proteins play a fundamental role in the recruitment and formation of clathrin-coated vesicles at the plasma membrane. The recruitment of transmembrane cargo receptors into clathrin-coated vesicles by AP-2 and the formation of a stable network of interactions between cargo, adaptors, and clathrin is a key step in vesicle formation (Maldonado-Báez and Wendland, 2006). For these reasons, the regulation of the interaction between adaptor proteins and cargo molecules is crucial to the process of clathrin-coated vesicle formation and trafficking. The ability of p120 to prevent cadherin recruitment into clathrin- and AP-2–enriched membrane domains reveals a previously unappreciated mechanism by which clathrin-mediated endocytosis is regulated. It remains to be determined whether this mechanism for endocytic regulation is unique to the cadherin tail-p120 interaction or if other receptors and clathrin cargo are similarly regulated by cytoplasmic-binding partners.

ACKNOWLEDGMENTS

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