PHOSPHORYLATION OF VACM-1/CUL5 BY PROTEIN KINASE A REGULATES ITS NEDDYLATION AND ANTIPROLIFERATIVE EFFECT.

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Expression of VACM-1/cul-5 gene in endothelial and in cancer cell lines in vitro inhibits cellular proliferation and decreases phosphorylation of mitogen-activated protein kinase (MAPK). Structure-function analysis of VACM-1 protein sequence identified consensus sites specific for phosphorylation by protein kinases PKA and PKC and a Nedd8 protein modification site. Mutations at the PKA specific site in VACM-1/cul-5 (S730AVACM-1) sequence resulted in increased cellular growth and the appearance of a Nedd8-modified VACM-1/cul5. The aim of this study was to examine if PKA dependent phosphorylation of VACM-1/cul5 controls its neddylation status, phosphorylation by PKC, and ultimately growth. Our results indicate that in vitro transfection of rat endothelial cells, RAMEC, with anti-VACM-1 specific siRNA oligonucleotides decreases endogenous VACM-1 protein concentration and increases cell growth. Western blot analysis of cell lysates immunoprecipitated with an antibody directed against PKA-specific phosphorylation site and probed with anti-VACM-1 specific siRNA oligonucleotides decreases endogenous VACM-1 protein concentration and increases cell growth. Western blot analysis of cell lysates immunoprecipitated with an antibody directed against PKA-specific phosphorylation site and probed with anti-VACM-1 specific antibody showed that PKA-dependent phosphorylation of VACM-1 protein was decreased in cells transfected with S730AVACM-1 cDNA when compared to the CMV transfected cells. This change was associated with increased modification of VACM-1 protein by Nedd8. Induction of PKA activity with forskolin, reduced modification of VACM-1 protein by Nedd8. Finally, RAMEC transfected with S730AVACM-1/cul5 cDNA and treated with Phorbol 12-myristate 13-acetate (PMA, 10nM and 100 nM) to induce PKC activity, grew significantly faster than the control cells. These results suggest that antiproliferative effect of VACM-1/cul5 is dependent on its posttranslational modifications and will help in the design of new anticancer therapeutics that target the Nedd8 pathway.

Vasopressin-activated calcium-mobilizing (VACM-1) protein (1), now identified as a cul5 gene product (2-4), is a 780 amino acids protein with a calculated Mr of 91 kDa. Transfection of various cell lines with VACM-1/cul5 cDNA attenuates cellular growth by a mechanism that involves inhibition of cAMP production, decreased phosphorylation of mitogen-activated protein kinase (MAPK) and a decrease in nuclear localization of early growth response gene (egr-1) product (5-7). In vivo, VACM-1/cul5 protein expression is specific to established endothelial cells (8) but is absent in sprouting capillaries (9), suggesting its involvement in the regulation of endothelium-specific growth. Interestingly, the human homolog of VACM-1 differs only in six amino acids from the rabbit VACM-1 and has been proposed to be a candidate for a tumor suppressor (2). Although expression of VACM-1/cul5 cDNA in a cancer derived cell line, T47D, decreased nuclear concentration of estrogen receptor, ERα, and inhibited cellular growth (6), the precise mechanism by which VACM-1/cul5 may regulate cell growth is not known. Like other culmins, however, VACM-1/cul 5 may serve as scaffold protein that allows the assembly of E3 ubiquitin ligase complexes involved in protein ubiquitination, and ultimately, degradation (10). Proteasome dependent protein degradation involves three ligases (E1-E3) which promote activation (E1), conjugation (E2) and ligation (E3) of ubiquitin to a substrate marked for degradation (11, 12). The E3 ligases are further divided into three groups based on their structure and substrate recognition (13-15). The most abundant group of the E3 ligases is characterized by the presence of a RING (Really Interesting New Gene) finger domain and uses cullin family members to recognize specific motifs on their substrates (16). The numerous E3 ligase complexes can be further
regulated by the posttranslational modifications of their components (11). For example, activation of E3 ubiquitin ligase *Itch* is regulated by phosphorylation-induced conformational changes (17, 18) and COP1 E3 ligase, which affects p53 ubiquitination, is phosphorylated by the ATM kinase (19). The specificity of the ubiquitin-proteasome degradation system is further controlled through modification of cullins by Nedd8 protein, which shares 58% identity and 79% similarity with ubiquitin (20-22). It is now proposed that cullins must be neddylated and form heterodimers to be an active component of the E3 ligase system (14). Conjugation of Nedd8 to cul1 enhances the ability of the complex to promote ubiquitin polymerization and is essential for proteolytic targeting of p27Kip1 (10, 22-24). Loss of Nedd8 system, on the other hand, leads to the dysfunction of tumor suppression by VHL (25) and compromises cul1 dependent regulation of eye development in Drosophila (26) while in mice it is essential for cell cycle progression (28). In *C. elegans* development, neddylated cul1 targets katanin, a microtubule severing complex, and thus acts as a negative regulator of contractility and cytokinesis (28, 29). Whether modification of cullins by Nedd8 is dependent on their phosphorylation has not been reported.

