The Cdc42 and Rac1 GTPases are required for capillary lumen formation in three-dimensional extracellular matrices

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Summary

Here we show a requirement for the Cdc42 and Rac1 GTPases in endothelial cell (EC) morphogenesis in three-dimensional extracellular matrices. Cdc42 and Rac1 specifically regulate EC intracellular vacuole and lumen formation in both collagen and fibrin matrices. *Clostridium difficile* toxin B (which blocks all three Rho GTPases) completely inhibited the ability of ECs to form both vacuoles and lumens, whereas C3 transferase, a selective inhibitor of Rho, did not. Expression of either dominant-negative (N17) or constitutively active (V12) Cdc42 using recombinant adenoviruses dramatically inhibited EC vacuole and lumen formation in both collagen and fibrin matrices. Both vacuole and lumen formation initiated in ECs expressing dominant-negative (N17) Rac1 but later collapsed, indicating a role for Rac1 during later stages of vessel development. Analysis of cultures using confocal microscopy revealed green fluorescent protein-V12Rac1, -Rac1 wild-type and -Cdc42 wild-type chimeric proteins targeted to intracellular vacuole membranes during the lumen formation process. Also, expression of the verprolin-cofilin-acidic domain of N-WASP, a downstream Cdc42 effector, in ECs completely interfered with vacuole and lumen formation. These results collectively reveal a novel role for Cdc42 and Rac1 in the process of EC vacuole and lumen formation in three-dimensional extracellular matrices.

Key words: Cdc42, Rac1, endothelial cell, and capillary morphogenesis

Introduction

Angiogenesis is the development of new capillaries from pre-existing blood vessels and is required for organ development, wound healing and pathological conditions including tumor growth (Folkman and D'Amore, 1996). Key components that regulate the angiogenic process include the extracellular matrix (ECM), integrins and angiogenic cytokines (Senger, 1996; Shattil and Ginsberg, 1997; Carmeliet and Jain, 2000). Angiogenesis occurs in both collagen and fibrin matrices through either the $\alpha_2\beta_1$ and $\alpha_1\beta_1$, or $\alpha_v\beta_3$, and $\alpha_5\beta_1$ integrins respectively (Brooks et al., 1994; Davis and Camarillo, 1996; Bloch et al., 1997; Senger et al., 1997; Bayless et al., 2000; Davis et al., 2000). It is clear that angiogenic stimuli elicit ECs to undergo morphogenesis including migration, lumen formation and branching during the formation of new capillaries (Carmeliet and Jain, 2000). However, little is known regarding the molecular events that regulate EC morphogenesis.

Previous work from our laboratory indicated that EC lumen formation in three-dimensional collagen and fibrin matrices is controlled by the formation and coalescence of pinocytic intracellular vacuoles (Davis and Camarillo, 1996; Salazar et al., 1999; Davis et al., 2000; Bayless et al., 2000; Bell et al., 2001). These structures have also been reported by others in vivo and in vitro (Speidel, 1933; Clark and Clark, 1939; Wolf and Bar, 1972; Dyson et al., 1976; Wagner, 1980; Folkman and Haudenschild, 1980; Montesano and Orci, 1985; Shimizu et al., 1986; Montesano and Orci, 1988; Konerding et al., 1992; Yang et al., 1999). Interestingly, formation of vacuolar and lumenal structures was completely blocked by the addition of cytochalasin B or nocodazole, actin and microtubule depolymerizing agents, respectively (Davis and Camarillo, 1996) (K.J.B. and G.E.D., unpublished). Thus, the formation of vacuoles that coalesce into lumenal structures is dependent on the EC cytoskeletal machinery.

The RhoA, Rac1 and Cdc42 GTPases regulate the activities of the actin cytoskeleton, along with gene transcription, cell cycle progression and adhesion (reviewed in Hall, 1998; Kaibuchi et al., 1999). They also regulate actin stress fiber, lamellipodia and filopodia formation (Nobes and Hall, 1995). In addition, they recently have been observed to influence both the microtubule and intermediate filament cytoskeletons (Liu et al., 1998; Inada et al., 1999; Goode et al., 2000; Meriane et al., 2000; Tian et al., 2000; Daub et al., 2001). Interestingly, Rho GTPases also control the process of phagocytosis, where phagocytic vesicles are formed (Ridley et al., 1992; Lamaze et al., 1996; Caron and Hall, 1998; Albert et al., 2000; Chimini et al., 2000; Garrett et al., 2000; Hotchin et al., 2000). Rho GTPases are well known to be activated downstream of integrin signaling pathways (Nobes and Hall, 1995; Clark et al., 1998; Albert et al., 2000; Chimini and Chavrier, 2000; Sastry and Burridge, 2000; Schwartz and Shattil, 2000; Kiosses et al., 2001), and integrin-ECM interactions are required for EC morphogenesis (Brooks et al., 1994; Senger, 1996; Davis and Camarillo, 1996; Senger et al., 1997; Salazar et al., 2000; Davis et al., 2000;

Bayless et al., 2000; Bell et al., 2001). Rho GTPases regulate processes required for EC morphogenesis, such as pinocytosis, endocytosis, cell migration, cell survival and actin dynamics (reviewed in Hall, 1998; Kaibuchi, 1999; Chimini and Chavrier, 2000; Ridley, 2001). One report indicates a role for Rho in EC morphogenesis on planar Matrigel substrates (Somlyo et al., 2000). However, little information exists concerning a role for Rho GTPases during EC morphogenesis in three dimensions.

Here, we address the hypothesis that Rho GTPases regulate EC morphogenic events, including vacuole and lumen formation in three-dimensional matrices. We utilize Rho-GTPase-specific toxins and modulate EC gene expression with recombinant adenoviruses expressing dominant-negative, constitutively active and green fluorescent protein chimeras to evaluate the role of RhoA, Rac1 and Cdc42 in EC vacuole and lumen formation in three dimensions. Our results demonstrate a requirement for Cdc42 and Rac1 in capillary lumen formation in three-dimensional collagen and fibrin matrices.

Materials and Methods

Bacterial toxins that affect GTPase function

Clostridium difficile Toxin B (toxin B) was obtained from TechLab (Blacksburg, VA). A full length cDNA clone of *Clostridium botulinum* C3 ADP ribosyltransferase was kindly provided by Brad McIntyre (MD Anderson Cancer Research Center). Gene-specific primers encoding the *Bam*H1 and *Sph*I restriction enzymes were used to amplify the C3 insert using polymerase chain reaction with the primers TAGGATCCGCTTATTCCATTAATCAAAAGGC and TAGCATGCT-TATTTAGGATTGATAGCTGTGCC with Advantage Taq polymerase and ultrapure dNTP mix (Clontech, Palo Alto, CA). Products were purified using glass milk protocol according to the manufacturer's instructions (ISC Bioexpress, Keysville, UT). Following purification, inserts and pQE30 vector (Qiagen, Valencia, CA) were digested at 37°C under appropriate conditions for 3 hours. Insert and vector were purified, analyzed and quantitated. Insert was ligated to the vector (50 ng) at a ratio of 4.5:1 overnight at 14°C before transforming into *E. coli* strain slyD– (Roof et al., 1994) and plating on selective ampicillin plates. Colonies were screened and those containing inserts were analyzed by sequence analysis (Lone Star Labs, Houston, TX). Recombinant protein was induced and isolated as described previously (Bayless and Davis, 2001).

Preparation of recombinant adenoviruses

Using the system previously described by Vogelstein and colleagues (He et al., 1998), recombinant adenoviruses were constructed to express both dominant-negative and constitutively active forms of the RhoA, Rac1 and Cdc42 GTPases. Full-length cDNA clones of dominant-negative N19RhoA, N17Rac1-myc and N17Cdc42-myc (G25K isoform) were obtained from A. Hall (Ridley et al., 1992; Nobes and Hall, 1998). Full-length constitutively active cDNA clones of V12Rac1 and V12Cdc42 were obtained from K. Kaibuchi (Kuroda et al., 1997), and V14RhoA clone was obtained from M. Negishi (Katoh et al., 1998). Dominant-negative constructs (N17RhoA, N19Rac1 and N19Cdc42) when introduced interfere and compete with endogenous GTPases for binding to guanosine nucleotide exchange factors (GEFs) that exchange GTP for GDP. Constitutively active mutants (V14RhoA, V12Rac1 and V12Cdc42) prevent intrinsic and GTPase-activating protein (GAP)-induced GTP hydrolysis (Feig, 1999). The following clones were amplified using the respective primer sets: N19RhoA and V14RhoA: AGCTCGAGGCCACCATGGCTGCCATCCGGAAG and AGTC-TAGATCACAAGACAAGGCAACCAGATTT; N17Rac1-Myc: AGC-TCGAGGCCACCATGGAACAAAAGCTGATCTCAG and AGTCT-AGATTACAACAGCAGGCATTTTCTCTTC; N17Cdc42-Myc: AG-GGTACCGCCACCATGGAACAAAAGCTGATCTC and AGTCTA-GATTAGAATATACAGCACTTCCTTTT; V12Rac1: AGCTCGA-GGCCACCATGCAGGCCATCAAGTGTGTG and AGTCTAG-ATTACAACAGCAGGCATTTTCTCTTC; V12Cdc42: AGGGT-ACCGCCACCATGCAGACAATTAAGTGTGTTG and AGTCTA-GATTAGAATATACAGCACTTCC (Genosys, The Woodlands, TX). Restriction digests of PCR products and the pAdTrack-CMV vector were carried out with *Xho*I and *Xba*I enzymes (Rho and Rac) and *Kpn*I and *Xba*I enzymes (Cdc42) for 3 hours. Digested vector and insert were purified, quantitated and ligated at an insert to vector ratio of 4.5:1 overnight at 14°C (Invitrogen Life Technologies). Positive clones were confirmed by restriction digest, sequence analysis at Lone Star Labs and Western blot analyses using transfected 293 cells. Recombination and virus production were carried out as described by He et al., 1998.

