Available online at www.sciencedirect.com
ScienceDirect
Microvascular Research 75 (2008) 155–168
Microvascular Research
www.elsevier.com/locate/ymvre

Regular Article

Expression of VACM-1/cul5 mutant in endothelial cells induces MAPK phosphorylation and maspin degradation and converts cells to the angiogenic phenotype

A. Buchwalter [a,1], C. Van Dort [a,1], S. Schultz [a,1], R. Smith [a], I.P. Le [a], J.L. Abbott [a], E. Oosterhouse [a], A.E. Johnson [a], F. Hansen-Smith [b], M. Burnatowska-Hledin [a,*]

[a] Department of Biology and Chemistry, Science Center, Hope College, Holland, MI 49422-9000, USA
[b] Oakland University, Rochester, MI 48309, USA

Received 17 October 2006; revised 3 July 2007; accepted 2 August 2007
Available online 4 September 2007

Abstract

Vasopressin-activated calcium mobilizing receptor (VACM-1) is a member of the cullin gene family involved in ubiquitin–proteosome dependent regulation of cellular functions. Expression of VACM-1 cDNA in cos-1 cells *in vitro* decreases basal cAMP levels and inhibits growth. The expression of ^{S730A}VACM-1 mutant cDNA, which removes PKA-dependent phosphorylation site in the VACM-1 cDNA sequence, reverses this phenotype. Since the expression of VACM-1 protein *in vivo* localizes largely to the vascular endothelial cells, in this study, we examined the effects of ^{S730A}VACM-1 cDNA expression on cellular signaling in the rat endothelial cell line RAMEC. Our results indicate that expression of ^{S730A}VACM-1 cDNA in RAMEC promotes cellular proliferation and induces angiogenic growth patterns. Western blot analyses indicate that ^{S730A}VACM-1 cDNA transfected cells express increased levels of Nedd8 modified VACM-1 and have higher levels of phosphorylated MAPK protein when compared to controls. Furthermore, expression of ^{S730A}VACM-1 cDNA induces translocation of the endogenous early response gene, egr-1, to the nucleus and leads to morphological changes that involve actin rearrangement. Finally, expression of ^{S730A}VACM-1 cDNA in RAMEC decreases concentration of maspin, a putative anti-angiogenic factor with a tumor suppressor activity. These results show that VACM-1 protein regulates endothelial cell growth and may modulate angiogenesis by a mechanism that involves MAPK phosphorylation, nuclear localization of egr-1, maspin expression, and actin polymerization.
© 2007 Elsevier Inc. All rights reserved.

Keywords: egr-1; Nedd8; Cellular proliferation; Actin

Introduction

Angiogenesis, the formation of blood vessels, involves endothelial cell migration, growth, and differentiation (Beck and D'Amore, 1997; Folkman and Haudenschild, 1980; Papetti and Herman, 2002; Senger et al., 1993; Yancopoulos et al., 1998). It is now apparent that the ubiquitin–proteosome degradation pathway can regulate many growth factors and structural proteins which control angiogenesis (Glickman and Ciechanover, 2001; Herrmann et al., 2004). Consequently, alterations in ubiquitination processes or mutations in components of ubiquitination complexes may compromise angiogenesis under physiological and pathological conditions (Herrmann et al., 2004). Ubiquitin conjugation to a protein takes place in three steps that involve ubiquitin (Ub) activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). Unlike E1 and E2, which are homologous families, E3 is comprised of many structurally unrelated subunits, thus allowing for the regulatory specificity of the process (Glickman and Ciechanover, 2001). Overall, hundreds of the mammalian E3 ligases can account for the diverse biological effects associated with the ubiquitin-dependent protein degradation (Glickman and Ciechanover, 2001; Herrmann et al., 2004). Interestingly, several classes of E3 complexes incorporate cullins as essential components of their ubiquitin–proteosome system (Lyapina et al., 1998; Mathias et al., 1996;

* Corresponding author. Fax: +1 616 395 7125.
E-mail address: hledin@hope.edu (M. Burnatowska-Hledin).
[1] Contributed equally to the work presented in this manuscript.

0026-2862/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.mvr.2007.08.004

Mukhopadhyay et al., 1997; Ou et al., 2002; Pause et al., 1997; Querido et al., 2001; Yu et al., 1998, 2003).

VACM-1, now recognized as a cul5 gene (Kipreos et al., 1996; Byrd et al., 1997), was first cloned from the rabbit kidney medullary cDNA library as a *V*asopressin *A*ctivated *C*alcium *M*obilizing protein (Burnatowska-Hledin et al., 1995). In an effort to assign a biological role to this protein, we have examined its involvement in signaling pathways previously associated with the mitogenic effect of vasopressin (Rozenghurt et al., 1981; Rupprecht et al., 1994). *In vivo*, the expression of VACM-1 protein is highest in the heart and skeletal muscle, and in all tissues examined to date VACM-1 protein localizes exclusively to the endothelial structures (Burnatowska-Hledin et al., 1999). Only in the kidney, in addition to its endothelial expression, VACM-1 is also present in the collecting tubule cells (Burnatowska-Hledin et al., 1999). When overexpressed *in vitro*, VACM-1 inhibited cAMP production, prevented mitogen activated protein kinase (MAPK) phosphorylation, and attenuated cellular proliferation independent of vasopressin activity (Burnatowska-Hledin et al., 2000, 2004; Van Dort et al., 2003). Interestingly, we have found that the expression of VACM-1 mutant, where the putative PKA-dependent phosphorylation site (^{727}KKI**S**^{730}) at serine 730 was mutated to alanine (^{S730A}VACM-1), resulted in a phenotype reversal when compared to cos 1 cells transfected with VACM-1 cDNA (Van Dort et al., 2003). ^{S730A}VACM-1 cDNA transfected cos 1 cells grew rapidly, appeared to lose contact inhibition, and had increased phosphorylated MAPK levels when compared to the CMV or to VACM-1 cDNA transfected cells (Van Dort et al., 2003). We reasoned, therefore, that the expression of mutated `VACM-1 in cells that express endogenous VACM-1 protein may create a dominant-negative phenotype. This strategy in which the function of a gene is altered so that it encodes a mutant product capable of inhibiting the wild type gene product in a cell has been useful in determining the mechanism of signaling specificity for many proteins (Herskowitz, 1987; Lagna and Hemmati-Brivanlou, 1998; Qian et al., 2003; Wu et al., 2004). The expression of a dominant-negative Rho GTPase, cdc42, for example, is sufficient to inhibit angiogenesis, whereas overexpression of a dominant-positive cdc42 can induce angiogenesis (Qian et al., 2003). We have hypothesized, therefore, that transfecting endothelial cells expressing endogenous VACM-1 protein with ^{S730A}VACM-1 cDNA may create a dominant-negative phenotype. We used a rat adrenal medullary endothelial cell line RAMEC (Papadimitriou et al., 1993), which expresses endogenous VACM-1 (Burnatowska-Hledin et al., 2001), to determine whether a dominant-negative phenotype can be established using the ^{S730A}VACM-1 cDNA mutant.

We report in this paper that stable overexpression of ^{S730A}VACM-1 cDNA in RAMEC resulted in a phenotype change. RAMEC stably transfected with the ^{S730A}VACM-1 cDNA had significantly higher growth rates compared to cells transfected with an empty vector or to cells transfected with VACM-1 cDNA. These effects appeared to involve increased MAPK phosphorylation, translocation of egr-1 protein to the nucleus, and a decrease in maspin concentration, all proteins recognized for their involvement in the regulation of angiogenesis (Beck and D'Amore, 1997; Davis, 1993; Khachigian and Collins, 1997; Zou et al., 1994). Importantly, ^{S730A}VACM-1 cDNA transfected cells exhibited an angiogenic growth pattern when compared to the vector transfected cells. The morphological changes were even more apparent when cells were grown on a Matrigel® support. These studies indicate that VACM-1/cul5 regulates cellular growth in endothelial cells and may control angiogenesis.

Materials and methods

Materials

All tissue culture media and reagents were purchased from Invitrogen (Grand Island, NY). The restriction enzymes were purchased from Promega Co. (Madison, WI). Collagen was purchased from Invitrogen and Matrigel® was purchased from BD Labs (Franklin Lakes, NJ). VACM-1 cDNA was subcloned into the pBK-CMV vector as described previously (Burnatowska-Hledin et al., 2000). Briefly, VACM-1 cDNA was cut with unique *Sal*I/*Xba*I restriction enzymes from the pSV-SPORT-1 vector and ligated into the *Sal*I/*Xba*I site in the polylinker region of the pBK-CMV vector (Stratagene Co., La Jolla, CA) (Burnatowska-Hledin et al., 2000; Van Dort et al., 2003).

Tissue culture

Cos-1 and RAMEC cells were grown in DMEM and DMEM-low glucose media, respectively, supplemented with 2% FBS and 8% HS (horse serum) at 37 °C under a water-saturated 5% CO_2 atmosphere as described previously (Burnatowska-Hledin et al., 2000, 2001). T47D cells (Keydar et al., 1979) were grown in RPMI medium supplemented with 10% FBS as described previously (Burnatowska-Hledin et al., 2004). All cell lines were plated at a density of 6–8×10^5 cells per 100 mm plate and cultured for 24 h before transfection. Cells were transfected with 5 μg VACM-1 cDNA (or ^{S730A}VACM-1 cDNA) per 100 mm culture dish using the FuGENE 6 (Roche Diagnostics Inc., Indianapolis, IN) (6) or with the Mirus reagent (Mirus, Madison, WI) as described by the manufacturers. After 24 h cells were split and incubated with fresh medium containing 600 μg/ml G418 (Gibco BRL Co., Gaithersburg, MD). The medium was changed every 3 days. Two to three weeks after transfection, G418 resistant cells were harvested and transferred into 6 well plates containing selective medium (250 μg/ml G418). Ultimately, 7–12 clones were selected for each cell line and their growth was monitored.