Analysis of VACM-1/cul5 protein structure revealed a putative modification sequence for Nedd8 at Lys724, protein kinase A (PKA) phosphorylation sequence at Ser730 and Thr426 and fifteen putative protein kinase C (PKC) dependent phosphorylation sites (5). The expression of a VACM-1 mutant where Ser730 has been changed to Ala (*S730AVACM-1*) significantly increased cellular growth and created a dominant negative phenotype (5). Further, overexpression of the mutant in rat endothelial cells, RAMEC, converted cells to the angiogenic phenotype when grown on a Matrigel® support (9). These results suggested, therefore, that cellular localization and the biological activity of VACM-1/cul5 protein may depend on its posttranslational modification status by PKA.

The *S730AVACM-1* mutant gives us a powerful tool to determine how PKA specific phosphorylation induces modification of VACM-1 by Nedd8 and to elucidate the mechanism of phenotype reversal when expressed in endothelial cells *in vitro* (5). This approach has been used by others to discover the mechanism by which PKA activity controls localization and activity of proteins that regulate cell growth and angiogenesis (30-33). For example, PKA dependent phosphorylation of an oncogene Gli increased its nuclear localization (32), while phosphorylation of a receptor GRK2 recruited the protein to the cell membrane (33). Since E3 ligases determine the specificity of the substrates being targeted for degradation, proteasome inhibitors are now marketed as drugs (15). Consequently, identifying VACM-1/cul5 as a component of the vasculature-specific E3 ligase, and determining how neddylation and/or phosphorylation affect its localization and biological activity, may be important in identifying specific targets for drugs to control cell growth and angiogenesis.

In this study we report several new findings that will help elucidate the mechanism of VACM-1/cul5 dependent cell growth. First, using siRNA oligonucleotides against VACM-1 mRNA, we confirmed the antiproliferative effects of VACM-1/cul5. Second, we demonstrated that cellular localization of VACM-1/cul5 proteins may be controlled by its posttranslational modifications. Third, we showed that the expression of *S730AVACM-1* cDNA, which lacks the PKA specific phosphorylation site, leads to increased neddylation of VACM-1. Finally, we found that PKC induced cell proliferation is significantly higher in cells transfected with *S730AVACM-1* cDNA when compared to the control group. Together, these results suggest that preferential phosphorylation of VACM-1/cul5 protein by these protein kinases regulates its modification by Nedd8 and may allow for the selective regulation of different cellular pathways (5, 6, 9).

**EXPERIMENTAL PROCEDURES**

**Materials-** All tissue culture media and reagents were purchased from Invitrogen (Grand Island, NY). VACM-1 cDNA was subcloned into the pBK-CMV vector as described previously (34). VACM-1/cul5 specific siRNAs were purchased from Ambion (Applied Biosystems Inc., Austin, Tx). Phorbol 12-myristate 13-acetate (PMA) (cat # P8139) and Gö6983 (cat # G1918) from Sigma Co. (St. Louis, Mo). Forskolin (FSK) was purchased from MP Biomedicals (Solon, OH). Polyclonal anti Nedd8 antibody was form and monoclonal anti-Ned8 Ab was purchased from Abcam (Cambridge, MA).

**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, sequencing was performed by Macromolecular Resources (Colorado State Univ., Ft. Collins, Co) as described previously (5).

**Tissue Culture-** RAMEC (Rat Adrenal Medullary Endothelial Cells) (35) were grown in low glucose DMEM supplemented with 2% FBS, 7% HS (horse serum), 89.6 U/mL penicillin, and 89.6 μg/mL streptomycin at 37°C under a water-saturated 5% CO₂ atmosphere as described previously (36). All cell lines were plated at a density of 6-8 x 10⁵ cells per 100 mm plate and cultured for 24 hours before transfection. Cells were transfected with 5 µg VACM-1 cDNA (or S730VACM-1 cDNA) per 100 mm culture dish using the FuGENE-6® (Roche Diagnostics Inc., Indianapolis, IN) (9) or with the Mirus reagent (Mirus, Madison, WI) as described by the manufacturer. After 24 hours cells were split and incubated with fresh medium containing 600 µg/ml G418 (Gibco BRL Co., Gaithersburg, MD). The medium was changed every three 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) (5).