In addition to producing adenoviruses that co-express green fluorescent protein (GFP), chimeric constructs were produced. Genes coding for both wild-type and constitutively active forms of RhoA, Rac1 and Cdc42 were cloned into the pEGFP-C2 vector (Clontech) along with a C-terminal verprolin-cofilin-acidic (VCA) domain of human N-WASP. Wild-type RhoA, Rac1, Cdc42 and VCA domain of N-WASP were amplified from human umbilical vein endothelial cell cDNA produced in our laboratory as previously described (Bell et al., 2001). Clones were amplified using the following primer sets: GFP-RhoA wt and GFP-V14RhoA: GAGATCTCTATGGCTGCC-ATCCGGAAG and AGAAGCTTTCACAAGACAAGGCAACCAG; GFP-Rac1wt and GFP-V12Rac1: AGAGATCTCTATGCAGGCCA-TCAAGTGTGTGGTG and AGAAGCTTTTACAACAGCAGGC-ATTTTCTCTTCC; GFP-Cdc42wt and GFP-V12Cdc42: AGA-GATCTCTATGCAGACAATTAAGTGTGTTG and AGAAGCTT-TTAGAATATACAGCACTTCCTTTTGGG; GFP-VCA: AGAGAT-CTCGGACCATCAGGTTCCAACTAC and AGAAGCTTTCAGTC-TTCCCACTCATCATC. Inserts and pEGFP-C2 vector were digested with *Bgl*II and *Hind*III enzymes as described above, and positive clones were confirmed with restriction digests, sequence analysis and western blot analysis using transfected 293 cells. Plasmids containing the EGFP-RhoA, -Rac1 and -VCA chimeric genes were then cloned into pShuttle-CMV using the common upstream primer for EGFP AGCTCGAGG-CCACCATGGTGAGCAAGGGC. The downstream primers used were AGTCTAGATCACAAGACAAGGCAACC-AGATTT, AGTCTAGATTACAACAGCAGGCATTTTCTCTTC, and AGAAGCTTTCAGTCTTCCCACTCATCATC respectively. The EGFP-Cdc42 primer set used was AGGGTACCGC-CACCATGGTGAGCAAGGGC and AGTCTAGATTAGAATAT-ACAGCACTTCCTTTT. Successful cloning was confirmed by sequence and restriction analyses.

Recombination of all clones with pAdEasy1 adenoviral backbone vector was accomplished using a previously described method (He et al., 1998). Briefly, 5 μg of Track-CMV (or Shuttle-CMV) plasmid was digested with *Pme*I (New England Biolabs, Beverly, MA) overnight at 37°C. Ethanol-precipitated linearized DNA was resuspended in 20 μl TE, and 4 μl was added to 100 ng pAdEasy1 before recombining into BJ5183 strain. Small colonies were screened using alkaline lysis and visualized on 0.8% agarose gels. Positive recombinants were transformed into DH5α cell line and the plasmids prepared at a large scale (Qiagen). Overnight digests (37°C) using *Pac*I (New England Biolabs) of 5 μg of plasmid were heated to 70°C before transfecting directly into 293 cell virus packaging line. 2 μg DNA were combined with 20 μl lipofectamine (Invitrogen Life Technologies) into 500 μl Optimem (Invitrogen Life Technologies) for 20 minutes. Mixture was added to 80% confluent 25 cm^2 flask containing 2.5 ml Optimem (Invitrogen Life Technologies) for 6 hours. The media was replaced every 3 days with 5 ml DMEM containing 10% FCS. Passage 1 viruses were harvested from 7-14 days after initial transfection.

Propagation of adenoviruses and preparation of viral extracts

293 cells were removed from flasks by scraping, collected in 50 ml

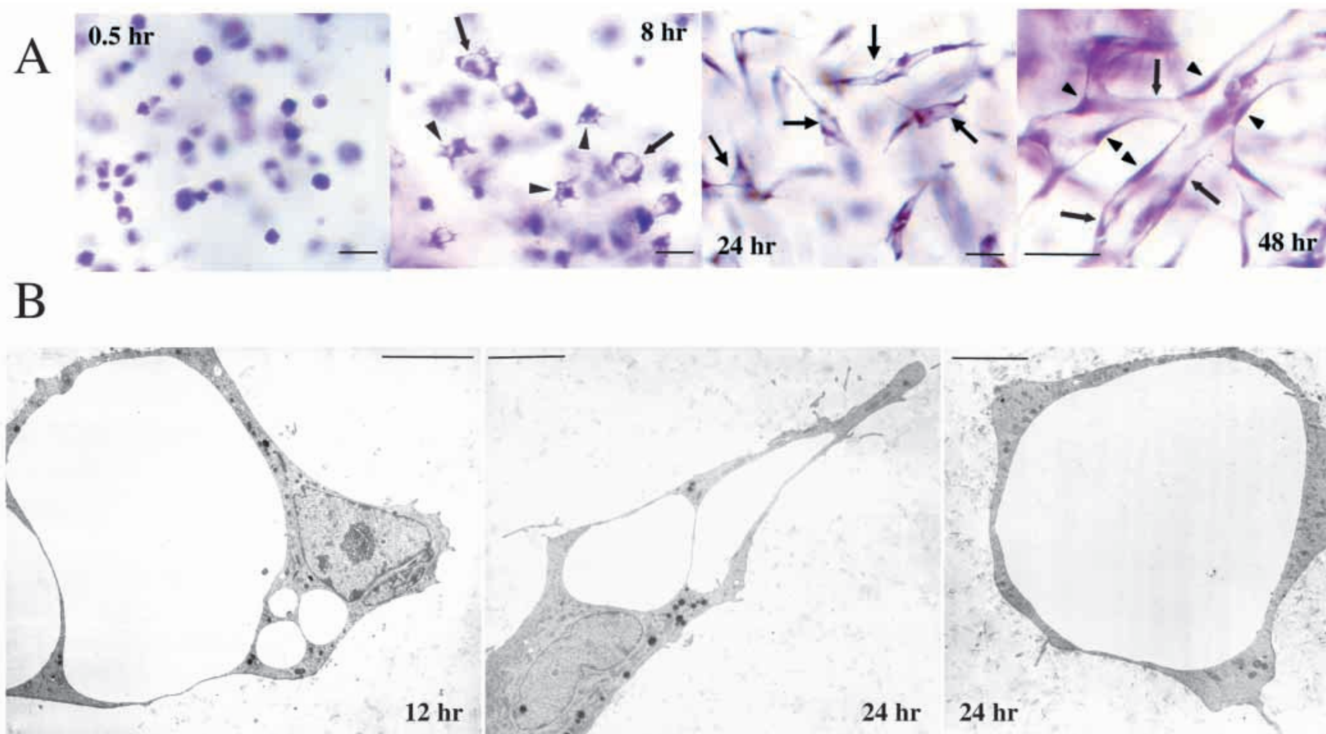


Fig. 1. (A) Time course of EC morphogenesis illustrating the formation of vacuoles and lumens in three-dimensional collagen matrices. Arrowheads indicate vacuole structures (8 hr) and nuclei (48 hr); arrows indicate lumen structures. Bar=50 μm. (B) Electron micrographic images of ECs undergoing morphogenesis in three-dimensional collagen matrices. Bar=5 μm.

polypropylene tubes, and spun at 350 ***g*** for 5 minutes. The media was aspirated and replaced with 2 ml cold PBS. Cells were vortexed before freezing in dry ice/methanol bath and thawed at 37°C. This cycle was repeated a total of four times before spinning at 350 ***g*** for 2 minutes. Supernatants were removed and aliquoted before storage at –80°C. Viruses were propagated by adding extracts in the absence of serum to freshly split confluent 75 cm^2 flasks of 293 cells for 6 hours before feeding. Cells were harvested when 30-50% of the monolayer illustrated cytopathic effects (CPE). All viruses (passage 3 or higher) described were screened to confirm the presence of nucleic acid inserts along with protein production using western blot analyses.

Three-dimensional assays with human endothelial cells in collagen and fibrin matrices

Human umbilical vein endothelial cells (HUVEC) were cultured (passage 2-6) as previously described (Davis and Camarillo, 1996). Cells were placed into 25 cm^2 tissue culture flasks overnight prior to infection with adenoviruses (10 PFU/cell). Monolayer cultures were rinsed twice with M199 (Invitrogen Life Technologies) before addition of adenoviruses in 2.5 ml M199. Cultures were placed at 37°C (5% CO_2) for 5-6 hours before media containing viral extracts was removed and replaced with growth medium. Cells were harvested 24 hours later and placed in three-dimensional (25 μl) collagen (3.5 mg/ml) or fibrin (10 mg/ml) matrices as described (Davis and Camarillo, 1996; Bayless et al., 2000). Cultures were fixed for 30 minutes with 2% paraformaldehyde in PBS at the time points indicated before being quantified and photographed using a Nikon Labophot microscope equipped with a Nikon FX-35A camera. Other cultures were fixed with glutaraldehyde and processed for electron microscopy as previously described (Davis and Camarillo, 1996).

Fluorescent labeling of intracellular vacuoles

Adenoviruses expressing GFP chimeric proteins coupled to Rho GTPases were administered to EC monolayers for 5-6 hours before removal of extracts and replacing with growth medium. After 36 hours, cells were trypsinized and placed into three-dimensional collagen gels (3.75 mg/ml). In these experiments, 1 μl gels were added to sterile coverslips and inverted onto 4- or 8-well (Nalgene, Rochester, NY) chambers containing culture media that was CO_2-equilibrated using methods previously described (Davis and Camarillo, 1996). In some cases, 2 μg/ml of mixed isomers of carboxytetramethyl rhodamine (Molecular Probes, Eugene, OR) was added to the culture media. Cultures were allowed to proceed for either 8 or 24 hours before being fixed and photographed. Those treated with carboxytetramethyl rhodamine were rinsed in 10 ml M199 for 1 hour at 37°C to remove free dye before being photographed live using a Nikon Labophot microscope equipped with a Nikon FX-35A camera. Cultures were fixed at 24 hour time points, mounted and sealed before image analysis using confocal microscopy. Images were captured using Radiance 2000 MP imaging system and LaserSharp 2000 software (Bio-Rad, Hercules, CA) at the Texas A&M University College of Veterinary Medicine Image Analysis Laboratory. Three-dimensional reconstruction was accomplished using Kitware software) at the Texas A&M University College of Veterinary Medicine Image Analysis Laboratory.