Western blot analysis

Total cell lysates and membrane fractions were prepared as described previously (Van Dort et al., 2003). Briefly, cells were grown to at least 70% confluency, washed in ice-cold PBS, and resuspended in 500 μl of buffer (50 mM Tris [pH 7.4], 0.1% Triton X-100, 150 mM NaCl, 1 M EDTA, 50 mM NaF) with 1 μg/ml apoprotein, 100 μM Pefabloc®SC, and 10 mM PMSF. All samples were homogenized with a Polytron homogenizer and protein concentration was determined using the Bradford method (BioRad Co., Richmond, CA). Cell membrane fractions prepared from total cell lysates were centrifuged at 45,000×*g* for 1 h at 4 °C. The membrane pellets were resuspended in the buffer and their protein concentrations were determined as described above. Samples were resuspended in 2× sample buffer (BioRad Co.), heated to 95 °C for 4 min and subjected to SDS polyacrylamide gel electrophoresis (PAGE) using a 10% running gel. The separated proteins were transferred to a nitrocellulose membrane (Osmotics Co. Trevose, PA) at 30 mV for 2 h. Nonspecific sites were blocked by membrane incubation for 30 min at room temperature with PBS containing 5% nonfat dry milk and 0.2% Tween-20. Membranes were incubated for 2 h at room temperature in buffer solution containing a 1:200 dilution of affinity purified polyclonal antibodies directed against the N-terminus (Ab-A) of VACM-1 protein (Burnatowska-Hledin et al., 1995, 2001). In some experiments, blots were stripped and reprobed with anti-Nedd8 specific Ab (1:500 dilution) developed in rabbit (Alexis Co.). (To probe for the presence of

maspin, membranes were incubated for 2 h at room temperature in a 1:2000 dilution of polyclonal anti-human maspin or a 1:1000 dilution of monoclonal anti-human maspin (BD/Pharmingen, Franklin Lakes, NJ.) To ascertain equal protein loading, blots were stripped and the membranes were incubated in a 1:10,000 dilution of monoclonal anti-rabbit GAPDH (Abcam Inc., Cambridge, MA) or 1:500 dilution of polyclonal anti-α-actin (Sigma Co. St. Louis, MO) for 1.5 h at room temperature. The membranes were next washed in the same buffer for 15 min and twice for 5 min and incubated for 2 h with a horseradish peroxidase (HRP) conjugated secondary antibodies (diluted at 1:2000 to 1:10,000) (Cell Signaling, Beverly, MA). The nitrocellulose membranes were washed as described above, exposed to the luminol detection reagents (Cell Signaling, Beverly, MA) for 1 min, and then exposed to the X-ray film (Amersham, Arlington Heights, IL).

Immunostaining

Affinity purified polyclonal antibodies directed against the N-terminus (Ab-A) of VACM-1 protein, characterized previously (Burnatowska-Hledin et al., 1995, 1999), were used to stain cells by indirect immunofluorescence. Cells grown on coverslips were fixed in 3% paraformaldehyde (in 1× PBS, pH 7.4) for 20 min, washed in PBS and incubated with a 1:20 dilution of Ab-A, or Ab-A preabsorbed with 10 μM peptide A, identical in sequence to the amino terminus sequence of VACM-1. Antibodies were diluted in PBS containing 0.1% BSA (PBS/BSA). Cells stained with anti-maspin or anti-Ned8 (Alexis Co.) antibodies were first permeabilized with 5% Tween-20 solution for 20 min, washed with PBS/2% BSA, and exposed to the antibody for 1.5 h. The primary antibodies were detected by incubating the sections in the presence of 1:40 dilution of FITC-conjugated goat anti-rabbit IgG (Vector Laboratories Inc, Burlingame, CA) in 1× PBS/2% BSA for 1 h. The sections were washed in 1× PBS/2% BSA, mounted with Vectashield® mounting medium, and viewed by epifluorescence microscopy (Eclipse E600, Nikon) equipped with Spot camera (Diagnostic Instruments, Sterling Heights, MI). The nuclear staining was achieved by DAPI found in the Vectashield® mounting medium (Vector Laboratories Inc, Burlingame, CA). The relative expression of specific proteins was calculated using the NIH Image program (rsb.info.nih.gov/ij/index.html).

Site-directed mutagenesis

Site-directed mutagenesis was performed using QuikChange™ Site-directed Mutagenesis Kit from Stratagene (La Jolla, CA). The mutagenesis primers were synthesized by Sigma-Genosys Inc. (Woodland, TX). To confirm the mutation site sequences, sequencing was performed by Macromolecular Resources (Colorado State Univ., Ft. Collins, CO) as described previously (Burnatowska-Hledin et al., 2000).

Cellular proliferation analysis

Clones expressing mutated VACM-1 cDNA and control cells were seeded in 100 mm plates at equal densities (2×10^4 cells/ml) in 10% FBS. After 24 h, medium was changed and both control and experimental cells were allowed to grow for either 4 or 5 days as indicated in the Results section. Cells were harvested by trypsinization and counted in a hemacytometer. In addition, some cells were grown on coverslips, stained with DAPI as described above, and nuclei visualized under epifluorescence microscopy were counted. Cells transfected with VACM-1 cDNA were grown for 8 days.

In vivo angiogenesis assay

Physiological angiogenesis was experimentally induced in rat extensor digitorum longus (EDL) muscles using the "increased contractile activity" (indirect electrical stimulation) and "stretch/overload" models as described previously (Hansen-Smith et al., 1992). Briefly, increased contractile activity of the EDL without concurrent systematic exercise effects was elicited by implantation of stainless steel multi-stranded teflon-insulated electrodes unilaterally in the vicinity of the peroneal nerve. Stimulation at 10 Hz, pulse width 0.3 ms, was maintained for 8 h/day, with no discomfort. In the "stretch/overload" model, the tibialis anterior muscle was unilaterally removed in rats, resulting in angiogenesis in the now stretched and overloaded synergist EDL muscle. Surgeries were performed on male Sprague–Dawley rats averaging 250–300 g under aseptic conditions, using fluothane or sodium pentobarbital anesthesia (30–40 mg/kg ip). Butnorphine (5 mg/kg i.m.) was administered postoperatively and animals were provided with regular chow and water *ad libitum*. EDL muscles from both intact and sham-operated animals of similar body weight were used as controls. The EDL muscles were removed at 4–14 days after treatment started and were snap-frozen in isopentane/dry ice slurry using an inert cryoprotectant (OCT medium, Miles Inc. Elkhart, IN). Cryostat sections from EDL blocks were cut at 6 μm thickness and fixed with 4% formalin before immunostaining. Sections were double-labeled with antibodies to VACM-1 or, in controls, pre-immune serum. After overnight exposure to the primary antibodies sections were then rinsed and exposed for 2 h to the FITC-labeled anti-rabbit antibody at 1:50 dilutions containing 25 μg/ml of the tetramethyl rhodamine derivative of the *Griffonia simplicifolia* I lectin (GSI; Vector laboratories, Burlingame, CA) to label capillaries and microvessels. The sections were examined by standard epifluorescence microscopy (Nikon Optiphot) and by laser scanning confocal microscopy (BioRad 600, Hemel Hampstead, UK). Capillaries were defined as GSI-positive vessels as previously reported (Hansen-Smith et al., 1992). Capillary density was evaluated using the mean number of capillaries around muscle fiber. Capillary density in EDL exposed to increased contractile activity was significantly higher than controls after 4–7 days and between 7 and 14 days in stretched muscles, as previously reported (Hansen-Smith et al., 1992).

In vitro tumorigenicity assays

Matrigel® invasion assay was performed in 24 well plates coated with the gel as described in the manufacturer's instructions. Approximately 2×10^4 cells were plated per well and cultured for 5 to 6 days. Cells were monitored daily and RAMEC tube formation was assessed by image analysis in 4–6 representative fields of view in four independent experiments as described in the literature (Dusbery et al., 2001).

Immunoprecipitation

Cell lysates (300 μg protein) from asynchronous cells were resuspended in 150 μl of solubilization buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 0.3% Triton X-100, 1 mM Pefabloc^{R}SC, and 10 μg/ml aprotinin) and incubated with a 1:250 dilution of affinity purified Ab-A (directed against the amino terminus sequence of VACM-1 protein), anti-Nedd8 Ab, or a nonspecific antibody. After 2 h of incubation at 4 °C, an equal volume aliquot of protein A/sepharose (Amersham Pharmacia Biotech) suspension was added and the incubation continued for another 2 h. The complex was centrifuged at 12,000 RPM for 2 min and washed two to three times in the solubilization buffer. Loading dye was added and the immunoprecipitates were separated using large, 10% SDS–PAGE gels, transferred to nitrocellulose and probed with an antibody directed against VACM-1 protein, as described above (Burnatowska-Hledin et al., 2001; Van Dort et al., 2003).

Microarray assay

Human PathwayFinder-1 GEArray™ Kit was obtained from SuperArray Inc. (Bethesda, MD), probed with biotin-16-dUTP-labeled VACM-1 cDNA fragment, and exposed to the AP-streptavidin chemiluminescent detection system, exactly as described in the manufacturer's instructions.

Statistical analysis

Data are expressed as mean ± one standard error (SE) of the mean. Student's *t*-test was used for data analysis. Significance was set at $p<0.05$.

Results

To test the hypothesis that VACM-1 is involved in the regulation of endothelial cell growth *in vivo*, we have examined

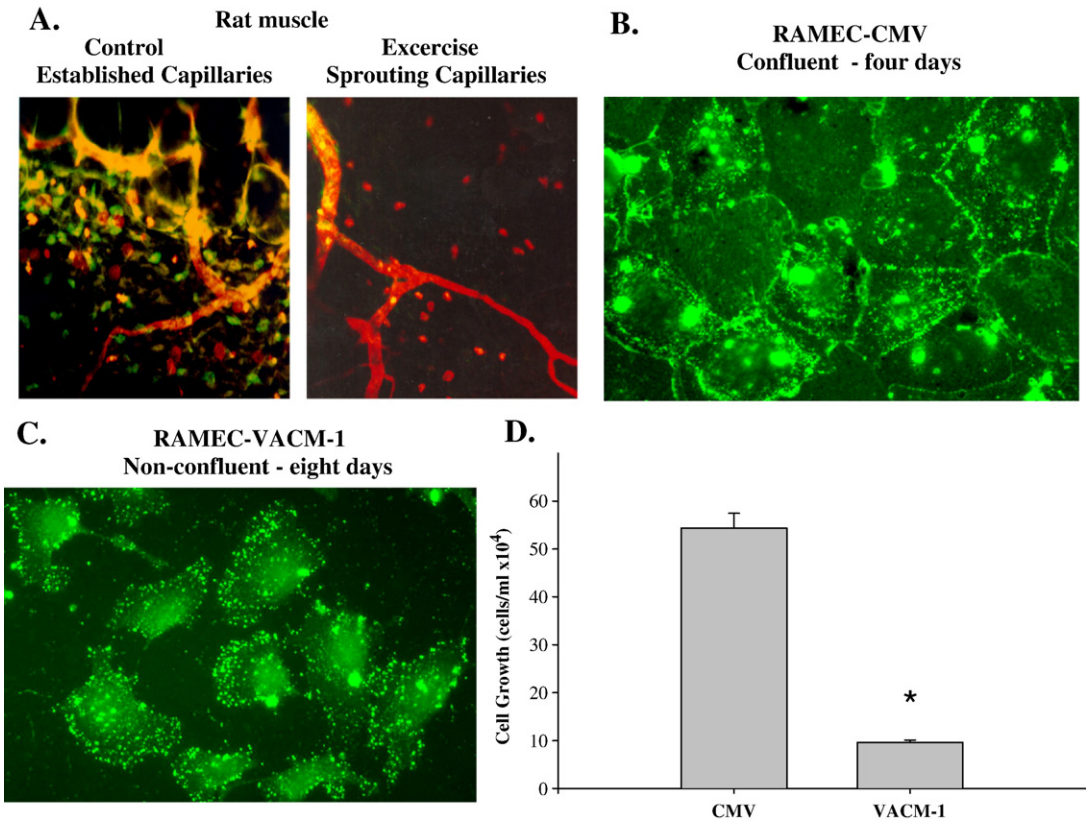


Fig. 1. VACM-1 protein expression *in vivo* and *in vitro*. (A) Lack of vascular VACM-1 expression in stress-induced angiogenesis in rat skeletal muscle *in vivo*. Control, nonproliferating vessels (left panel) and sprouting capillaries (right panel) were identified by GSI lectin stain (red) and immunostained with anti-VACM-1 antibody (green) and examined under confocal microscopy after 7 days of induced angiogenesis. The yellow color represents co-localization of VACM-1 and GSI stain. (B) VACM-1 protein expression in confluent cultures of RAMEC grown for 4 days and immunostained with anti-VACM-1 specific Ab (magnification, 40×). (C) VACM-1 protein expression in VACM-1 cDNA transfected RAMEC grown for 8 days and immunostained with anti-VACM-1 specific Ab (magnification, 40×). (D) Overexpression of VACM-1 cDNA in RAMEC inhibits growth. CMV and VACM-1 transfected cells were seeded at equal density (5×10^4 cells/plate) and their growth monitored as described in Materials and methods for 4 days ($n=4$, *: $p<0.01$).