**Immunostaining-** Affinity purified rabbit polyclonal antibody directed against the N-terminus (Ab-A) of VACM-1 protein (1, 8) were used to stain cells by indirect immunofluorescence. Cells grown on coverslips were fixed in 3% paraformaldehyde (in 1xPBS, pH 7.4) for 20 minutes, washed in PBS, permeabilized with 5% Tween-20 solution for 20 minutes, washed with PBS/2% BSA, and incubated for two hours 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 detected by incubating cells in the presence of 1:40 dilution of either FITC-conjugated goat anti-rabbit IgG or Texas Red conjugated anti-rabbit Ab (Vector Laboratories Inc, Burlingame, CA) in 1X PBS/2% BSA for 1 hr. The slides were washed with 1x PBS with 0.2 % 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).

**Cellular proliferation analysis-** RAMEC transfected with VACM-1 cDNA, S730VACM-1 cDNA, and with CMV vector were seeded in 100 mm plates at equal densities (2x10⁵ cells/mL) harvested at specified time points and counted in a hemacytometer. Alternatively, cells were seeded at equal densities in 12-well plates and after three days photographs were taken using Image-Pro Express® at 10x magnification. To quantitate density, cells were counted in at least three random one-square centimeter areas. In addition, cells were grown on coverslips, stained with DAPI as described above, and nuclei visualized under epifluorescence microscopy were counted.

**Wound healing growth assay-** Cells were plated on 24-well tissue culture plates at 2x10⁴ cells/mL per well. After cells reached confluency, the cell layer was scratched using a 1-200 μL pipette tip (38). Cell cultures were photographed at time zero and at sixteen hours after the appropriate treatment, and Image-Pro Express® was used to measure the cell monolayer wound distances. Stock solutions of 1 mM Forskolin (FSK), PMA and Gö6983 were prepared in dimethyl sulfoxide (DMSO) and diluted to appropriate concentrations in tissue culture media immediately before use. The control cells were treated with media containing 0.1% DMSO.

**SiRNA-** Transfections with anti-VACM-1 siRNAs targeting different regions of the VACM-1/cul5 sequence were performed according to the protocol in the Silencer® siRNA Starter Kit purchased from Ambion® (cat # AM16708A). Three specific antisense oligonucleotides in the Silencer® pre-designed siRNA included: siRNA#1, 5'-AGAUUCCUGGCGUAAAAGCtt-3' (ID 192207), siRNA#2 5'-CCACGUAUCAAGCAUGAGCtt-3' (ID 192208) and siRNA#3 5'-UAGCAUCAUAAACACUGCtt-3'(ID 192209). The control cells were sham transfected. For the negative control, cells were transfected with siRNAs that did not target any gene sequences provided in the starter kit. The positive control used was GAPDH siRNA provided in the starter kit. In some experiments double transfection was
performed after 24 hours and cell lysates were collected at 48 hours (9).

**Immunoprecipitation-** Total lysates (100-300 µg protein) were prepared 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® 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, anti-phospho-PKA substrate (RRXS/T) Ab (Cellular Signaling Technology) (30) or a nonspecific antibody. After 2 hours of incubation, proteinA/sepharose (Amhersham Pharmacia Biotech) suspension was added and the incubation continued for another 2 hours. The complex was centrifuged at 12,000 RPM for 2 minutes and washed two to three times in the solubilization buffer. Loading dye was added and the immunoprecipitates were heated to 95º C for 5 minutes, separated using 12.5% SDS-PAGE gels, transferred to nitrocellulose and probed with an antibody directed against VACM-1 protein, as described below (5, 36).

**Western blot analysis-** Total cell lysates and membrane fractions were prepared as described previously (5). 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, 1M EDTA, 50 mM NaF) with 1 µg/ml aprotinin, 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). Nuclear and membrane fractions were isolated as described previously (35). Both nuclear and membrane pellets were resuspended in the buffer and their protein concentrations were determined as described using Bradford assay (34). All samples were resuspended in 4X sample buffer (Invitrogen® Co.), heated to 95º C for 5 min, and subjected to SDS polyacrylamide gel electrophoresis (PAGE) using a 12.5% running gel. The separated proteins were transferred to a nitrocellulose membrane (Osmotics Co. Trevose, PA) at 30 mV for 2 hours. Nonspecific binding was blocked by incubating membrane temperature with PBS containing 5% nonfat dry milk and 0.2% Tween-20 for 30 min at room temperature. When probing with anti-phospho-PKA phosphorylation specific Ab, blots were blocked with 5% BSA solution. Membranes were next incubated for 2 hours 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 (1, 36). In some experiments, blots were stripped and reprobed with anti-Nedd8 specific Ab (1:500 dilution) developed in rabbit (Alexis Co.). To ascertain equal protein loading, blots were stripped and incubated in a 1:10,000 dilution of monoclonal anti-mouse GAPDH (Abcam Inc., Cambridge, MA) for 1.5 hours at room temperature. The membranes were next washed in the same buffer for 15 min and twice for 5 min and incubated for 2 hours with a horse radish 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 or longer, if appropriate, and exposed to the X-ray film (Amhersham, Arlington Heights, Il).