Digesting cells from collagen matrix

Cells undergoing morphogenesis were digested out of collagen matrices to image vacuoles and lumens in two dimensions and also demonstrate that cells digested from the matrix have the ability to attach in an integrin-dependent manner. Duplicate collagen gels (25 μl volume) were placed into 200 μl M199 (37°C) with 5 μg collagenase (Sigma-Aldrich Corp., St. Louis, MO) for 10 minutes with gentle agitation. After digestion of gels, cells were layered onto coverslips coated with 10 μg/ml pronectin F, a recombinant Arg-Gly-Asp containing protein. Cells were allowed to attach for 15-20

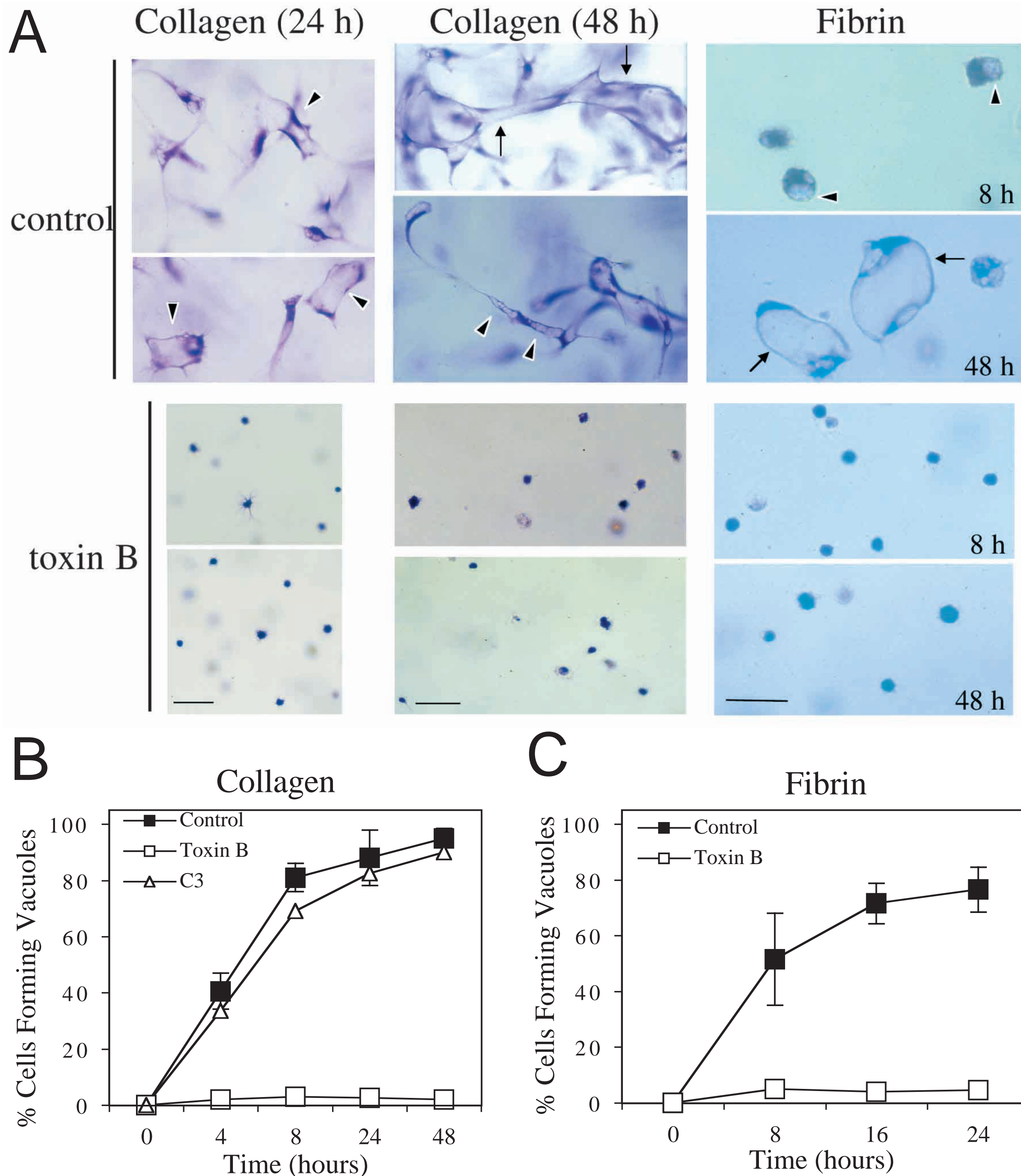


Fig. 2. *C. difficile* toxin B completely inhibits EC morphogenesis in both collagen and fibrin matrices. (A) Photographs of three-dimensional cultures fixed and stained with toluidine blue. ECs were allowed to undergo morphogenesis in collagen and fibrin matrices for 8, 24 or 48 hours as indicated. Arrowheads indicate vacuoles; arrows indicate developing lumins. Cells pretreated with toxin B are shown in lower panel. Note the complete lack of vacuoles and lumenal structures in toxin B group. Quantification of cells forming vacuoles and lumens in three-dimensional collagen (B) and fibrin matrices (C). Cultures were fixed and stained at various time points following pretreatment with toxin B and C3 ribosyltransferase (collagen only). Data are expressed as a percentage of cells forming vacuoles±s.d. (n=3). Bar=30 μm.

minutes before being gently aspirated and rinsed. Cells were photographed live or fixed with 2% paraformaldehyde before being imaged using fluorescence and phase microscopy.

Western blot analysis

Monoclonal antibodies specific for Rho (Cytoskeleton, Inc., Denver, CO) and Cdc42 (BD Transduction Laboratories, Lexington, KY), along with a polyclonal antibody specific for Rac (Cytoskeleton, Inc.), were tested using western blot analysis to rule out crossreactivity using recombinantly produced His-tagged Rho, Rac and Cdc42 proteins. Extracts from three-dimensional cultures were made as described (Salazar et al., 1999) before performing western blot analyses with antibodies diluted 1:1000. Secondary antibodies

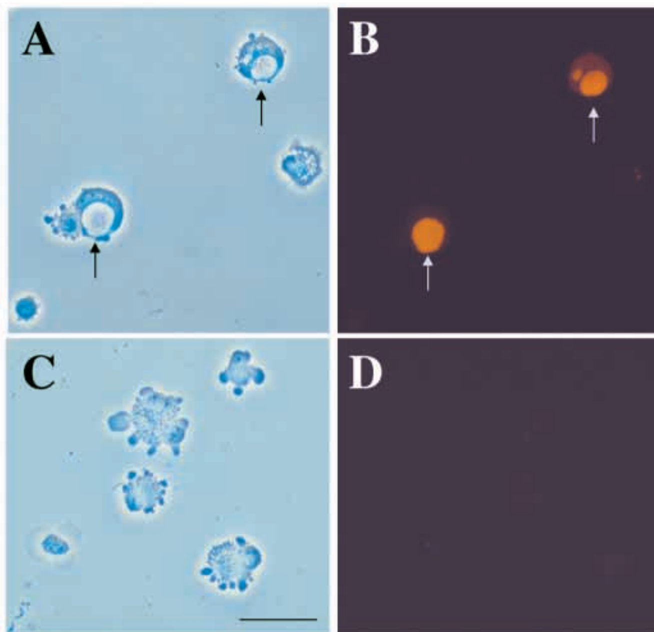


Fig. 3. *C. difficile* toxin B completely inhibits pinocytic uptake into intracellular vacuoles during EC morphogenesis. Three-dimensional EC cultures were allowed to develop in the presence of carboxytetramethyl rhodamine added to culture media (2 μg/ml). After 4 hours, collagen matrix was digested with collagenase for 10 minutes at 37°C in M199. Cells were plated onto pronectin-F coated coverslips and allowed to attach before rinsing off the free dye. Coverslips were mounted onto culture media and photographed live under phase contrast (A,C) and fluorescence (B,D) microscopy. The control group (A,B) are shown with toxin-B-treated cells (C,D). Bar=50 μm.

conjugated to horseradish peroxidase (Dako, Carpinteria, CA) were added 1:2000 prior to development using chemiluminescence (Amersham Biosciences, Piscataway, NJ). To detect GFP, rabbit antiserum was produced by Bethyl Laboratories (Montgomery, TX) using recombinant His-tagged GFP as an antigen.

Results

Inhibition of Rho GTPases with toxin B results in complete blockade of EC vacuole and lumen formation

To characterize the role of Rho GTPases during EC morphogenesis (Davis and Camarillo, 1996; Bayless et al., 2000), ECs were pre-treated with *C. difficile* toxin B and then resuspended in three-dimensional collagen or fibrin matrices. Under normal EC culture conditions (Fig. 1A) (Davis and Camarillo, 1996), we observe that ECs develop vacuolar structures (0-8 hours) that coalesce to form the developing lumenal compartment (after 24 hours). Branching morphogenesis follows to allow neighboring cells to interconnect and form multicellular capillary tubes (48 hours). These EC networks contain multiple cells. Arrowheads indicate nuclei labeled after toluidine blue staining of cultures. Cross-sectional analysis of these structures is illustrated by electron micrograph data shown in Fig. 1B. These data illustrate vacuoles (12 hours) that merge into lumenal structures (24 hours) that are devoid of extracellular matrix, which is located on the abluminal EC surface. Our observations both from the present study and previous work (Davis and Camarillo, 1996; Bayless et al., 2000) indicate that interference with the vacuole formation process prevents further EC morphogenesis and formation of a lumenal compartment. Consequently, EC vacuoles are precursors to lumen formation in ECs.