its expression in a model of induced angiogenesis in adult rat skeletal muscle (Hansen-Smith et al., 1992). In this model, EDL muscle stimulation leads to structural changes in 3–4 days and a significant increase in capillary density by 7 days compared to the collateral control. VACM-1 protein was identified with the green (FITC) immunofluorescent marker and capillaries were identified with GSI lectin stain (red). After 7 days of induced angiogenesis, VACM-1 protein was present in the established capillaries (left panel) where the green FITC signal co-localized with the red GSI lectin stain (yellow) but not in the sprouting capillaries (right panel) where little VACM-1 could be detected.

To examine the significance of the endothelium-specific VACM-1 expression *in vivo*, we next turned to an *in vitro* rat endothelial cell (RAMEC) model (Papadimitriou et al., 1993) we have used previously to study the endogenous expression of VACM-1 protein (Burnatowska-Hledin et al., 2001). To confirm the antiproliferative effect of VACM-1 observed in cos 1 cells (Van Dort et al., 2003), we transfected RAMEC with VACM-1 cDNA. Our results, summarized in Figs. 1C–D, show that overexpression of VACM-1 cDNA in RAMEC significantly inhibited cell growth when compared to the CMV vector transfected cells. Unlike control cells grown to confluency at four days (Fig. 1B), VACM-1 transfected cells were not confluent at 8 days after plating (Fig. 1C).

Since RAMEC express endogenous VACM-1, we proposed to create a dominant-negative phenotype by expressing ^{S730A}VACM-1 mutant cDNA. The expression of ^{S730A}VACM-1 mutant cDNA in RAMEC led to a significant increase in cellular growth and a clear phenotype change in 8 cell lines originally isolated when compared to the CMV vector transfected RAMEC (data not shown). Thus, one clone was chosen at random for the studies described here. VACM-1 protein expression in CMV vector and in ^{S730A}VACM-1 cDNA transfected RAMEC was confirmed by Western blot analysis with an antibody directed against the amino terminus of VACM-1 protein (Fig. 2A). Our results revealed that, in cells stably transfected with the ^{S730A}VACM-1 cDNA, VACM-1 protein signal was increased (Fig. 2A–C, VACM-1: GAPDH; 1.54±0.18 vs. 1.0±0.15, $n=6$, $p<0.055$). The immunocytochemistry data indicated that unlike the membrane and/or cytosolic localization of VACM-1 in RAMEC cells (Figs. 1B and 2D, left panels), VACM-1 protein localization in ^{S730A}VACM-1 cDNA transfected cells appeared to be largely nuclear (Fig. 2D, right panel). The immunofluorescence signal was significantly reduced in ^{S730A}VACM-1 cDNA transfected cells incubated

with the peptide preabsorbed Ab (Fig. 2D, bottom panel). Western blot analysis further confirmed lower expression of VACM-1 protein in cell membranes isolated from the ^{S730A}VACM-1 cDNA transfected cells when compared to the CMV vector transfected cells (Fig. 2E). Interestingly, a larger M_r species identified on a Western blot of cell lysates from ^{S730A}VACM-1 cDNA transfected cells when compared to the control cells (Fig. 2A) was not observed in the cell membrane fractions (Fig. 2E).

Previous immunoprecipitation studies in cos 1 cells transfected with ^{S730A}VACM-1 cDNA identified the upper band as Nedd8 modified VACM-1 (Van Dort et al., 2003). Consequently, blots shown in Fig. 2A were stripped and reprobed with anti-Nedd8 specific antibody (Figs. 3A–B). The upper band recognized by anti-VACM-1 Ab in ^{S730A}VACM-1 cDNA transfected cells (Fig. 2A) was also recognized by the anti-Nedd8 Ab (Fig. 3A). The signal intensities of the Nedd8 probed blots were significantly higher when compared to the control group (Fig. 3B, $n=4$, $p<0.05$). The identity of the Nedd8-modified protein at ~60 kDa is not known. The specificity of Nedd8 signal in ^{S730A}VACM-1 cDNA transfected cells was further confirmed using the immunostaining and immunoprecipitation approaches. As shown in a representative immunostaining experiment in Fig. 3C ($n=3$), Nedd8 signal was higher in ^{S730A}VACM-1 cDNA transfected RAMEC when compared to the CMV or VACM-1 cDNA transfected RAMEC. To ascertain that the increase in the nuclear intensity of Nedd8 signal represented increased VACM-1 protein neddylation, we next immunoprecipitated VACM-1 protein from the CMV vector and ^{S730A}VACM-1 cDNA transfected RAMEC with the anti-VACM-1 protein specific antibody and probed the blots with anti-Nedd8 specific antibody. Our results indicate that, in lysates from ^{S730A}VACM-1 cDNA but not the CMV transfected RAMEC, the protein immunoprecipitated with anti-VACM-1 Ab can be detected with anti-Nedd8 antibody (Fig. 3D, $n=3$). A signal observed in the control lanes without primary antibody but not in the control samples incubated with anti-VACM-1 Ab-A may reflect a nonspecific binding of Nedd8 protein to sepharose A.

The expression of ^{S730A}VACM-1 cDNA affected cellular structure and induced changes in RAMEC growth pattern (Fig. 4A–E). Cells looked smaller and elongated. When con-

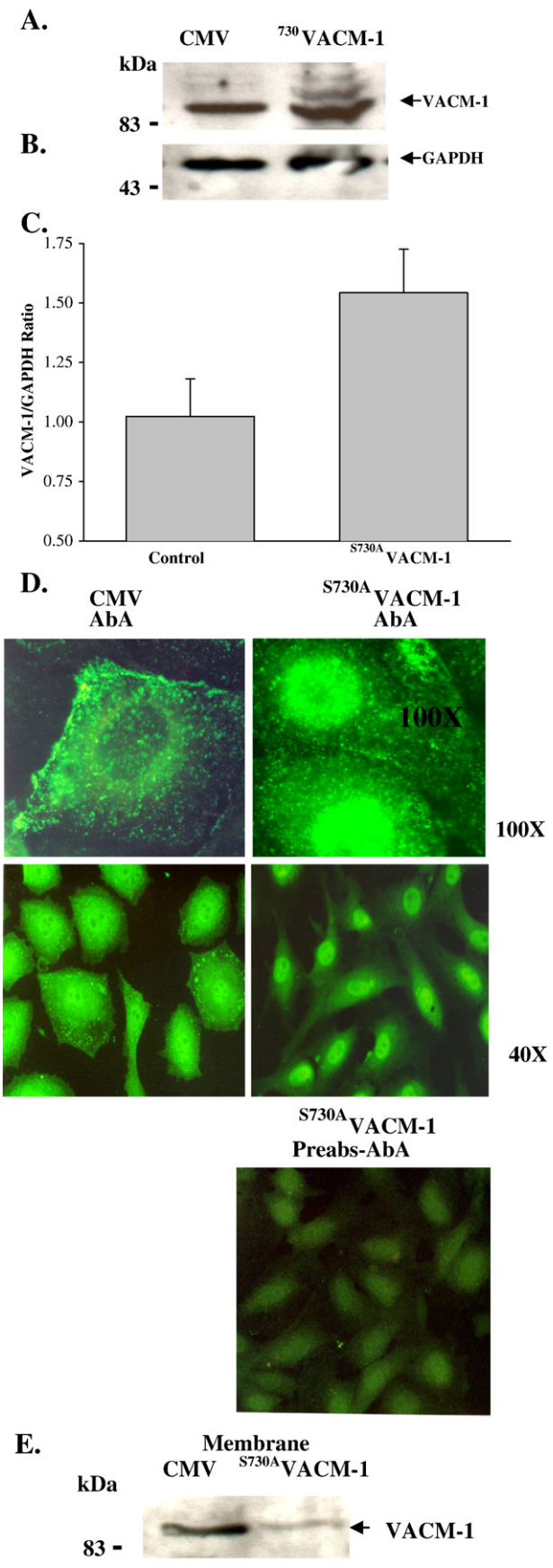


Fig. 2. Effects of ^{S730A}VACM-1 expression in endothelial cells (RAMEC). (A) A representative Western blot analysis of RAMEC and ^{S730A}VACM-1 cDNA transfected RAMEC probed with an Ab generated against the amino terminus of VACM-1 (upper panel). (B) To correct for unequal protein loading, blots were reprobed with anti-GAPDH antibody. (C) The intensities of VACM-1 and GAPDH specific signals shown in A and B were quantified using NIH imaging and results were expressed as a ratio of the VACM-1:GAPDH signal intensity. (D) Immunocytochemistry of CMV and ^{S730A}VACM-1 cDNA transfected RAMEC. Cells were grown on coverslips, fixed, permeabilized, and immunostained with anti-VACM-1 antibody directed against its amino terminus sequence (Ab-A) as described in Materials and methods. Virtually no VACM-1 signal was detected in ^{S730A}VACM-1 cells immunostained with the antibody preincubated with 10 μg of the peptide (magnification, 40×). (E) Western blot analysis of cell membrane fractions from CMV and ^{S730A}VACM-1 cDNA transfected RAMEC. Samples were resolved by SDS–PAGE and probed with anti-VACM-1 specific Ab.