**Statistical Analysis-** Data are expressed as mean ± one standard error (SE) of the mean. SYSTAT® t-tests were used for data analysis. Significance was set at p<0.05 unless noted otherwise.

**RESULTS**

To confirm the antiproliferative effects of VACM-1/cul5 in vitro, RAMEC expressing endogenous VACM-1 protein (36) were transfected with VACM-1 cDNA. The growth of these cells was monitored as described in Experimental Procedures (9). The CMV vector transfected RAMEC were confluent three days after plating compared to the minimal growth of the VACM-1 cDNA transfected cells (Fig. 1A). When cell growth was quantitated, there was a significant difference in growth rates between the two groups. The VACM-1 cDNA transfected RAMEC failed to reach confluency one week after plating (data not shown).

To further ascertain the antiproliferative effect of VACM-1, we transfected cells with three anti-VACM-1/cul5 specific siRNA oligonucleotides (Ambion® Inc). Time (24 hrs and 48 hrs) and dose (0 nM control, 15 nM and 30 nM) dependent effects of the anti-VACM-1/cul5 specific oligonucleotides were examined using light microscopy and Western blot analysis. Our results (Fig. 2) demonstrate that RAMEC transfected with
any of the three anti-VACM-1 specific siRNA oligonucleotides for 24 hours, grew significantly faster than the control cells (Fig. 2A, n=3 each, *p<0.05). A representative photograph for cells treated with siRNA #2 is shown in Fig. 2B (upper panel). There was no significant difference in RAMEC growth rate between control cells and those cells transfected with the GAPDH siRNA, which was used as a positive siRNA control (Fig. 2B, middle panel). The negative control siRNA, which did not target any particular sequence, was used to evaluate the transfection efficiency, and it had no effect on cell growth (Fig. 2A, bottom panel). The growth promoting effects of anti-VACM-1 specific siRNA oligonucleotides were further confirmed at 48 hours after transfection using methylene blue staining technique (Fig. 3A).

To establish that the increase in cell growth was associated with changes in VACM-1 protein concentration, cell lysates from control and anti-VACM-1 specific siRNA oligonucleotide transfected cells were examined for VACM-1 protein expression using the Western blot approach. VACM-1/cul5 signal was quantified and corrected for GAPDH protein concentration. Our results (Fig. 3B and 3C) show a decrease in VACM-1 protein concentration in RAMEC treated with all oligonucleotides at 24 hours post transfection, but the decrease was statistically significant only in the group treated with siRNA#2 (n=4, p<0.05). The decrease in VACM-1/cul5 protein expression after treatment with siRNA oligonucleotides hours was time dependent. A representative blot for lysates prepared from cells transfected with siRNA#2 and collected at different time points is shown in Fig. 3D. The highest decrease in VACM-1/cul5 protein concentration was observed in RAMEC treated with all oligonucleotides at 24 hours post transfection, but the decrease was statistically significant only in the group treated with siRNA#2 (n=3, *p<0.05).

The appearance of a larger Mr species on Western blot was previously identified as Nedd8 modified VACM-1 protein (5, 9). Consequently, wound healing assay described above was used to examine Ned8 signal in proliferating and nonproliferating cells. Our results indicate that in the wound area where cells are proliferating, Ned8 signal is very intense and localizes to the nucleus, while in the nonproliferating regions of the cell culture, Ned8 signal is reduced or absent (Fig. 5A). The coimmunostaining experiments using anti-VACM-1/cul5 specific Ab (green) and anti-Nedd8 specific Ab (red) indicate that VACM-1 and Nedd8 signal colocalize in the nuclear region but not in the cell membrane (Fig. 5B and 5C).
study where anti-Nedd8 Ab was used to “pull-down” proteins and anti-VACM-1 Ab was used to probe the blot, showed that only the higher Mr species was detected (Fig. 6B). These results suggested therefore, that neddylation of VACM-1 protein may be regulated by PKA dependent phosphorylation at Ser730.