Toxin B is known to inhibit Rho, Rac and Cdc42 GTPases (Sehr et al., 1998). Toxin B completely blocked EC morphogenesis by interfering with vacuole and lumen formation (Fig. 2). Photographs of treated versus untreated cultures in three-dimensional collagen and fibrin matrices are shown in Fig. 2A. In these experiments, we observe that pretreatment of ECs with toxin B results in failure of the lumenal compartment and interconnecting structures to form at both 24 and 48 hour time points. Similarly, toxin B blocks vacuole (8 hours) and lumen formation (48 hours) in fibrin matrices. Quantitation of these results in both three-dimensional collagen (Fig. 2B) and fibrin (Fig. 2C) matrices reveals a dose-dependent increase in the formation of vacuoles and lumens over time in control cultures, whereas complete blockade of these responses is observed with toxin B treated EC cultures. The effects of toxin B could not be explained by the induction of cell death. EC monolayers treated for 24 hours with toxin B retained the ability to exclude trypan blue (96.4%±0.6) compared with the control (97.4%±0.9) and were capable of attaching to ECM substrates (Fig. 3C) and collagen matrices (data not shown). Further experiments revealed that pretreatment with the exoenzyme C3 transferase, which selectively inhibits Rho GTPases, had no effect on vacuole and lumen formation (Fig. 2B), but did ribosylate Rho in vitro (not shown) and inhibited Rho function, as we have reported previously (Verma et al., 2000). These results indicate that the Rho GTPase alone was not involved in formation of EC vacuoles and lumenal structures. In contrast, toxin B treatment completely inhibited EC morphogenesis at all stages, indicating that Rac and Cdc42 were the likely candidates for regulating these processes. These data strongly support the hypothesis that Rho GTPases are required for EC vacuole and lumen formation during morphogenesis in three-dimensional matrices.

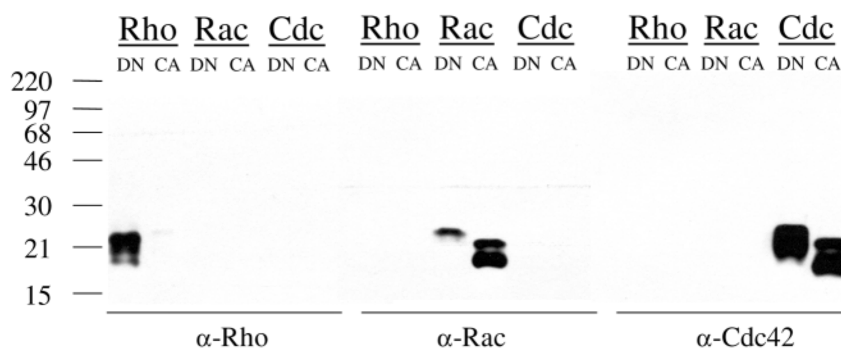


Fig. 4. Adenoviruses expressing mutant forms of Rho GTPases induce specific expression of each protein in ECs. ECs were infected with either DN or CA Rho, Rac or Cdc42 adenoviruses. Cells were allowed 24 hours to express proteins before placing them in three-dimensional collagen matrices. Cultures were allowed to proceed for 24 hours prior to preparing extracts. Samples were loaded onto 12% SDS-PAGE gels, blotted to PVDF and probed with GTPase-specific antibodies. Infection with GFP control adenovirus showed no signal (not shown).

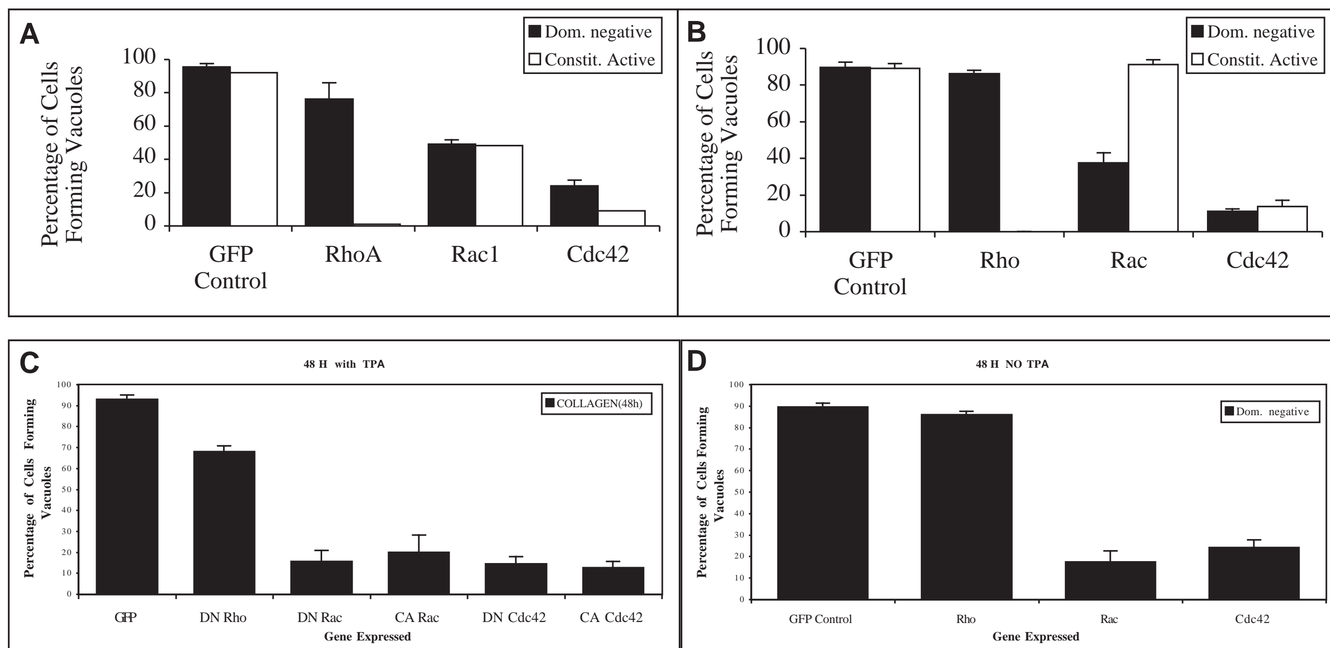


Fig. 5. Influence of Rho GTPase mutants on EC vacuole and lumen formation in three-dimensional collagen matrices. Endothelial cells were infected for 24 hours prior to resuspension in three-dimensional collagen matrices. Cultures were allowed to proceed for 24 (A,B) or 48 (C,D) hours in the presence (A,C) or absence (B,D) of TPA before fixation in paraformaldehyde. Quantitation was carried out using fluorescence microscopy. The average vacuole formation expressed as a percentage of cells forming vacuoles is shown±s.d. (200 cells counted per group; *n*=3).

Endothelial cell lumen formation occurs through pinocytic intracellular vacuoles

The process of endothelial cell vacuole formation has previously been shown to be an integrin-dependent pinocytic event (Davis and Camarillo, 1996). Mixed isomers of carboxytetramethyl rhodamine were incubated with endothelial cell cultures during vacuole formation. This dye is taken up into vacuoles by control ECs. After 4 hours in culture, ECs were digested out of a collagen matrix and plated onto coverslips before being rinsed to remove free dye and photographed (Fig. 3A,B). Pretreatment of endothelial cells with toxin B, which completely blocked the ability of vacuoles and subsequent lumens to form, inhibited the uptake of rhodamine dye into vacuolar structures (Fig. 3C,D). Toxin-B-treated ECs were able to attach to coverslips despite the complete blockade of vacuole formation. Identical uptake of rhodamine dye occurs in ECs infected with control adenoviruses expressing green fluorescent protein (GFP) (see later), indicating transfection with control adenoviruses does not affect the pinocytic process or the ability of ECs to form vacuoles.

Expression of Cdc42 and Rac1 mutants in ECs inhibits vacuole and lumen formation in three dimensions.

We analyzed the ability of the RhoA, Rac1 and Cdc42 GTPases to regulate EC vacuole formation using recombinant adenoviruses to deliver dominant-negative (DN) and constitutively active (CA) forms of each GTPase. The adenoviral delivery system utilized here is designed to co-express GFP as an indicator of viral transfection (He et al., 1998). Preliminary results indicated that treatment with control GFP adenovirus had no effect on the ability of ECs to form vacuoles as compared to previous studies. Also, EC monolayers infected identically to cells resuspended in three-dimensional matrices were monitored over the same period of time (3-4 days) to rule out toxicity (not shown). The CA Rho virus induced EC death (not shown), whereas the other viruses had no toxic effects on EC monolayers during the 3-4 day period following expression of Rho GTPase mutants. Adenovirally transfected ECs were allowed 24 hours to express mutant forms of Rho GTPases prior to being placed in collagen matrices. After 24 hours, cultures were analyzed for selective expression of the RhoA, Rac1 and Cdc42 proteins using western blot analysis (Fig. 4). Our results indicate that both the dominant-negative and constitutively active viruses elicited select induction of RhoA, Rac1 or Cdc42 compared with GFP-infected cultures. Although DN Rac1 and Cdc42 proteins migrate slightly higher due to the presence of a c-myc epitope tag, the CA RhoA signal is weak because ECs expressing CA RhoA undergo cell death. The expression of CA RhoA was confirmed using a control 293 cell line (not shown). The ability of cells expressing mutant forms of Rho GTPases to maintain interactions with the extracellular matrix was tested using adhesion assays (Bayless and Davis, 2001). ECs were allowed to express proteins 24 hours prior to testing in adhesion assays. Our results indicate that adhesion of ECs expressing GFP, CA Cdc42, CA Rac1, DN RhoA, DN Rac1 and DN Cdc42 proteins were similar to both collagen type I and osteopontin substrates, which are β1- and β3-integrin-dependent interactions (not shown). Further experiments were conducted with ECs transfected with DN and CA RhoA, Rac1 and Cdc42 adenoviruses undergoing morphogenesis at the 24 or 48 hour