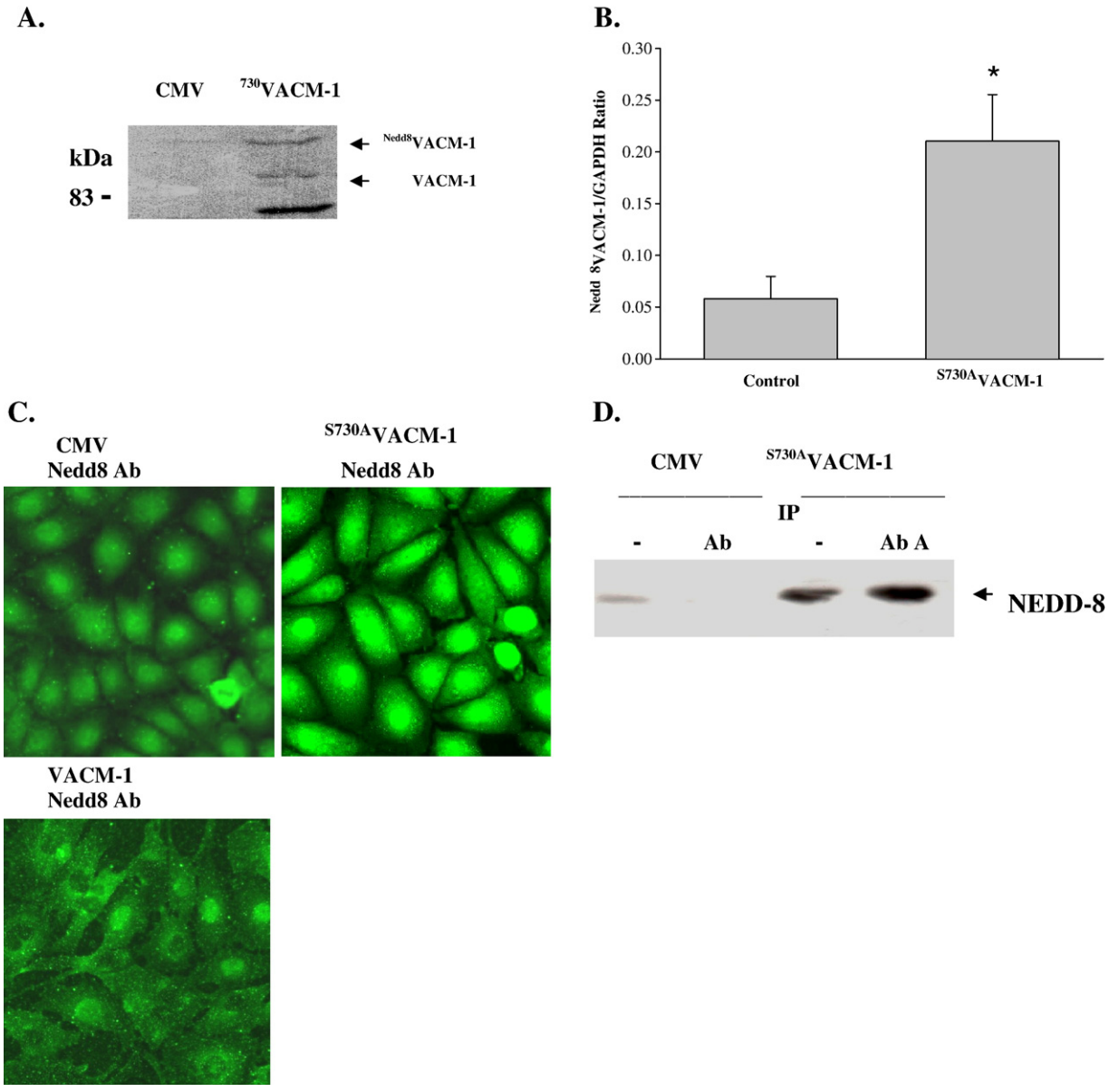
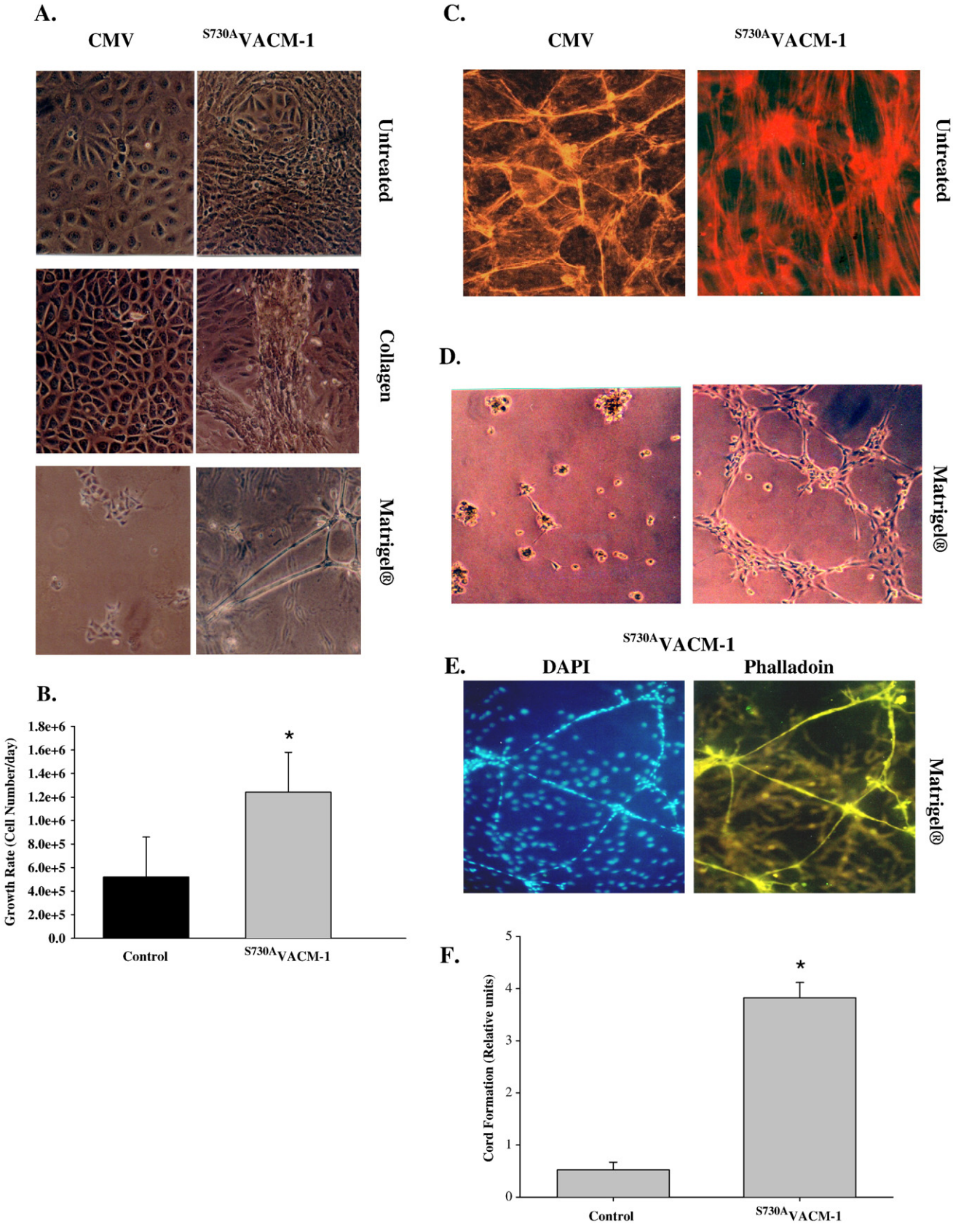


Fig. 3. Effects of ^{S730A}VACM-1 expression RAMEC on VACM-1 protein neddylation. (A) To determine the identity of the upper band seen in blot 2A, nitrocellulose membranes were stripped and reprobed with anti-Nedd8 specific antibody as described in Materials and methods. (B) The intensities of Nedd8 signal shown in A were quantified using NIH Imaging and results were expressed as a ratio of the ^{Nedd8}VACM-1:GAPDH signal intensity (*, $p<0.05$). (C) CMV, VACM-1, and ^{S730A}VACM-1 cDNA transfected RAMEC were immunostained with anti-Nedd8 specific Ab as described in Materials and methods. (D) Immunoprecipitates (IP) from CMV and ^{S730A}VACM-1 cDNA transfected RAMEC isolated with anti-VACM-1 specific Ab (Ab-A) were resolved by SDS–PAGE and probed with anti-Nedd8 A ($n=3$). No Ab was added to the control samples (−).

fluent, ^{S730A}VACM-1 cDNA transfected cells formed a "swirling" pattern when compared to the "cobblestone" appearance of the CMV transfected cultures (Fig. 4A, top panel). These morphological changes were even more apparent when cells were grown either on a collagen or on Matrigel® support (Fig. 4A, middle and bottom panels, respectively). Cells grown

Fig. 4. Stable expression of ^{S730A}VACM-1 in endothelial cells (RAMEC) affects cell morphology and induces growth. (A) CMV transfected control and ^{S730A}VACM-1 cDNA transfected RAMEC were seeded at equal density on polystyrene, collagen (magnification, 20×), and on Matrigel® (magnification, 4×) support and their growth was monitored. (B) Growth rates of a representative CMV and ^{S730A}VACM-1 transfected RAMEC clones. Cells were seeded at equal density (5×10^4 cells/plate) and their growth monitored over time as described in Materials and methods ($n=5$, *: $p<0.05$). (C–E) Stable expression of ^{S730A}VACM-1 in endothelial cells (RAMEC) compromised actin polymerization. CMV and ^{S730A}VACM-1 cDNA transfected RAMEC were seeded at equal density on untreated plates (C, magnification 40×) or on Matrigel® (D, magnification 20×) support, and their growth was monitored. (E) DAPI and phalloidin staining of ^{S730A}VACM-1 cDNA transfected RAMEC grown on Matrigel® (magnification, 4×). All cells were grown on glass coverslips and stained with rhodamine labeled phalloidin (10^{-8} M). Phalloidin signal was very intense and was clearly visible with the green filter (magnification, 4×). (F) Endothelial tube formations were quantitated by image analysis in 4 independent experiments ($n=4$, *: $p<0.0001$).

on collagen formed elongated patterns while cells grown on Matrigel® formed structures/cords previously described as "angiogenic" (Folkman and Haudenschild, 1980) and observed in NIH 3T3 cells transformed with *ras* oncogene or with a dominant-negative mutant of cdc42 protein (Dusbery et al., 2001; Qian et al., 2003). These morphological changes in



RAMEC transfected with ^{S730A}VACM-1 mutant (Fig. 4A) were associated with a significant increase in the growth rate when compared to CMV-transfected cells ($n=5$, $p<0.05$, Fig. 4B). To further confirm these observations, we stained the control and ^{S730A}VACM-1 cDNA transfected cells with an actin-specific stain, phalloidin (10^{-8} M). Our data indicate that, in RAMEC transfected with ^{S730A}VACM-1 cDNA, actin organization appeared to be random; cells lost the stress fibers and became elongated (Fig. 4C). The angiogenic phenotype (Dusbery et al., 2001; Folkman and Haudenschild, 1980) of RAMEC transfected with ^{S730A}VACM-1 was even more apparent when cells were grown on Matrigel® and stained with DAPI and phalloidin (Fig. 4D–E) ($n=5$). When quantitated, the relative tube formation was significantly higher in ^{S730A}VACM-1 cDNA transfected cells when compared to controls (Fig. 4F).