Thus, we next explored the hypothesis that the rapid translocation of VACM-1 protein form the nuclear region in non-confluent cells to the cytosol and/or membrane in the confluent cells (Fig. 4) depends on modification of VACM-1 by Nedd8 and is regulated by PKA. To induce PKA activity, CMV and S730AVACM-1 cDNA transfected cells were treated with 10 μM forskolin for 5 or 15 min as described in literature (30, 37). Total cell lysates were collected and immunoprecipitated with anti-VACM-1 specific Ab. Samples were resolved on SDS-PAGE and after transfer to nitrocellulose, blots were probed with an antibody that recognizes PKA phosphorylated proteins (30, 37). Our results show that PKA dependent phosphorylation of VACM-1/cul5 is higher in the CMV-transfected cells when compared to the S730AVACM-1 cDNA transfected cells (Fig. 6C, first two lanes). PKA specific signal was increased in all experiments from control cells treated with FSK for 5 min. (3787+849 vs 7559+2915, n=4, p<0.2). No change in signal intensity was observed in S730AVACM-1 cDNA transfected cells (2111±703 vs 2024±844, n=3, NS). Interestingly, only the 91 kDa VACM-1/cul5 and not the higher Mr species identified in Fig. 6B as the neddylated VACM-1/cul5, was immunoprecipitated with the anti-phospho-PKA substrate specific antibody (Fig. 6C and D). To extend these experiments even further, we used the immunocytochemistry approach. The representative data shown in Fig. 7A indicate that treatment of control cells with forskolin (FSK, 10 μM) to increase PKA activity, reduced nuclear localization of VACM-1/cul5 protein. This effect was rapid, as VACM-1/cul5 signal intensity was decreased in 45 min and totally disappeared at 2 and 4 hours after treatment. Similarly, we observed that in the proliferating cells (wound area) treated with forskolin for 45 min and stained with anti-Nedd8 specific Ab, Nedd8 signal was reduced as well (Fig. 7B).

To ascertain the specificity of this effect, these experiments were repeated using double immunostaining approach. Our results shown in Fig. 8A indicated that in control cells Nedd8 and VACM-1/cul5 signal in the proliferating cells is largely overlapping. Induction of PKA activity with FSK resulted in a decreased signal intensity. Interestingly, in cells expressing the mutated VACM-1 cDNA lacking the PKA phosphorylation site, treatment with Fsk for 45 min did not appear to affect localization of either protein (Fig. 8B, lower panel).

In addition to the PKA specific phosphorylation site at Ser730 and the neddylation site at Lys724, 15 putative PKC-specific phosphorylation sites can be identified in VACM-1 sequence (5). To further examine VACM-1 phosphorylation status in S730AVACM-1 cDNA transfected RAMEC, we examined whether VACM-1 signal intensity was affected by treating cell lysates with either 300 μM okadaic acid (OKA) to inhibit endogenous phosphatases, or by treatment with 100U/mL calf-intestinal phosphatase (CIP) to decrease protein phosphorylation (38). Lysate samples pretreated with CIP had lower signal intensity compared to the control or to the OKA treated lysates (Fig. 9A), suggesting that in the absence of PKA-phosphorylation site, VACM-1 may still be phosphorylated.

To examine whether PKC plays a role in the regulation of VACM-1 dependent cell growth, cells were treated with PMA to induce PKC activity and with Gö6983 to inhibit its activity (37). The time-dependent and dose-dependant effect of PMA and Gö6983 were established (data not shown). Representative light microscopy results of the wound-healing assay for the CMV control and S730AVACM-1 cDNA transfected cells treated with 10 and 100 nM PMA and 7 nM Gö6983 are shown in Fig. 9B. When data shown in Fig. 9B were quantitated, we observed a significant increase in cell growth in both groups treated with PMA. However the increase in S730AVACM-1 cDNA transfected RAMEC was significantly higher when compared to the control group (n=5, *p<0.05). Further, when PKC activity was inhibited with 10 nM Gö6983, cell growth was decreased in S730AVACM-1 cDNA transfected cells significantly more than in the control group (n=5, *p<0.05).