time points. These experiments were conducted in the presence and absence of phorbol ester (a powerful stimulator of vacuole formation) to determine whether expression of mutant forms of Rho GTPases have the ability to stimulate or inhibit vacuole formation. Quantitation of EC vacuole formation was conducted under fluorescence so that only transfected cells (~90-95%) were included. Experiments were performed under normal culture conditions (Fig. 5A,C) (in the presence of TPA, a phorbol ester) and in the absence of TPA (Fig. 5B,D). Marked inhibition of EC vacuole formation was observed following expression of CA or DN Cdc42. Also, DN Rac1 expression markedly blocked these events as well, whereas DN RhoA virus had minimal to no effect. CA Rac1 had inhibitory effects with TPA, but not in its absence. The CA RhoA virus blocked EC morphogenesis by inducing EC death (probably through an apoptotic mechanism). A more detailed analysis of a time course showing EC vacuole formation revealed similar conclusions. Experiments were conducted in both collagen and fibrin three-dimensional matrices in the presence and absence of phorbol ester (Fig. 6A,B, respectively). Expression of mutated forms of Cdc42 markedly inhibited vacuole formation throughout the time course, whereas expression of DN Rac1 mutant particularly blocked at later stages of morphogenesis and appeared to induce regression of formed vacuoles in the presence of TPA. In the absence of TPA, expression of DN Rac1 completely inhibited vacuole formation, whereas CA Rac1 has no effect. DN RhoA expression had a slight inhibitory effect with TPA stimulation, while without TPA, effects were similar to expression of GFP control. Photographs of the blocking effects of Cdc42 mutants are shown in Fig. 7. In panels A-D, after 4 hours of morphogenesis, ECs expressing GFP or CA Cdc42 were digested out of collagen gels and plated to allow imaging in two dimensions. As shown in Fig. 7A,B, GFP control cells form intracellular vacuoles whereas ECs expressing CA Cdc42 do not (Fig. 7C,D). Interestingly, although morphogenesis is blocked, cells expressing CA Cdc42 were able to attach to coverslips coated with Pronectin F (Fig. 7C,D). Later cultures (24 hours) of ECs showed a marked inhibitory effect of CA Cdc42 on EC vacuole formation in three-dimensional collagen matrices (Fig. 7F) versus GFP control (Fig. 7E). Thus, Cdc42 appears to play a critical role in the EC vacuole formation process. Additional photographs of cultures expressing GFP, CA Rac1 and CA Cdc42 are shown in Fig. 7G-I. Together, these data indicate that Cdc42 appears to play a dominant role in these events, whereas Rac1 plays a distinct but also necessary role in EC vacuole and eventual lumen formation. In contrast, the toxin data, combined with the adenoviral expression data, do not support a major role for Rho in EC vacuole and lumen formation in three-dimensional matrices.

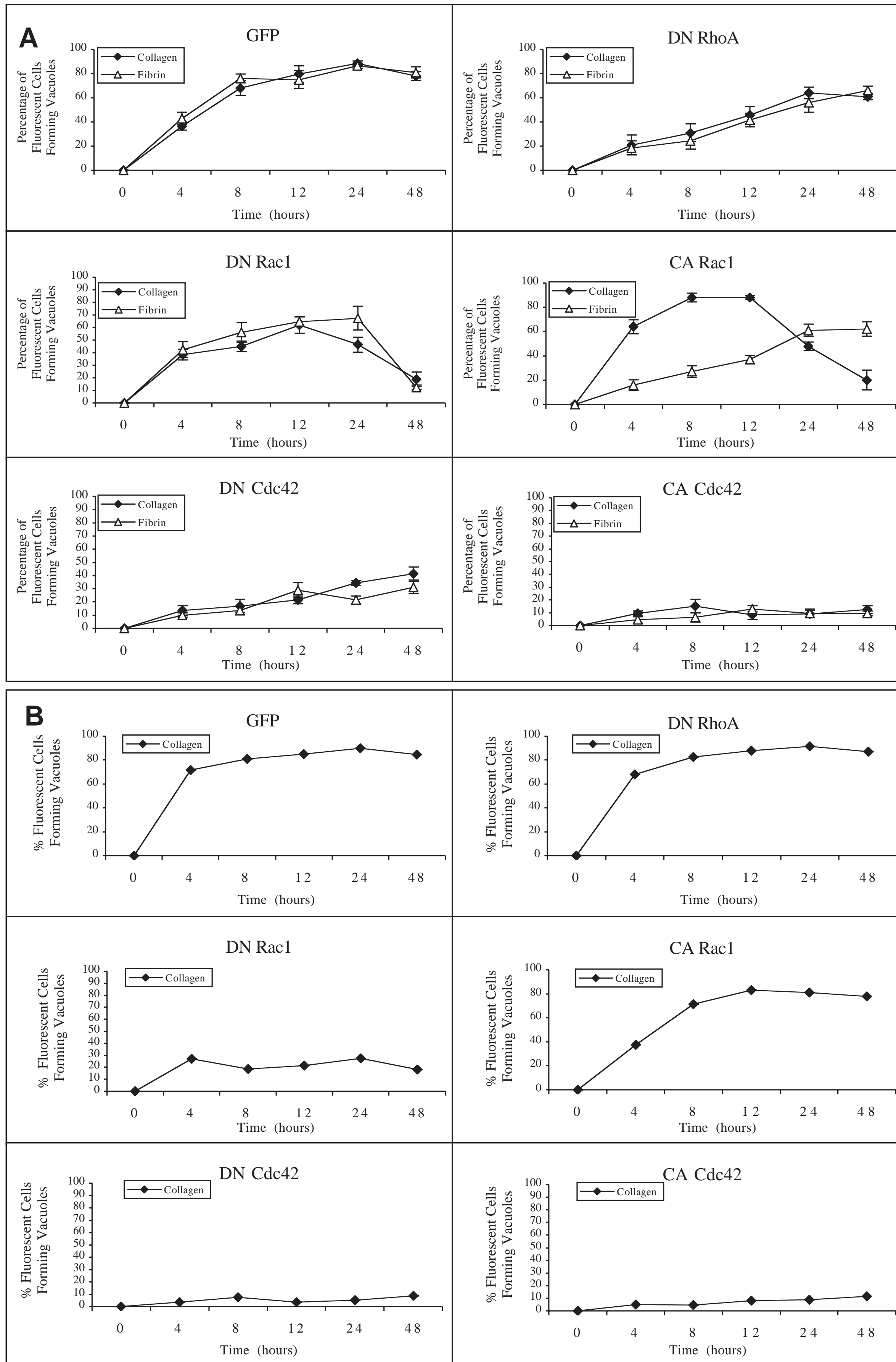
Induction of Cdc42 protein during EC morphogenesis

Because of the dominant role of Cdc42 in EC vacuole and lumen formation, western blots were performed to examine the expression levels of this protein during EC morphogenesis in collagen matrices. As shown in Fig. 8, Cdc42 protein is markedly induced between 12 and 48 hours of morphogenesis. In contrast, the control protein, G3PDH, shows stable expression. These data correspond to previously unreported findings using DNA microarray analysis (Bell et al., 2001), comparing mRNA levels at 0, 8, 24 and 48 hours of morphogenesis. Our data indicate that compared with reference mRNA (0 hour time point), both Cdc42 and Cdc42 effector protein-2 (Hirsch et al., 2001) (CEP 2) mRNA levels are upregulated over the time course. Cdc42 levels were increased 1.2, 1.6 and 1.5 fold, whereas CEP 2 increased 1.6, 1.4 and 1.6 fold at 8, 24 and 48 hour time points, respectively. The timing of Cdc42 induction coincides with a marked expansion of the EC lumenal compartment during morphogenesis (Fig. 1A). As we show later, expression of Cdc42 wild type in ECs resulted in an increased number of small EC vacuoles. We observe that fusion of vacuoles appears to increase lumenal diameter (Davis and Camarillo, 1996; Bayless et al., 2000), so increases in Cdc42 levels during 12-48 hours of morphogenesis may directly stimulate the lumen expansion process through increased vacuole formation and fusion.

Cdc42 and Rac1 target to pinocytic vacuolar membranes in ECs during morphogenesis

The ability of DN and CA Rac1 and Cdc42 constructs to interfere with vacuole and lumen formation raised the question of whether or not these molecules may be targeted to vacuolar membranes. To address this question, adenoviruses were constructed to express chimeric GFP-Rac1 that was constitutively active (CA), GFP-Cdc42 wild type (wt) and GFP-Rac1wt. Previous studies have indicated an ability of Rac1 to localize to pinocytic vesicles (Ridley et al., 1992) and interact with endocytic vacuoles of epithelial cells, which had been either stimulated with the VacA toxin of *Helicobacter pylori* or had its cadherin-based intercellular junctions disrupted (Hotchin et al., 2000; Akhtar and Hotchin 2001). Western blots were performed using antisera raised against recombinant GFP to confirm that these viruses express each respective fusion protein (Fig. 9A). Also, GFP-RacV12, GFP-Cdc42wt and GFP-Rac1wt showed specific reactivity with Rac1 and Cdc42 antibodies (not shown). We observed that EC vacuoles are derived from pinocytic events as carboxyrhodamine added to the culture medium strongly labels the vacuole compartment in cells expressing GFP alone (Fig. 9B), GFP-Rac1V12, GFP-Rac1wt or GFP-Cdc42wt (Fig. 9C). GFP-Rac1V12, GFP-Rac1wt and GFP-Cdc42wt localized to the vacuolar membranes surrounding carboxyrhodamine dye (arrowheads). Photographs of other experiments (without carboxyrhodamine addition) revealed labeling of vacuolar membranes with GFP-Rac1V12 (Fig. 9D), GFP-Cdc42wt (Fig. 9E) and GFP-Rac1wt proteins (not shown). Arrowheads indicate labeling of vacuolar membranes with GFP-chimeric Rac1 and Cdc42 proteins (Fig. 9C-E). Interestingly, we observed that expression of GFP-Cdc42wt resulted in an obvious increase in the number of EC vacuoles compared with the GFP control (Fig. 9E). We have been unable to show that GFP-Cdc42V12 targets to vacuoles because this chimeric protein blocked morphogenesis (not shown).