To study a possible mechanism of ^{S730A}VACM-1 mutant induced growth in RAMEC, we next examined its effects on MAPK phosphorylation. Our data indicate that, in RAMEC transfected with ^{S730A}VACM-1 cDNA, MAPK (Erk1/Erk2) phosphorylation was significantly higher than in the CMV vector transfected cells (Figs. 5A–B). Furthermore, the levels of MAPK phosphorylation in ^{S730A}VACM-1 cDNA transfected cells were directly correlated to the ratio of modified VACM-1/VACM-1 signal intensity ($r^2=0.98$) (Fig. 5C). Since the regulation of multiple genes implicated in angiogenesis and in the pathogenesis of vascular disease that involve MAPK activation leads to increased expression of egr-1 (Beck and D'Amore, 1997; Davis, 1993; Khachigian and Collins, 1997), we next examined whether ^{S730A}VACM-1 expression in RAMEC compromises egr-1 signaling. The immunocytochemistry results indicate that largely cytosolic localization of egr-1 in control RAMEC becomes nuclear in ^{S730A}VACM-1 but not VACM-1 cDNA transfected RAMEC (Figs. 6A–B). These observations were further confirmed in cos 1 cells transfected with either VACM-1 or ^{S730A}VACM-1 cDNAs. In control cos 1 cells lacking VACM-1, egr-1 localization appeared to be nuclear, whereas it was virtually absent in VACM-1 transfected cells and became nuclear in ^{S730A}VACM-1 mutant transfected cells (Fig. 6C).

To determine if VACM-1 mutant overexpression will compromise any antiangiogenic gene, we chose to examine its effects on maspin expression at the protein level. Our Western blot results, summarized in Fig. 7, indicate that in RAMEC transfected with ^{S730A}VACM-1 cDNA the endogenous maspin levels were significantly decreased or abolished (Figs. 7A and B). These observations were confirmed at the cellular level using the immunostaining approach. As shown in a representative experiment in Fig. 7C and summarized in Fig. 7D, the intense immunostaining with anti-maspin Ab observed in the control cells was largely absent in ^{S730A}VACM-1 cDNA transfected cells ($n=4$). To ascertain that the ^{S730A}VACM-1 mutant has a dominant-negative effect on maspin, we examined the effect of wild type VACM-1 overexpression on maspin concentration in T47D cancer derived cell line. As shown in a representative Western blot in Fig. 7E and summarized in Fig. 7F, little expression of endogenous maspin can be detected in those cells, while stable expression of VACM-1 cDNA led to a significant increase in maspin protein levels ($n=5$, $p<0.05$).

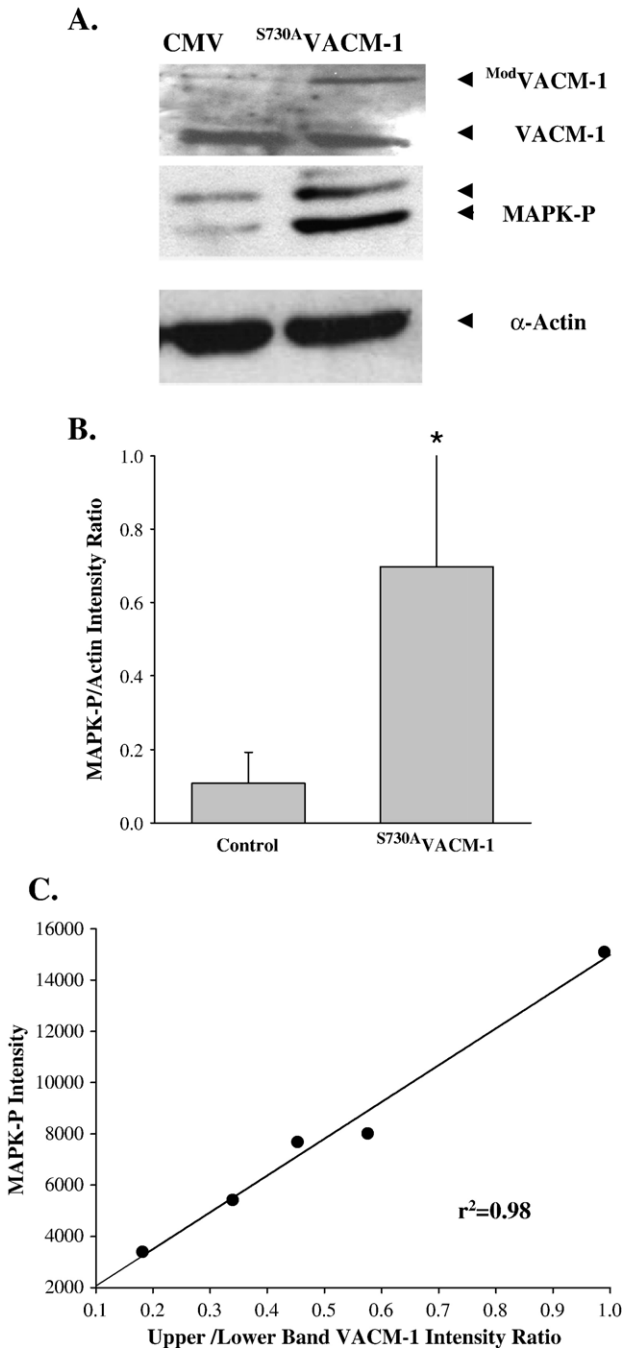


Fig. 5. Expression of ^{S730A}VACM-1 cDNA mutant induces MAPK phosphorylation. (A) A representative Western blot analysis of VACM-1, MAPK phosphorylation and α-actin levels in control and ^{S730A}VACM-1 transfected RAMEC. (B) Data from experiments shown in A were quantitated using Scion Imaging Program and corrected for α-actin levels ($n=5$, *: $p<0.05$). (C) The phosphorylated MAPK levels were correlated with the levels of ^{Mod}VACM-1/VACM-1 ($r^2=0.98$).

Furthermore, maspin levels were directly correlated to VACM-1 protein concentration (Fig. 7G, $r^2=0.80$, $p<0.001$).

Finally, to identify steps either preceding or subsequent to MAPK/egr-1 activation, and maspin degradation, *Angiogenesis GEArray® Q* series blots (Superarray Inc), containing 96 representative genes known to be involved in modulating various biological processes of angiogenesis, were probed with cRNA-

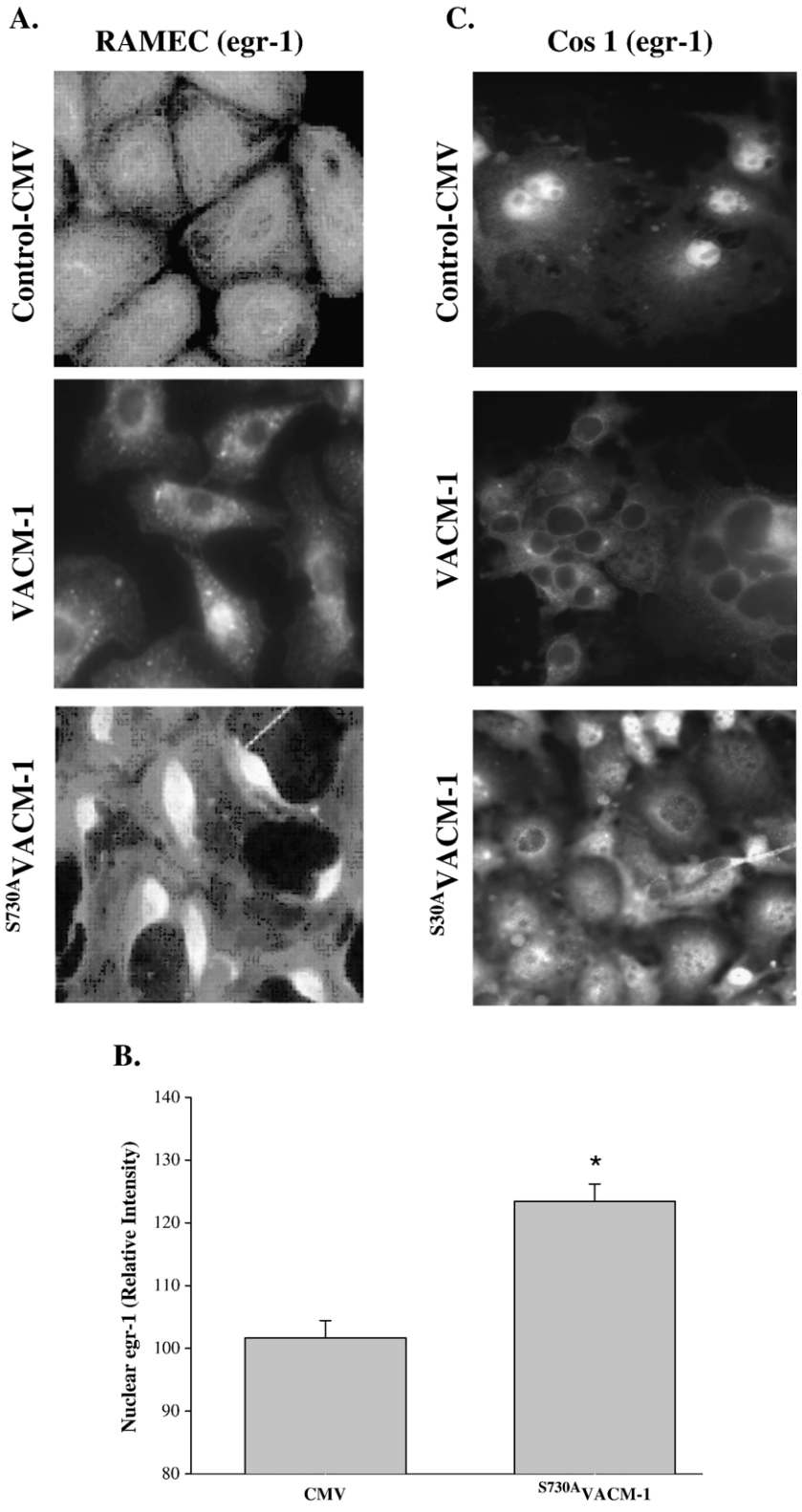
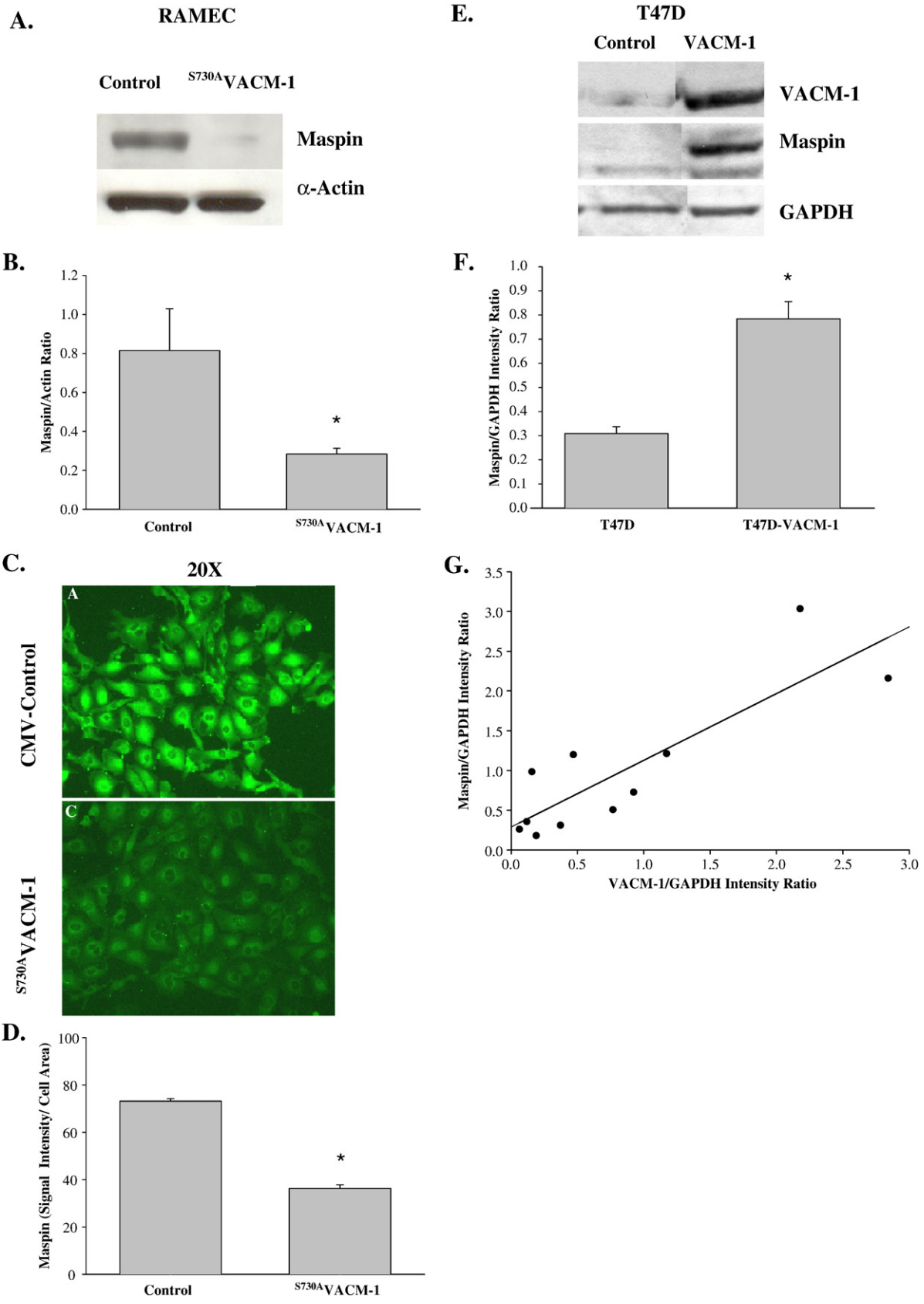