Discussion

In this study we report several new findings that enhance our understanding of the mechanism by which VACM-1/cul5 protein regulates cellular
growth. First, we confirmed that overexpression of VACM-1/cul5 cDNA in rat endothelial cell line inhibits cellular growth, whereas treatment of RAMEC with siRNA oligonucleotides targeting endogenous VACM-1/cul5 leads to an increased cell growth (Figs. 1-3). Second, we demonstrated that the subcellular localization of VACM-1/cul5 protein may be controlled by its posttranslational modifications (Fig. 4). Further, we showed that the nuclear but not cytosol or membrane-localized VACM-1/cul5 protein is modified by Nedd8 protein (Fig. 5). Third, we showed that neddylation of VACM-1/cul5 protein is controlled by PKA specific phosphorylation at S730 (Figs. 6-8). Finally, we found that stimulation of PKC activity with PMA induced cell proliferation significantly higher in cells transfected with S730A VACM-1 cDNA, when compared to the control where PKA-specific phosphorylation site was intact (Fig. 9). Our results suggests for the first time that neddylation of VACM-1/cul5, itself controlled by the PKA dependent phosphorylation, may regulate its subsequent phosphorylation by PKC.

Phosphorylation is a rapid and effective way to change cellular localization and the function of a protein (39). Both PKA and PKC specific phosphorylation regulates cellular function of many proteins in health and disease (30-33, 40). For example, PKA regulates nucleo-cytoplasmic shuttling of a transcription factor Id1 during angiogenesis (32) and is identified as an inhibitory component in the Gli protein translocation to the nucleus (33). Activation of PKA leads to the retention of Gli in the cytoplasm, while inhibition of PKA activity promotes its nuclear localization. Similarly, PKC dependent phosphorylation of specific proteins and its role in cellular processes like angiogenesis, a crucial step in tumor development, has been established (41). Further, the transition from the phosphorylated to the non-phosphorylated form of the Fas-associated death domain-containing protein, associated with carcinogenesis (42), has been used as a marker for cancer progression (43). Although the phosphorylation of proteasome’s subunits by specific kinases has been reported (12, 16), our results are the first to show that posttranslational modifications of VACM-1/cul5 protein by Nedd8 is controlled by PKA-specific phosphorylation.

VACM-1/Cul5 is the least conserved member of the cullin family and its biological significance is only emerging. To date, cul 5 dependent E3 ubiquitin ligase complexes have been shown to control the adenovirus-induced p53 degradation in vitro and the degradation of proteins essential for the prevention of HIV infectivity (44, 45). In vivo, expression of VACM-1/cul5 protein is largely endothelium-specific (8, 9). When expressed in several cell lines in vitro, VACM-1/cul5 inhibits MAPK phosphorylation and nuclear localization of egr-1, signaling molecules recognized for their role in the regulation of cellular proliferation (5, 9). These effects appear to be dependent on the state of VACM-1 modification by Nedd8 as in cells transfected with the S730A VACM-1 cDNA, phosphorylation of MAPK was directly correlated to the level of neddylated VACM-1 protein (5). Interestingly, Nedd8 conjugation to cullins is believed to be fundamental for the activity and stability of numerous E3 ligases (46). Further, it has been reported that cullins must be neddylated and form heterodimers to be an active component of the E3 ligase complex (14). This proposed model supports the dominant negative phenotype observed in S730A VACM-1 cDNA transfected endothelial cells in vitro (5). Importantly, a recent report targeting NEDD8-specific pathway for development of anti-cancer drugs (47), further underscores the significance of our findings.

In summary, this study shows that PKA dependent phosphorylation of VACM-1/cul5 regulates its neddylation and subsequent phosphorylation by PKC. Since the neddylation process is now a target for development of new drugs to regulate excessive cellular proliferation (47), understanding the underlying mechanism at the cellular level is critical.

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REFERENCES


Figure Legends

**Figure 1.** Overexpression of VACM-1 cDNA in rat endothelial cells, RAMEC inhibits cellular growth. A. A representative phase contrast microscopy showing decreased cell growth in VACM-1 transfected cells when compared to the CMV transfected cells at three days. RAMEC expressing endogenous VACM-1 transfected with CMV vector or VACM-1 cDNA were seeded at equal density and growth was monitored and quantitated as described in Experimental Procedures. B. Cells shown in A were counted in triplicate in six independent culture plates. Values are expressed as mean ± SE (* indicates p<0.05).

**Figure 2.** Downregulation of VACM-1/cul5 with anti-VACM-1 specific siRNA oligonucleotides induces cellular proliferation in RAMEC. A. Growth rates in RAMEC, expressing endogenous VACM-1/cul5 protein and transfected with three siRNA oligonucleotides targeting VACM-1/cul5 mRNA. All three anti-VACM-1 siRNA oligonucleotides significantly increased cell growth when compared to the control RAMEC at 24 hours. (n=3, *p<0.05). B. A representative phase contrast microscopy showing increased cell growth in RAMEC expressing endogenous VACM-1 protein and transfected with 0, 15 and 30 nM siRNA #2 (top panel). Anti-GAPDH siRNA (middle panel) or a scrambled siRNA (bottom panel) were used as controls.