Further analysis of this localization using confocal microscopy is shown in Fig. 10. Cultures (1 μl) were analyzed at 24 hour time points. Data shown are from ECs expressing GFP-Rac1V12 and GFP-Rac1wt proteins. Arrows indicate vacuolar membranes. Serial sections (1 μm) through EC structures revealed targeting of GFP-Rac1wt (Fig. 10A) and GFP–Rac1V12 constructs (Fig. 10B) to intracellular vacuoles.


A
GFP
DN RhoA
DN Rac1
CA Rac1
DN Cdc42
CA Cdc42
Collagen
Fibrin
Percentage of Fluorescent Cells Forming Vacuoles
Time (hours)
B
% Fluorescent Cells Forming Vacuoles
0
4
8
12
24
48

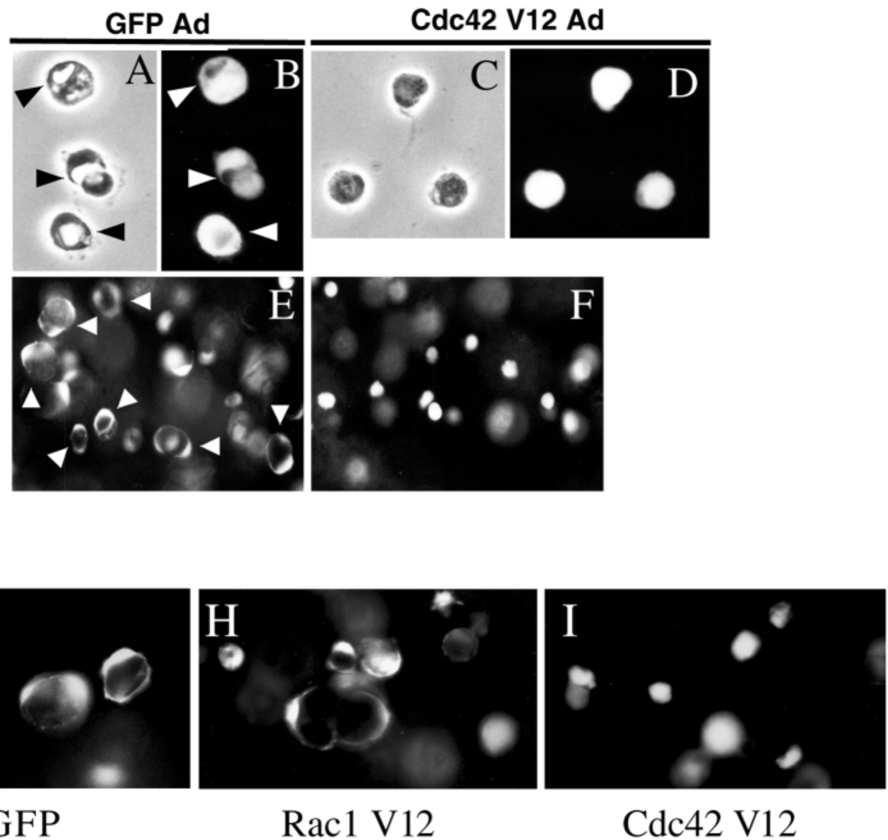


Fig. 7. Expression of constitutively active Cdc42 blocks EC vacuole formation in three-dimensional collagen matrices. Upper panel: ECs were photographed using phase contrast (A,C) and fluorescence microscopy (B,D-F). ECs were treated with control GFP adenovirus (A,B,E) or Cdc42 CA adenovirus (C,D,F). Arrowheads indicate the presence of EC vacuoles. Shown in A-D are ECs digested from collagen assays (4 hours) and plated onto coverslips. Arrowheads indicate the presence of EC vacuoles. (E,F) Fluorescent images of intact cultures fixed at 24 hours of morphogenesis. Arrowheads indicate vacuole and lumen formation. Lower panel: magnified images of intact cultures using ECs infected with GFP, CA Rac and CA Cdc42 adenovirus at 24 hours in three-dimensional collagen matrices.

It is observed that many intracellular vacuoles (arrowheads) are directly adjacent to or appear to be fusing with the developing lumenal membrane (open arrows). It is evident that the lumenal membrane appears irregular in areas where vacuoles may have recently fused (arrows). The increased sensitivity and resolving power of confocal fluorescence microscopy further confirm the targeting of Rac1 to vacuole membranes to regulate lumen formation. Identical confocal imaging experiments with GFP-Cdc42wt constructs revealed similar data (Fig. 11A). Four separate sections of a multicellular complex are shown. An increased number of intracellular vacuoles was observed with expression of the GFP-Cdc42wt construct (arrowheads). Interestingly, we observed that this structure contained two nuclei, indicating that these cells are capable of forming multicellular structures. Three-dimensional reconstruction of a GFP-Cdc42wt-labeled capillary structure is shown in Fig. 11B. After fixation (24 hours), cultures expressing the GFP-Cdc42wt construct were labeled with propidium iodide (PI) to label nuclei. Interestingly, the collagen matrix showed some affinity for the propidium iodide dye, whereas the central lumenal compartment did not. As shown in the Fig. 11B (left panel), four nuclei (arrowheads) could be observed in this multicellular structure along with a negatively stained lumenal compartment (L). Analysis of GFP-Cdc42wt signal (converted to red here) revealed a multicellular structure of ECs surrounding a central lumenal compartment (outlined by arrows). Overall, these data show that GFP-Cdc42wt, GFP-Rac1V12 and GFP-Rac1wt constructs target to EC vacuole membranes, which regulate the lumen formation process. In this system, individual cells form multicellular capillary structures, which arise through intracellular vacuole formation, coalescence and fusion within individual ECs. These events are followed by branching and sprouting events to form interconnecting multicellular lumenal structures.

Fig. 6. The Cdc42 and Rac1 GTPases are required for EC morphogenesis in three-dimensional extracellular matrices. Recombinant adenoviruses were used to express either dominant-negative (DN) or constitutively active (CA) Rho GTPases in ECs. ECs were cultured in either collagen or fibrin matrices, and were quantified by counting the percentage of fluorescent ECs with vacuoles over time. (A) Cultures formed with phorbol ester; (B) Cultures formed without phorbol ester. The average vacuole formation expressed as a percentage of cells forming vacuoles is shown±s.d. (200 cells counted per group; *n*=3).

Expression of the verprolin-cofilin-acidic (VCA) domain of human N-WASP, a downstream effector of Cdc42, in ECs blocks lumen formation

Our results reveal a role for Cdc42 in EC lumen formation, on

Fig. 8. Induction of Cdc42 protein during EC morphogenesis in three-dimensional collagen matrices. ECs were cultured in collagen matrices for various times and extracts were prepared at the time points indicated. Samples were run on SDS-PAGE gels, blotted to PVDF membranes and probed with monoclonal antibodies specific to either Cdc42 or control G3PDH.