Fig. 6. Expression of ^{S730A}VACM-1 mutant induces nuclear localization of egr-1 protein. (A) Immunocytochemistry of egr-1 expression in CMV (upper panel), VACM-1 cDNA (middle panel), and in ^{S730A}VACM-1 transfected RAMEC (lower panel). (B) The intensities of egr-1 specific signal for CMV (upper panel) and in ^{S730A}VACM-1 transfected RAMEC (lower panel) shown in A were quantified using NIH Imaging ($n=3$, *: $p<0.005$). (C) Immunocytochemistry of egr-1 in CMV, VACM-1, and ^{S730A}VACM-1 cDNA transfected cos 1 cells. All cells were grown on coverslips, fixed, permeabilized and immunostained with anti-egr-1 Ab as described in Materials and methods. Magnification, 40×.

derived probes from CMV vector and ^{S730A}VACM-1 transfected RAMEC. Our results indicate that at least twelve genes known to regulate cytoskeletal functions and signaling during angiogenesis were compromised. For example, urokinase plasminogen activator (uPA), which is required for tumor invasion and metastasis and involved in tissue remodeling (Papetti and



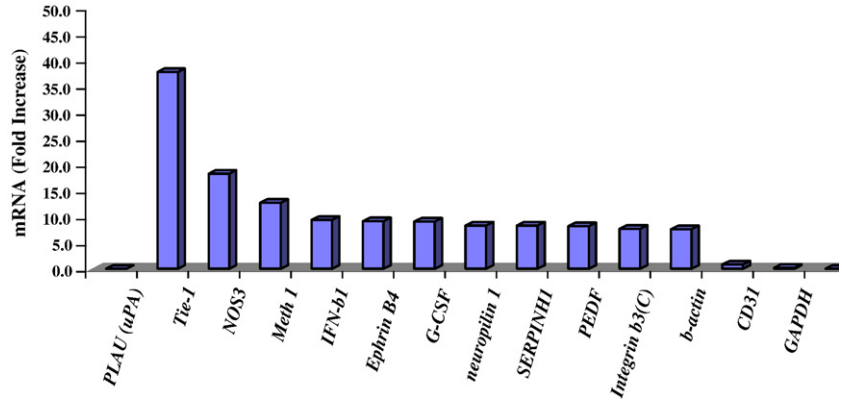


Fig. 8. Effects of ^{S730A}VACM-1 expression on gene mRNA induction in RAMEC. Angiogenesis *GEArray® Q* series blots containing 96 genes known to be involved in modulating various biological processes of angiogenesis was probed with cRNA-derived probes from CMV vector and ^{S730A}VACM-1 transfected RAMEC. Data are expressed as the ratio of the signal from ^{S730A}VACM-1 and CMV transfected RAMEC ($n=1$). The intensity of each signal was corrected for the level of GAPDH mRNA.

Herman, 2002; Yancopoulos et al., 1998), was increased almost 40-fold. Tie-1, a receptor tyrosine kinase specific to endothelial cells and important for the integrity and survival of endothelial cells during angiogenesis (Yancopoulos et al., 1998), was increased almost 20-fold. The mRNA for EphB4, a receptor tyrosine kinase which mediates the contact and signaling between arterial and venous compartments, integrin β3, which enhances survival of angiogenic endothelial cells, and neuropilin-1, a neuronal receptor for the semaphorin family involved in angiogenesis, were increased almost 10-fold (Fig. 8). The expression of numerous growth factors present on the blot was not affected (data not shown). The endothelial cell marker CD31 (PECAM) and GAPDH genes were not changed either (Fig. 8). Subsequent Western blot analyses confirmed that expression of integrin β3/GAPDH signal intensity was significantly increased in ^{S730A}VACM-1 transfected RAMEC when compared to the CMV transfected cells (1.02±0.12 vs. 0.58±0.09, respectively, $n=4$, $p<0.05$, data not shown).

Discussion

In the studies presented here we demonstrate that, in a rat endothelial cell line *in vitro*, signal transduction pathways that regulate transcription of genes involved in angiogenesis, vascular remodeling, and in the pathogenesis of vascular disease (Blacque and Worrall, 2001; Davis, 1993; Kaufmann et al., 2001) may be regulated by VACM-1/cul5. Our observation that, in a model of exercise induced angiogenesis in adult rat skeletal muscle (Hansen-Smith et al., 1992), VACM-1 protein is present in the established but not in the sprouting capillaries (Fig. 1A), implies its involvement in the regulation of angiogenesis and supports the *in vitro* results described below.

Our *in vitro* results show that overexpression of VACM-1 cDNA in RAMEC, which express endogenous VACM-1 protein (Burnatowska-Hledin et al., 2001), significantly decreased cellular proliferation (Fig. 1D). Control RAMEC and RAMEC transfected with VACM-1 cDNA were plated at equal density and their growth was monitored. Whereas control cells reached confluency at 4 days, cells transfected with VACM-1 cDNA were not confluent after 8 days of culture. These results confirm our previous studies which demonstrated that VACM-1 expression in cos 1 and T47D cancer cell line inhibits growth (Van Dort et al., 2003; Johnson et al., 2007). Importantly, our data show that overexpression of ^{S730A}VACM-1 cDNA mutant, where the putative PKA dependent phosphorylation site was eliminated, confers a dominant-negative effect (Herskowitz, 1987; Lagna and Hemmati-Brivanlou, 1998; Qian et al., 2003; Wu et al., 2004) on the endothelial cell growth and signaling pathways (Figs. 2 and 3). It remains to be established whether these changes are a consequence of the change in cellular localization of mutated VACM-1 protein (Figs. 2E and D).

Interestingly, expression of this mutant in RAMEC leads to the appearance of a larger M_r protein on Western blots, suggesting that the mutated VACM-1 protein may be further modified. Since the putative PKA-specific phosphorylation site (underlined) in VACM-1 protein sequence (**AI**IQ**IMKMRK**KLS) overlaps the consensus Nedd8 conjugation region (bold) (Hori et al., 1999; Wada et al., 2000), we reprobed these blots with an anti-Nedd8 specific antibody. Our results indicate that the upper band indeed represents VACM-1 modified by Nedd8 (Figs. 2F–I). Nedd8 is a novel 81 amino acid peptide, 60% identical and 80% homologous to ubiquitin (Hori et al., 1999; Wada et al., 2000). Unlike ubiquitin, which targets diverse proteins for degradation (Glickman and Ciechanover, 2001; Herrmann et al., 2004; Pan et al.,

Fig. 7. Stable expression of ^{S730A}VACM-1 cDNA in RAMEC and VACM-1 cDNA in T47D cell affects maspin protein levels. (A) Western blot analysis of cell lysates from control and ^{S730A}VACM-1 cDNA transfected RAMEC showing maspin expression. (B) Data shown in A were quantitated using the NIH Scion imaging program ($n=4$, *; $p<0.05$). (C) A representative immunocytochemistry of CMV and ^{S730A}VACM-1 cDNA transfected RAMEC using anti-maspin antibody ($n=3$) (magnification, 20×). (D) The intensities of maspin specific signal shown in C were quantified using NIH Scion Imaging and corrected for the cell area. (E) VACM-1 (upper panel) and maspin (middle panel) protein expression in control and VACM-1 cDNA transfected T47D cancer cells. To correct for unequal protein loading, membranes were stripped and reprobed with anti-GAPDH antibody (lower panel). (F) Data shown in D were quantitated and expressed as maspin/GAPDH ($n=5$, *, $p<0.05$). (G) Correlation between maspin and VACM-1 protein levels in T47D cells ($r^2=0.80$).