**Figure 3.** VACM-1 specific siRNA oligonucleotides induce cell growth and attenuate VACM-1 protein expression in RAMEC. A. RAMEC seeded at equal density were transfected with a random siRNA (first panel), siRNA targeting GAPDH (second panel) and siRNAs targeting VACM-1, (# 2 and #3, third and fourth panels) and stained with methylene blue at 48 hours after transfection. B. Western blot analysis of RAMEC transfected with anti-VACM-1 specific siRNA oligonucleotides #1, #2 and #3 and anti GAPDH siRNA. Lysates were collected at 48 hours, quantitated for protein concentration and resolved by SDS-PAGE electrophoresis. After transfer to the nitrocellulose membrane, blots were probed with anti-VACM-1 specific antibody as described in Experimental Procedures. To ascertain equal protein loading, membranes were stripped and re-probed with anti-GAPDH Ab (not shown). C. The intensity of the signal shown in B was quantitated using Image J® as described in Experimental Procedures (n=4, *p<0.05). D. Time dependent effect of 15 nM siRNA #2 on VACM-1 protein expression at 12, 24 and 48 hours. In addition, VACM-1 protein concentration was examined in cells double transfected with the 15 nM siRNA #2 at 24 hours, and collected at 48 hours (48-db). E. A representative immunocytochemistry result demonstrating the effect of 15 nM siRNAs on VACM-1 protein expression at 48 hours after transfection. Magnification 40X. (n=3)

**Figure 4.** Cellular localization of endogenous VACM-1 protein in RAMEC is dependent on cell cycle. A-B. RAMEC grown to confluency on glass coverslips were “wounded” with a pipette tip and allowed to grow into the “wound.” Cellular growth was monitored microscopically. Cells were fixed and immunostained with anti-VACM-1 specific antibody as described in the Methods. B. A representative immunostaining of
different regions from the same cover slip shown in A: the immunostaining: in the wound (i), close to the wound (ii), and distant from the wound (iii). C. Western blot analysis of nuclear, membrane and cytosolic cell fractions collected from asynchronous RAMEC grown for 48 hours and resolved by SDS-PAGE, transferred to nitrocellulose and probed with anti-VACM-1 Ab. D. Signal intensity for VACM-1/cul5 protein and modified VACM-1/cul5 were quantitated and presented as a ratio of the upper to lower band. (n=3, *p<0.05).

**Figure 5.** Nedd8- specific modification of VACM-1/cul5 protein is localized to the nucleus. A. RAMEC grown to confluency on glass cover slips were “wounded” with a pipette tip and allowed to grow into the “wound.” Immunostaining experiments described in Fig. 4A were repeated using anti-Nedd8 specific Ab. The immunostaining in the wound and in a confluent region distant from the wound is shown. Magnif., 40X. B. Cells were fixed and immunostained with anti-VACM-1 specific Ab (green) and anti-Nedd8 specific antibody (red) as described in the Experimental Procedures. Nuclear staining was performed using DAPI (Magnif., 100x). B. Overlay of VACM-1/cul5 and Nedd8 proteins localization in the nuclear but not membrane region in control RAMEC.

**Figure 6.** Nedd8- specific modification of VACM-1/cul5 protein is regulated by PKA activity. A. Immunostaining of CMV and S730AVACM-1 cDNA transfected RAMEC with anti-Nedd8 antibodies showed increased Nedd8 signal in S730AVACM-1 cDNA transfected RAMEC when compared to controls. B. Removal of PKA phosphorylation site in VACM-1/cul5 protein (S730A ) sequence increases modification of VACM-1 by Nedd8. Lysates from S730AVACM-1 transfected cells were immunoprecipitated with the polyclonal anti-Nedd8 antibodies and probed with anti-VACM-1 Ab as described in Experimental Procedures. Total cell lysates from CMV transfected RAMEC were used a control. C. PKA dependent phosphorylation of VACM-1/cul5 is attenuated in S730AVACM-1cDNA transfected cells when compared to controls. CMV and S730AVACM-1 cDNA transfected RAMEC were treated with 10 μM FSK for 5 or 15 min. Total cell lysates (100 μg) were immunoprecipitated with anti-VACM-1 specific antibody and protein A/sepharose as described in Experimental Procedures. The immunoprecipitates were resolved by SDS-PAGE electrophoresis. After transfer to the nitrocellulose membrane, blots were probed with anti-phospho-PKA substrate specific Ab as described in Experimental Procedures. Lysate from CMV transfected RAMEC were used as a control. D. Signal intensity shown in C was quantitated and compared (n=3, *; p<0.2).