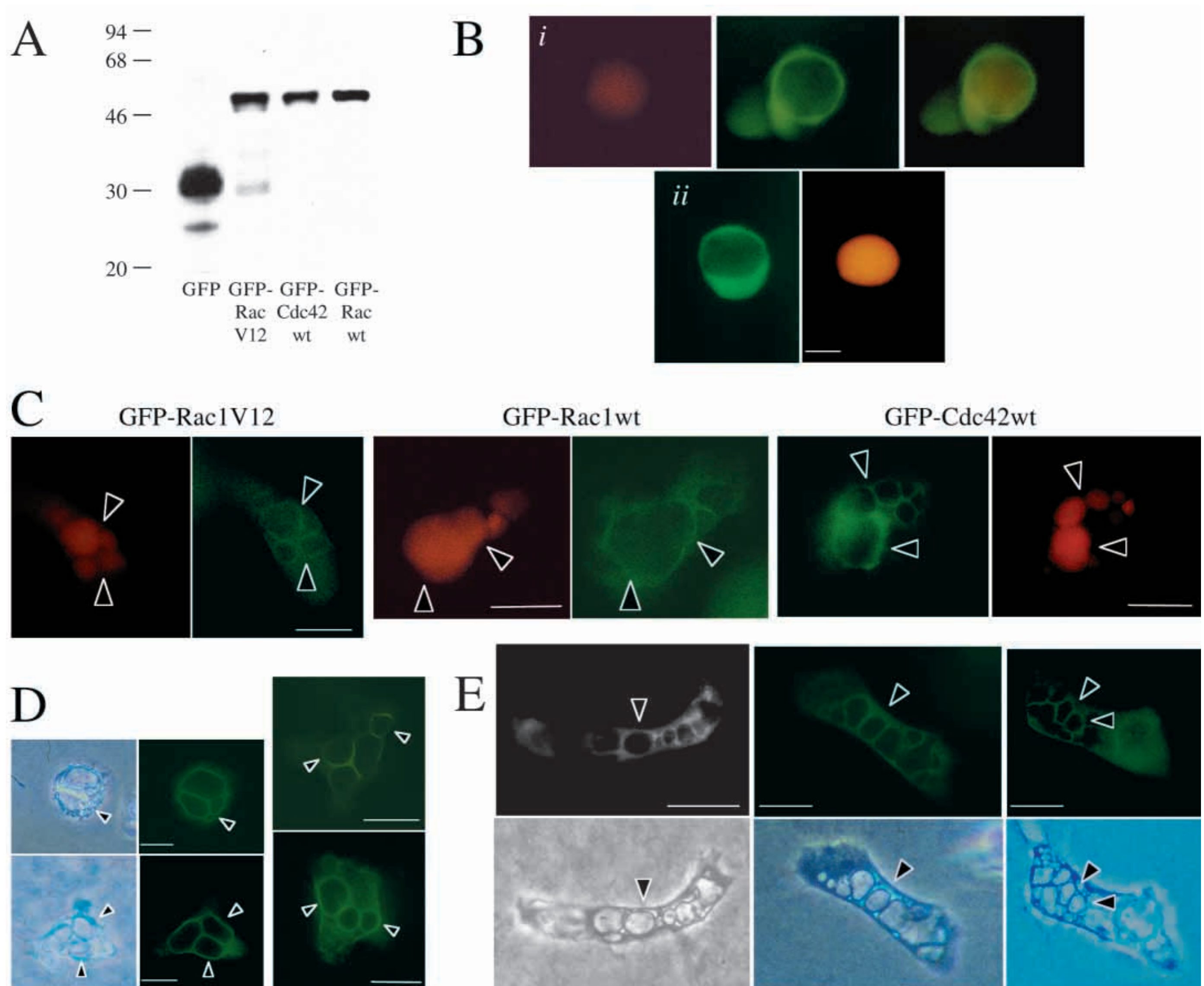


Fig. 9. Targeting of GFP-Rac and -Cdc42 fusion proteins to EC intracellular vacuole membranes during morphogenesis in three-dimensional collagen matrices. (A) Western blot analysis showing production of GFP-Rac1 and GFP-Cdc42 fusion proteins. (B) ECs expressing GFP alone were cultured in collagen matrices (24 hours) in the presence of carboxyrhodamine in the culture media to label EC lumens after rinsing out free dye. (i) Left panel: a photograph with a rhodamine filter; middle panel: a photograph with a fluorescein filter; right panel: merged image. (ii) Dual images indicating rhodamine labeling of vacuoles and lumens of ECs expressing GFP. (C) ECs expressing GFP-Rac1V12, GFP-Rac1wt and GFP-Cdc42wt were cultured in collagen matrices for 8-24 hours in the presence of carboxyrhodamine to label pinocytic intracellular vacuoles. After washing, ECs were photographed with the rhodamine or fluorescein filters. Note the labeling of vacuoles with GFP-Rac1 or GFP-Cdc42 constructs. ECs expressing either GFP-Rac1V12 (D) or GFP-Cdc42 wt (E) were cultured in collagen matrices and photographed to indicate the targeting of Cdc42 and Rac to vacuole membranes (D,E). Arrowheads indicate labeling of vacuolar membranes. Bar=20μm in panel B, 30 μm in panels C-E.

the basis of the ability of both DN and CA constructs to markedly interfere with this process. N-WASP is a known downstream effector specific for Cdc42 (Symons et al., 1996). Recent studies have indicated that a C-terminal VCA domain in N-WASP is responsible for actin polymerization, and recombinant production of this domain stimulates actin polymerization through the Arp2/3 complex (Rohatgi et al., 1999; Higgs and Pollard, 2000; Prehoda et al., 2000). On the basis of these studies, we constructed an adenovirus to express GFP-VCA in ECs to determine the effect of this construct on lumen formation. This experiment was designed to mimic the influence of CA Cdc42 expression in ECs since it possesses the greatest inhibitory effect (Fig. 6). The western blot analysis shown in Fig. 12A shows the production of the chimeric protein. Expression of GFP-VCA in ECs placed in three dimensional collagen matrices resulted in nearly complete blockade of vacuole and lumen formation over 24 hours (Fig. 12B). Photographs illustrating the morphological effects of this construct on ECs in three dimensions are shown in Fig. 12C. Interestingly, although blocking lumen formation, GFP-VCA

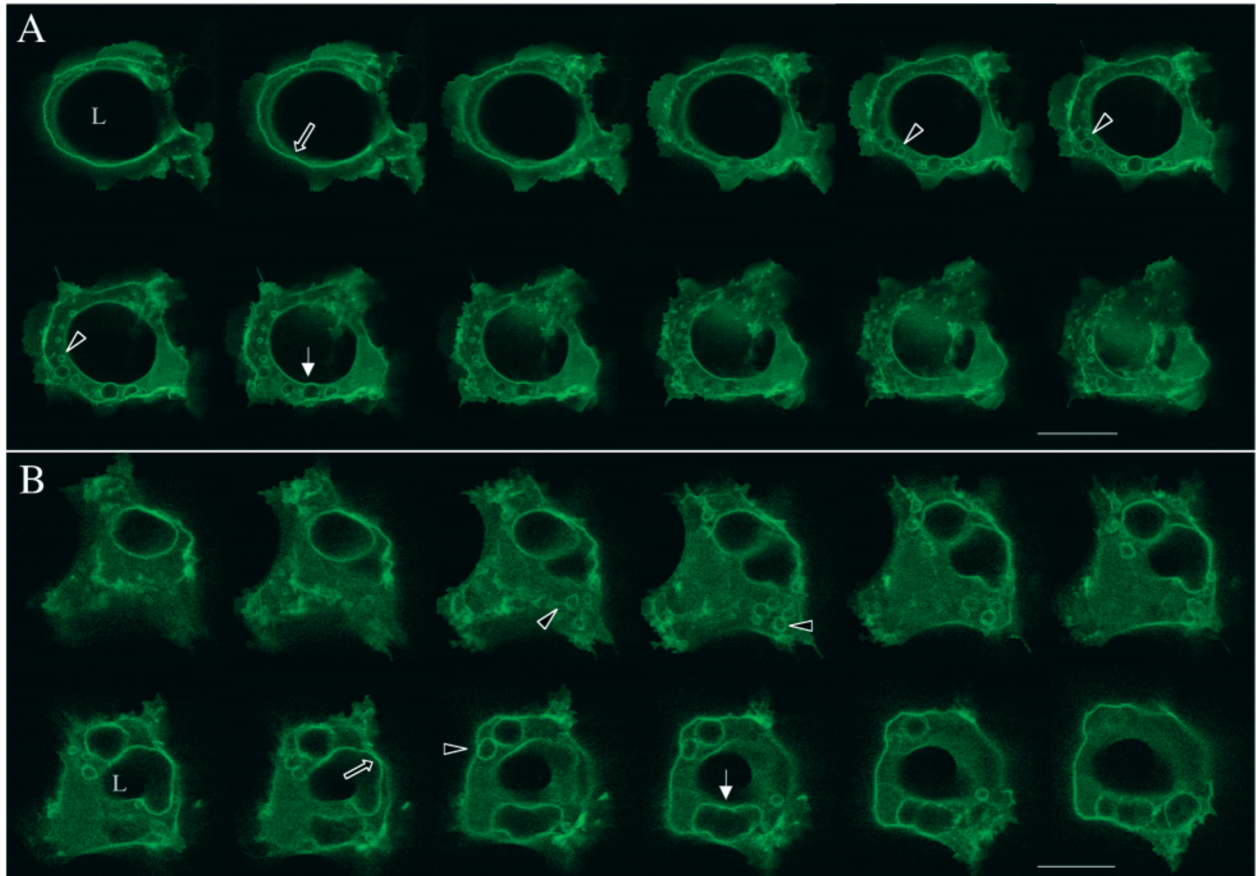


Fig. 10. Confocal analysis of GFP-Rac constructs targeting to vacuolar and lumenal membranes. (A) GFP-Racwt and (B) GFP-Rac1V12 localize to vacuolar EC membranes (arrowheads) as well as lumenal membranes (open arrows). Vacuolar fusion events (arrows) appear to occur between vacuoles and the lumenal membrane. Serial sections are 1 μm thick. Bar=20 μm.

induced numerous filopodia-like extensions from ECs. The VCA domain of N-WASP was previously linked downstream of Cdc42 and shown to stimulate Arp2/3-dependent actin polymerization (Rohatgi et al., 1999; Higgs and Pollard, 2000; Prehoda et al., 2000).

Discussion

Relationship of EC vacuole and lumen formation to phagocytosis and macropinocytosis

The formation of EC vacuoles that eventually coalesce to form a lumenal compartment is a pinocytic process (this paper) (Davis and Camarillo, 1996) that resembles macropinocytosis and phagocytosis, where the involvement of RhoA, Rac1 and Cdc42 have been well documented (Ridley et al., 1992; Lamaze et al., 1996; Caron and Hall, 1998; Albert et al., 2000; Chimini and Chavrier, 2000; Garrett et al., 2000; Hotchin et al., 2000). We find here that Rac1 and Cdc42 are involved in regulating EC vacuole and lumen formation, whereas Rho seems to play a minimal role in the process. Our data are consistent with previous studies showing distinct roles for Rac1 and Cdc42 versus Rho in various cellular events (Burridge, 1999; Wojciak-Stothard et al., 2001). Rac1 and Cdc42 have been reported to mediate FcγR-mediated phagocytosis, whereas Rho regulates C3-complement-mediated phagocytosis (Caron and Hall, 1998). Here, we find that DN and CA Rac1 and Cdc42 expression interferes with EC vacuole formation in three-dimensional matrices, whereas inhibitors of Rho have little or no effect. The effects are more pronounced in the absence of phorbol ester, a stimulant of vacuole and lumen formation (Davis and Camarillo, 1996; Bayless et al., 2000) and macropinocytosis (Swanson and Watts, 1995). Expression of CA Rac1 fails to induce lumenal collapse without phorbol ester stimulation, indicating that Rac1 may play a stimulatory role in the process, as is suggested by our data showing that CA Rac1-treated cells also exhibited larger lumenal diameters compared with the GFP control (not shown). These findings correlate with previous reports of phorbol ester stimulation of Rac-dependent signaling events (Ridley et al., 1992). Together, our data agree with what has previously been reported for Rho, Rac and Cdc42 in pinocytic events.