2004), Nedd8 selectively modifies cullins but does not appear to mark these proteins for degradation (Wu et al., 2000). For example, conjugation of Nedd8 to cul1 enhances the ability of ROC1–cul complex to promote ubiquitin polymerization (Wu et al., 2000) and is essential for cul1 involvement in *Drosophila* eye development *in vivo* (Ou et al., 2002). The report that mutation in the Nedd8 activating enzyme causes yeast cells to undergo multiple S phases without intervening mitosis further supports the importance of Nedd8 dependent modification of cullins (Hori et al., 1999). The immunostaining data and Western blot probing of VACM-1 immunoprecipitates from ^{S730A}VACM-1 cDNA transfected cells with anti-Nedd8 specific antibody indicate that neddylation of VACM-1 is increased in the mutant cell line (Fig. 3). Consequently, the changes in cellular localization of VACM-1 and its biological activity may be regulated by its neddylation status. Our results also suggest that the modification of VACM-1 by Nedd8 may be regulated by its PKA-dependent phosphorylation status. Such mechanism of action, although new for cullins, is not unprecedented as ubiquitination of p53, for example, is also dependent on its phosphorylation status (Glickman and Ciechanover, 2001). Finally, Wimuttisuk and Singer (2007) reported recently that the biological activity of cul3 depends on heterodimer formation that involves Nedd8-dependent modification, thus validating the "dominant-negative" phenotype observed in our study.

The expression of ^{S730A}VACM-1 cDNA not only increased cell growth, it also induced changes in RAMEC growth pattern. Cells looked smaller and elongated and formed a "swirling" pattern when compared to the "cobblestone" appearance of the CMV transfected cultures. These morphological changes were even more apparent when cells were grown on collagen where they formed elongated patterns or on Matrigel® where they formed structures/cords previously described as "angiogenic" (Folkman and Haudenschild, 1980) and observed in NIH 3T3 cells transformed with *ras* oncogene (Dusbery et al., 2001). Furthermore, in RAMEC transfected with ^{S730A}VACM-1 cDNA, actin organization appeared to be random; cells lost the stress fibers and became elongated (Fig. 4). The relative "tube" formation was significantly higher in ^{S730A}VACM-1 cDNA transfected cells when compared to controls. The actin-specific phalloidin staining data (Fig. 4) imply that VACM-1 may regulate cytoskeletal actin organization. These observations are not totally surprising as actin filaments are instrumental in a variety of cell processes including maintenance of cell structure, motility, cell cycle control, and signaling that will lead to tube formation during angiogenesis (Kurz et al., 2002; Usatyuk and Natarajan, 2004). In the endothelium, actin rearrangements are regulated by many factors, including antiangiogenic proteins like endostatin and maspin, which decrease stress fiber formation and focal adhesions (Blacque and Worrall, 2001; Calderwood et al., 2000; Odero-Marah et al., 2003; Wickstroom et al., 2003). Interestingly, in *Caenorhabditis elegans*, cytoskeletal rearrangements during embryogenesis are regulated by cul1 and appear to be Nedd8 dependent (Kurz et al., 2002). In those studies, Nedd8 modified cul1 targeted katanin, a microtubule-severing complex, and acted as a negative regulator of contractility and cytokinesis. Consequently, the loss of stress fiber and focal adhesion formation, along with the appearance of random actin polymerization in the cells overexpressing mutated VACM-cDNA, suggests that the control of cytoskeletal actin arrangement may depend on VACM-1 phosphorylation/neddylation status.

The expression of a dominant-negative phenotype in the ^{S730A}VACM-1 mutant transfected cells induced MAPK phosphorylation when compared to the CMV vector transfected cells. The increase in MAPK phosphorylation was directly correlated with the levels of modified VACM-1 protein, again implying the importance of the phosphorylation/neddylation status in the control of MAPK activity (Fig. 5). Since MAPK activity in angiogenesis is often associated with the induction of egr-1 gene (Beck and D'Amore, 1997; Kaufmann et al., 2001; Rupprecht et al., 1994), we next examined changes in egr-1 localization. The immunocytochemistry results indicate that in RAMEC, egr-1 localization remains largely cytosolic whereas in ^{S730A}VACM-1 mutant transfected cells it localizes to the nucleus (Figs. 6A–B). Similarly, in cos 1 transfected with VACM-1 cDNA, egr-1 expression is largely absent from the nucleus when compared to control or ^{S730A}VACM-1 mutant transfected cells (Fig. 6C). These observations are also in agreement with our previous report that overexpression of VACM-1 in T47D cancer cells leads to a decrease in egr-1 mRNA levels and a significant decrease in nuclear egr-1 protein localization when compared to control cells (Burnatowska-Hledin et al., 2004). Since egr-1 plays a key role in linking "injurious stimuli" to the induction of genes that are responsible for vascular damage (Khachigian and Collins, 1997), identification of factors that may regulate either MAPK and/or egr-1 activation is important in the identification of novel drug targets.

Should VACM-1 form proteosome complexes expected of the cullin family (Wormald and Hilton, 2003), its induction/overexpression is expected to degrade specific proteins (Glickman and Ciechanover, 2001). Consequently, the effect of VACM-1/^{S730A}VACM-1 mutant product on MAPK phosphorylation, egr-1 localization, and actin polymerization, but not on their concentrations, implied an indirect effect. In an attempt to identify antiangiogenic genes that are affected by VACM-1/cul5, we next focused our attention on maspin, a member of the serpin family of inhibitory proteins, previously implicated in the control of tumor growth, cell motility, invasion, and angiogenesis (Bass et al., 2000; Blacque and Worrall, 2001; Ou et al., 2002). High levels of maspin protein are present in a healthy but not in aggressively cancerous breast epithelium (Bass et al., 2000), and maspin expression leads to decreased tumor induction and metastasis of aggressive breast tumor cells *in vitro* (Bass et al., 2000; Odero-Marah et al., 2003; Zou et al., 1994). The observation that maspin levels decreased significantly in ^{S730A}VACM-1 cDNA transfected RAMEC when compared to the CMV vector transfected cells identifies VACM-1 as a novel regulator of maspin expression. The involvement of VACM-1 in the regulation of maspin levels was further confirmed by the studies in T47D cancer cell line, where overexpression of the wild type VACM-1 protein increases maspin levels (Fig. 7E–G). Whether the changes in maspin levels precede changes in MAPK phosphorylation and/or egr-1 localization in response to changes in VACM-1 activation remains to be determined.

Finally, to identify other proteins that may be affected by ^{S730A}VACM-1 in steps either preceding or subsequent to MAPK/egr-1 activation, *Angiogenesis GEArray® Q* series blots containing 96 genes known to be involved in modulating various biological processes of angiogenesis were probed with cRNA-derived probes from CMV vector and ^{S730A}VACM-1 transfected RAMEC. Our results indicate that mRNA levels for several genes known to regulate cytoskeletal/matrix forming functions and signaling during angiogenesis (Yancopoulos et al., 1998) were increased in ^{S730A}VACM-1 transfected RAMEC (Fig. 8). Interestingly, numerous growth factors present on the blot were not affected (data not shown). Although the increase in mRNA levels for integrin β3 was confirmed at the protein level (data not shown), the mRNA array was only probed once and consequently these results, although interesting, need to be interpreted with caution. Unfortunately, further investigation into these changes was beyond the scope of this study.

This novel role of VACM-1/cul5 in the regulation of endothelial cell growth and angiogenesis is further supported by a report indicating that a targeted disruption of p185/cul7, a cul1 related gene that forms an SCF-like multi-protein complex, leads to abnormal vascular morphogenesis (Arai et al., 2003). Similarly, a mouse cul1-dependent complex involved in Notch degradation may be critical in normal vascular development (Tsunematsu et al., 2004). Since the specificity of the ubiquitin–proteosome degradation system resides with the hundreds of the E3 ligases which recognize specific motifs on their substrates that have to be targeted for ubiquitination (Glickman and Ciechanover, 2001), the identity of the E3 ligase forming proteins like VACM-1/cul5 that may be vasculature specific is important.

In summary, the work presented here indicates that the VACM-1/cul5 gene product, which is specific to endothelial cells *in vivo*, regulates cell growth by a mechanism that may involve regulation of maspin protein concentration, MAPK phosphorylation, and egr-1 translocation to the nucleus and cytoskeletal rearrangements. Furthermore, these activities are dependent on VACM-1 protein phosphorylation by PKA and subsequent neddylation. Since the ubiquitin–proteosome system, the major nonlysosomal pathway for protein degradation, can affect cardiovascular function and is a target for drug development, understanding VACM-1/cul5 involvement in the regulation of angiogenesis under physiological and pathophysiological conditions is critical.

Acknowledgments

We want to thank Dr. P. Lelkes for the gift of the RAMEC. This work was supported by NIH grant RO1-DK47199, R15-CA1-4014, NSF-REU grants #DBI-982057 and CHE-9619667, and The Camille and Henry Dreyfus Foundation Teacher Scholar Award to M. Burnatowska-Hledin.

References

Arai, T.J., Kasper, S., Skaar, J.R., Ali, S.H., Takahashi, C., DeCaprio, J.A., 2003. Targeted disruption of p185/cul7 gene results in abnormal vascular morphogenesis. Proc. Natl. Acad. Sci. 100, 9855–9860.

Bass, R., Moreno-Fernandez, A.M., Ellis, V., 2000. Maspin inhibits cell migration in absence of protease inhibitory activity. J. Biol. Chem. 277, 46845–46848.

Beck Jr., K.L., D'Amore, P.A., 1997. Vascular development: cellular and molecular regulation. FASEB J. 11, 365–373.

Blacque, O., Worrall, M., 2001. Evidence for a direct interaction between the tumor suppressor serpin, maspin, and types I and III collagen. J. Biol. Chem. 277, 10783–10788.

Burnatowska-Hledin, M., Spielman, W.S., Smith, W.L., Shi, P., Meyer, J.M., Dewitt, D.L., 1995. Expression cloning of an AVP-activated calcium-mobilizing receptor from rabbit kidney medulla. Am. J. Physiol. 268, F1198–F1210.

Burnatowska-Hledin, M., Lazdins, I.B., Listenberger, L., Zhao, P., Sharangpani, A.M., Folta, V., Card, B., 1999. VACM-1 receptor is specifically expressed in rabbit vascular endothelium and renal collecting tubule. Am. J. Physiol. 276, F199–F209.

Burnatowska-Hledin, M., Capps, B., Folta, V., Zhao, P., Mungall, C., Sharangpani, A.M., Listenberger, L., 2000. VACM-1 a cullin gene family member, regulates cellular signaling. Am. J. Physiol. 279, 1266–1273.