**Figure 7.** Nuclear localization of VACM-1 and Nedd-8 is regulated by PKA. A. Control cells treated with 10 μM FSK for at least 45 min, 2hrs and 4 hrs were fixed and immunostained with anti-VACM-1 specific antibody as described in the Experimental Procedures. Nuclear staining was monitored with DAPI. To show disappearance of nuclear localization of VACM-1 protein after treatment with FSK, the three signals were
overlayed. The edge of the “wound” area is at the top of the slide. B. Cells treated with 10 μM FSK for at least 45 min were fixed and immunostained with polyclonal anti-Nedd8 specific antibody as described in the Experimental Procedures. Nuclear staining was monitored with DAPI. To show disappearance of nuclear localization of VACM-1 protein after treatment with FSK, the signals were overlayed. The edge of the “wound” area is at the top of the slide.

Figure 8. Forskolin induced activation of PKA reduces nuclear localization of VACM-1 and Nedd8 in control but not S730AVACM-1 cDNA transfected cells. A. Control cells treated with 10 μM FSK for at least 45 min, were fixed and immunostained with anti-VACM-1 (green) and anti-Nedd8 (red) specific antibodies as described in the Experimental Procedures. Nuclear staining was monitored with DAPI. To show disappearance of nuclear localization of VACM-1 protein after treatment with FSK, the signals were overlayed. The edge of the “wound” area is at the top-left of the slide. B. Control and S730AVACM-1 cDNA transfected cells treated with 10 μM FSK for at least 45 min were fixed and immunostained as described above (A). To show disappearance of nuclear localization of VACM-1 protein after treatment with FSK, the signals were overlayed. The edge of the “wound” area is at the top-left of the slide.

Figure 9. Regulation of VACM-1/cul5 dependent cell growth by PKC. A. Total lysates from S730AVACM-1 cDNA transfected RAMEC were divided equally and incubated with either OKA (300 μM) or CIP (100 U/mL) for 30 min. Lysates were resolved by SDS-PAGE electrophoresis and after transfer to the nitrocellulose membrane, blots were probed with anti-VACM-1 specific antibody as described in Experimental Procedures. B. Wound healing assay from CMV transfected control and S730AVACM-1 cDNA transfected RAMEC. Confluent cell cultures were wounded with a pipette tip and cell re-growth was observed under inverted phase-contrast microscope (Nikon). Photographs were taken at time 0, and 16 hours after treatment with the vehicle, PMA (10 and 100 nM) and Gö6983 (7 nM), as described in Experimental Procedures. C. The effects of PMA and Gö6983 shown in A were quantitated. (n=5,*p<0.001).
**Figure 1**

**A.**

*RAMEC-CMV*  
*RAMEC-VACM-1*

**B.**

![Bar chart showing cell population (per cm²)](chart.png)

Cell Population (per cm²)
Figure 2
A.

B.
Figure 3

A. 48 hrs post-transfection

B. Control siRNA #1 siRNA #2 siRNA #3 GAPDH

C. Neg Control GAPDH siRNA VACM-1 siRNA #2

D. VACM-1/GAPDH 0.0 0.5 1.0 1.5 2.0 2.5

E. VACM-1 Expression

Control siRNA

siRNA #1

siRNA #2

siRNA #3
Figure 4

A. VACM-1 localization

C.

D.

B.
Figure 5.

A. Nedd8 localization

Wound Area  Confluent

B. VACM-1  Nedd8

C. VACM-1 and Nedd8 localization in CMV-RAMEC
Figure 6
A. 

B. 

C. 

D.
Figure 7

A.

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B.

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Legend:
- **Overlay**: CMI
- **DAPI**: Nedd8 Ab

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Figure 8

A.

Control  FSK-45'

VACM-1

Ned8

Overlay

B.

Control  Fsk 45'

CMV

VACM-1
Figure 9

A. Total Cell Lysate

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B. RAMEC

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C.

- Control
- PMA (10 nM)
- PMA (100 nM)
- G66983 (10 nM)

% Growth
Phosphorylation of VACM-1/cul5 by protein kinase A regulates its neddylation and antiproliferative effect
Shirley E. Bradley, Alyssa E. Johnson, Isabelle P. Le, Elizabeth Oosterhouse, Michael P. Hledin, Gabriel A. Marquez and Maria Burnatowska-Hledin

J. Biol. Chem. published online November 16, 2009

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