GFP-Cdc42 and GFP-Rac1 constructs target to vacuolar and lumenal membranes and regulate EC morphogenesis

Expression of wild-type Cdc42 and CA Rac1 GFP-chimeric proteins in ECs revealed that these constructs target to vacuolar membranes during the morphogenic process. These findings are similar to a previous report where a GFP-Rac construct targeted to vacuoles in epithelial cells (Hotchin et al., 2000).

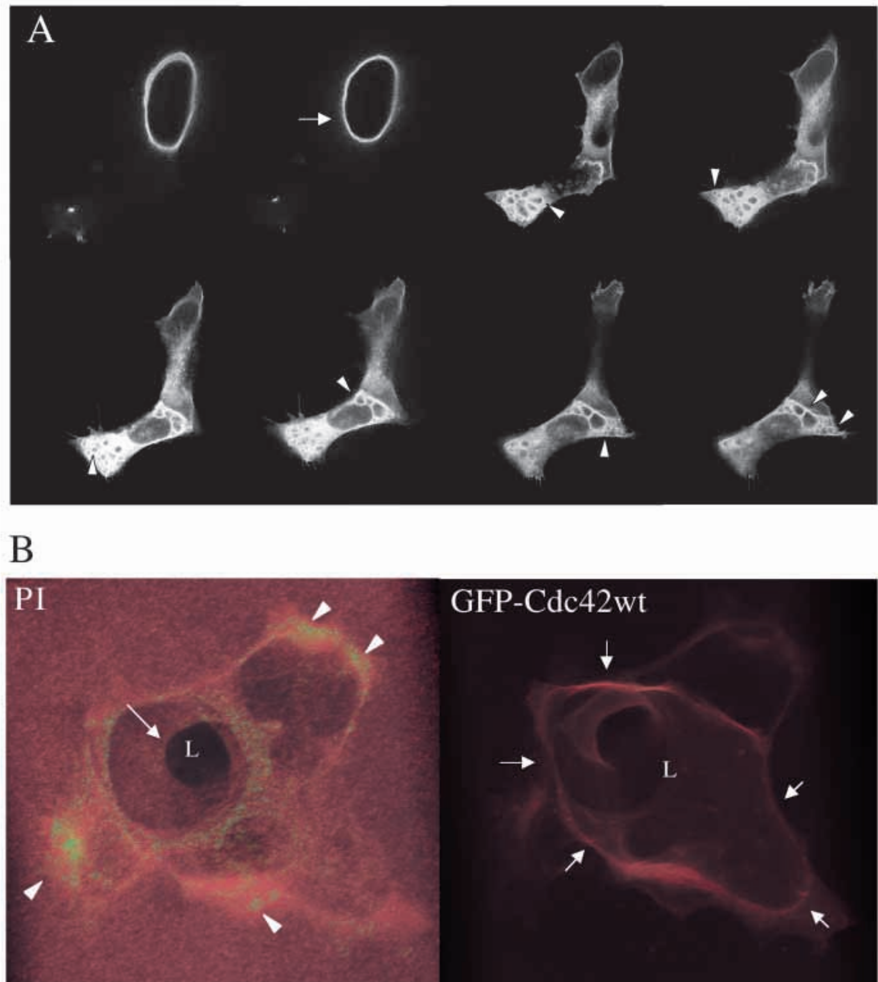


Fig. 11. Confocal analysis of GFP-Cdc42wt localization to EC vacuolar membranes and lumenal structures. (A) Targeting of GFP-Cdc42wt to vacuolar (arrowheads) and lumenal membranes (arrows). (B) Three-dimensional reconstruction of a multicellular structure containing a lumen (L). Left: propidium iodide (PI) staining of EC cultures indicating multiple cells interconnect to form a lumenal compartment. Nuclei are indicated with arrowheads. Right: GFP-Cdc42wt (shown in red) localizes to lumenal membranes (arrows).

Our observation that expression of GFP-Cdc42 wt led to an increase in vacuolar numbers suggests that Cdc42 may be rate limiting, which has been previously reported for Cdc42-mediated endocytosis in dendritic cells (Garrett et al., 2000). Further support for this idea is provided by the upregulation of Cdc42 protein during EC morphogenesis (Fig. 8). The GFP chimeric protein targeting data correlate with vacuole formation data, with ECs expressing DN and CA Rac1 and Cdc42 constructs. The ability of Rac1 and Cdc42 mutants to interfere with vacuole formation and block morphogenesis supports their direct involvement in the lumen formation process. Additionally, both Rac and Cdc42 chimeras target to vacuolar membranes (Figs 9-11). Intracellular vacuole structures enlarge and coalesce to eventually form a lumenal compartment. These preliminary lumens appear to open up or interconnect, allowing for EC junction formation and interconnection through branching events with neighboring ECs. Eventually, the lumenal structure is lined by multiple ECs to form tubes (Figs 1, 11). How these ECs are physically arranged during these events to form tubes in three dimensions remains unclear. Further studies are necessary to address this question, although the technologies utilized in this study (i.e. image analysis and fluorescent EC labeling) should allow detailed investigation of this question.

Here we present data that clearly indicate a role for Cdc42 in regulating the pinocytic and integrin-dependent process of vacuole and lumen formation (Davis and Camarillo, 1996; Bayless et al., 2000). A previous study reported that actin polymerization occurred at the leading edge of pinosomes in

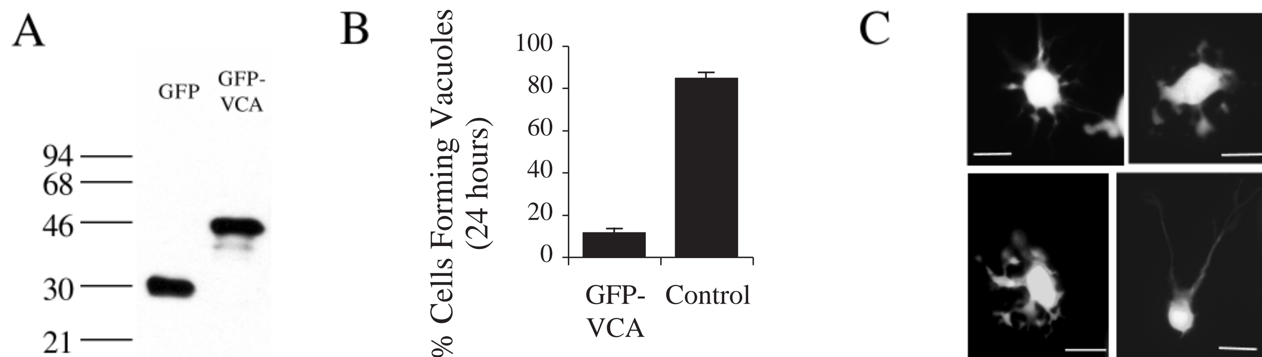


Fig. 12. Expression of a GFP fusion protein containing the VCA domain of N-WASP interferes with EC vacuole formation. (A) ECs were induced to express GFP control or GFP-VCA (a C-terminal domain of N-WASP that activates the Arp 2/3 complex). Extracts were made and samples were run on SDS-PAGE, blotted to PVDF membranes and probed with anti-GFP antibodies. (B) Quantitation of the effects of GFP-VCA chimera on EC vacuole formation at 24 hours of EC morphogenesis. (C) ECs expressing GFP-VCA were cultured in collagen matrices and photographs were taken after 24 hours of culture. Images show ECs in three-dimensional collagen expressing GFP-VCA that are unable to form lumenal structures but form numerous processes. Bar=25 μm.

mast cells (Merrifield et al., 1999), suggesting the same may be true for EC vacuoles. The Arp2/3 complex has also been linked to downstream Rho GTPase regulation of phagocytosis (May et al., 2000). In addition, actin polymerization through the Arp2/3 complex was mediated through the C-terminal VCA domain of N-WASP (Rohatgi et al., 1999; Higgs and Pollard, 2000; Prehoda et al., 2000), a downstream effector of Cdc42. Our results indicate that expression of the N-WASP VCA domain in ECs markedly blocked vacuole and lumen formation but not branching events. Interestingly, analysis of EC morphology revealed the presence of numerous EC processes, some of which resemble filopodia (Fig. 12). These data are consistent with results from the expression of CA Cdc42 in ECs, which strongly implicate the Cdc42-N-WASP pathway in EC morphogenesis during lumen formation events.

This pathway appears to act downstream of the $\alpha_2\beta_1$ or $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins, which regulate EC morphogenesis in collagen and fibrin three-dimensional extracellular matrices, respectively (Davis and Camarillo, 1996; Bayless et al., 2000). In addition, previous studies indicated a critical role for the extracellular matrix, integrin, cytoskeletal (MIC) signaling axis during EC morphogenesis in three dimensions (Salazar et al., 1999; Bell et al., 2001) and angiogenesis in vivo (Brooks et al., 1994; Bloch et al., 1997; Senger et al., 1997). Rho GTPases have emerged as crucial regulators of intracellular signaling pathways, and in response to extracellular stimuli, Rho proteins regulate both vesicular and membrane trafficking events (Ridley, 2001). The work reported here supports these concepts and begins to identify critical downstream regulators of the MIC signaling axis pathway in EC morphogenesis.

Overall, the data presented here investigate the molecular pathways required for EC vacuoles and lumen formation in three-dimensional collagen or fibrin matrices. It is clear from our studies that both Cdc42 and Rac1 play a critical role in these events. Future studies will aim to identify the signaling pathways and EC molecules relevant to this Cdc42- and Rac1-dependent process.

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