Burnatowska-Hledin, M., Zeneberg, A., Roulo, A., Grobe, J., Zhao, P., Lelkes, P.L., Clare, P., Barney, C., 2001. Expression of VACM-1 protein in cultured rat adrenal endothelial cells is linked to the cell cycle. Endothelium 8, 49–63.

Burnatowska-Hledin, M., Kossoris, J., Van Dort, C., Murrey, D., Abbott, J., Kan, C., Barney, C., 2004. VACM-1 expression in T47D human breast cancer cell line. Biochem. Biophys. Res. Commun. 319, 817–825.

Byrd, P.J., Stankovic, T., McConville, C.M., Smith, A.D., Cooper, P.R., Taylor, A.M.R., 1997. Identification and analysis of expression of human VACM-1, a cullin gene family member located on chromosome 11q22–23. Genome Res. 7, 71–75.

Calderwood, D.A., Shattil, S.J., Ginsberg, M.H., 2000. Integrins and actin filaments: reciprocal regulation of cell adhesion and signaling. J. Biol. Chem. 275, 22607–22610.

Davis, R.J., 1993. The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. 268, 14553–14556.

Dusbery, N., Resau, J., Webb, C., Koochekpour, S., Koo, H.M., Leppla, S.H., Vande Woude, G.F., 2001. Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways. Proc. Natl. Acad. Sci. 98, 4089–4094.

Folkman, J., Haudenschild, C., 1980. Angiogenesis *in vitro*. Nature 288, 551–556.

Glickman, M.H., Ciechanover, A., 2001. The ubiquitin–proteosome proteolitic pathway: destruction for the sake of construction. Physiol. Rev. 82, 373–428.

Hansen-Smith, F., Banker, K., Morris, L., Joswiak, G., 1992. Alternative histochemical markers for skeletal muscle capillaries: a statistical comparison among three muscles. Microvasc. Res. 44, 112–116.

Herrmann, J., Ciechanover, A., Lerman, L.O., Lerman, A., 2004. The ubiquitin–proteosome system in cardiovascular diseases—a hypothesis extended. Cardiovasc. Res. 61, 11–21.

Herskowitz, I., 1987. Functional inactivation of genes by dominant negative mutations. Nature 329, 219–222.

Hori, T., Osaka, F., Miyamoto, C., Okabayashi, K., Shimbara, N., Kato, S., Tanaka, K., 1999. Covalent modification of all members of human cullin family proteins by NEDD8. Oncogene 18, 6829–6834.

Johnson, A.E., Le, I.P., Buchwalter, A., Burnatowska-Hledin, M.A., 2007. Estrogen dependent growth and estrogen receptor (ER)-α concentration in T47D breast cancer cells are inhibited by VACM-1, a cul-5 Gene. Mol. Cell. Biochem. 301, 13–20.

Kaufmann, K., Bach, K., Thiel, G., 2001. The extracellular signal-regulated protein kinases Erk1/Erk2 stimulate expression and biological activity of the transcriptional regulator Egr-1. J. Biol. Chem. 382, 1077–1081.

Keydar, I., Chen, L., Karby, S., Weiss, F.R., Delarea, J., Radu, M., Chaitcik, S., Brenner, H.J., 1979. Establishment and characterization of a cell line of human breast carcinoma origin. Eur. J. Cancer 15, 659–670.

Khachigian, L., Collins, T., 1997. Inducible expression of Egr-1-dependent genes. A paradigm of transcriptional activation in vascular endothelium. Circ. Res. 81, 457–461.

Kipreos, E.T., Lander, L.E., Wing, J.P., He, W.W., Hedgecock, E.M., 1996. Cul-1 is required for cell cycle exit in *C. elegans* and identifies a novel gene family. Cell 85, 829–839.

Kurz, T.L., Pintard, J.H., Willis, D.R., Hamill, P., Gonczy, M.P., Bowerman, B., 2002. Cytoskeletal regulation by the Nedd8 ubiquitin-like protein modification pathway. Science 295, 1294–1298.

Lagna, G., Hemmati-Brivanlou, A., 1998. Use of dominant negative constructs to modulated gene expression. Curr. Top. Dev. Biol. 36, 74–98.

Lyapina, S.A., Correll, C.C., Kipreos, E.T., Deshaies, R.J., 1998. Human CUL1 forms an evolutionarily conserved ubiquitin ligase complex (SCF) with SKP1 and an F-box protein. Proc. Natl. Acad. Sci. 95, 7451–7456.

Mathias, N.S., Johnson, L., Winey, M., Adams, A.E.M., Goetsch, L., Pringle, J.R., Byers, B., Goebl, M.G., 1996. Cdc53p acts in concert with Cdc4p and Cdc34p to control the G_1- to S-phase transition and identifies a conserved family of proteins. Mol. Cell. Biol. 16, 6634–6643.

Mukhopadhyay, D., Knebelmann, B., Cohen, H.T., Ananth, S., Sukhatme, V.P., 1997. The von Hippel–Lindau tumor suppressor gene product interacts with Sp1 to suppress vascular endothelial growth factor promoter activity. Mol. Cell. Biol. 17, 5629–5639.

Odero-Marah, V.A., Khalkhali-Ellis, Z., Chunthapong, J., Amir, S., Seftor, R.E. B., Seftor, E.A., Hendrix, M.J.C., 2003. Maspin regulates different signaling pathways for motility and adhesion in aggressive breast cancer cells. Cancer Biol. Ther. 2, e67–e72.

Ou, C.-Y., Lin, Y.-F., Chen, Y.-J., Chien, C.-T., 2002. Distinct protein degradation mechanisms mediated by Cu1 and Cul3 controlling Ci stability in *Drosophila* eye development. Genes Dev. 16, 2403–2414.

Pan, Z.-Q., Kentsis, A., Dias, D.C., Yamoah, K., Wu, K., 2004. Nedd8 on cullin: building an expressway to protein destruction. Oncogene 23, 1985–1997.

Papadimitriou, E., Unsworth, B.R., Maragoudakis, M.E., Lelkes, P.I., 1993. Time-course and quantification of extracellular matrix maturation in the chick chorioallantoic membrane and in cultured endothelial cells. Endothelium 1, 207–219.

Papetti, M., Herman, I.M., 2002. Mechanism of normal and tumor-derived angiogenesis. Am. J. Physiol. 282, C946–C970.

Pause, A., Lee, S., Worrell, R.A., Chen, D.Y.T., Burgess, W.H., Linehan, W.M., Klausner, R.D., 1997. The von Hippel–Lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins. Proc. Natl. Acad. Sci. 94, 2156–2161.

Qian, Y., Luo, J., Leonard, S.S., Harris, G.K., Millecchia, L., Flynn, D.D.C., Shi, X., 2003. Hydrogen peroxide formation and actin filament reorganization by Cdc42 are essential for ethanol-induced in vitro angiogenesis. J. Biol. Chem. 278, 16189–16197.

Querido, E., Blanchette, P., Yan, Q., Kamura, T., Morrison, M., Boivin, D., Kaelin, W.G., Conaway, R.C., Conaway, J.W., Branton, P.E., 2001. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a cullin-containing complex. Genes Dev. 15, 3104–3117.

Rozenghurt, E., Legg, A., Strang, G., Courtenay-Luck, N., 1981. Cyclic AMP: a mitogenic signal for Swiss 3T3 cells. Proc. Natl. Acad. Sci. 78, 4392–4396.

Rupprecht, H., Sukhatme, V., Rupprecht, A., Sterzel, R., Coleman, D., 1994. Serum response elements mediate protein kinase C dependent transcriptional induction of early growth response gene-1 by arginine vasopressin in rat mesangial cells. J. Cell. Physiol. 159, 311–323.

Senger, D.R., Van De Water, L., Brown, L.F., Nagy, J.A., Yeo, K.T., Yeo, T.K., Berse, B., Jackman, R.W., Dvorak, A.M., Dvorak, H.F., 1993. Vascular permeability factor (VPF, VEGF) in tumor biology. Cancer Metastasis Rev. 12, 303–324.

Tsunematsu, R., Nakayama, K., Oike, Y., Nishiyama, M., Ishida, N., Hatakeyama, S., Bessho, Y., Kageyama, R., Suda, T., Nakayama, K., 2004. Mouse Fbw7/Sel-10/Cdc4 is required for Notch degradation during vascular development. J. Biol. Chem. 279, 9417–9423.

Usatyuk, P., Natarajan, V.V., 2004. Role of mitogen-activated protein kinases in 4-hydroxy-2-nonenal-induced actin remodeling and barrier function in endothelial cells. J. Biol. Chem. 279, 11789–11797.

Van Dort, C., Zhao, P., Parmelee, K., Capps, B., Poel, A., Listenberger, L., Wasilevich, B., Murrey, J.D., Kossoris, J., Clare, P., Burnatowska-Hledin, M., 2003. VACM-1, a cullin gene family member, attenuates cellular growth *in vitro*. Am. J. Physiol. 285, C1386–C1396.

Wada, H., Yeh, E.T.H., Kamitani, T., 2000. A dominant-negative UBC12 mutant sequesters NEDD8 and inhibits NEDD8 conjugation in vivo. J. Biol. Chem. 275, 17008–17015.

Wickstroom, S.A., Alitalo, K., Keski-Oja, J., 2003. Endostatin associates with lipid rafts and induces reorganization of the actin cytoskeleton via down-regulation of RhoA activity. J. Biol. Chem. 278, 37895–37901.

Wimuttisuk, Wananit, Singer, Jeffrey D., 2007. The Cullin3 ubiquitin ligase functions as a Nedd8-bound heterodimer. Mol. Biol. Cell 18, 899–909.

Wormald, S., Hilton, D.J., 2003. Inhibitors of cytokine signal transduction. J. Biol. Chem. 279, 821–824.

Wu, K., Chen, A., Pan, Z.-Q., 2000. Conjugation of Nedd8 to CUL1 enhances the ability of the ROC1–CUL1 complex to promote ubiquity polymerization. J. Biol. Chem. 275: 41, 32317–32324.

Wu, Y.-L., Hooks, S.B., Harden, T.K., Dohlman, H.G., 2004. Dominant-negative inhibition of pheromone receptor signaling by a single point mutation in the G protein a subunit. J. Biol. Chem. 279, 35287–35297.

Yancopoulos, G.D., Klagsbrun, M., Folkman, J., 1998. Vasculogenesis, angiogenesis and growth factors: ephrins enter the fray at the boarder. Cell 93, 661–664.

Yu, H., Peters, J.M., King, R.W., Page, A.M., Hieter, P., Kirschner, M.W., 1998. Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science 279, 1219–1222.

Yu, X.Y.Y., Liu, B., Luo, K., Kong, W., Yu, X.F., 2003. Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif–Cul5–SCF complex. Science 302, 1056–1060.

Zou, Z., Anisowicz, A., Hendrix, M.J.C., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E., Sager, R., 1994. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science 263, 526–529.