Aquaporin-2 Levels in vitro and in vivo are Regulated by VACM-1, a Cul 5 Gene

Isabelle P. Le, Sarah Schultz, Bradley T. Andresen, Gary L. Dewey, Ping Zhao, Laura Listenberger, Peter M. Deen, Abby Buchwalter, Christopher C. Barney, Maria A. Burnatowska-Hledin

Departments of Biology and Chemistry, Schaap Science Center, Hope College, Holland, MI 49422-9000; a Van Andel Research Institute, 333 Bostwick NE, Grand Rapids, MI 49503; c Department of Physiology, UMC Nijmegen Nijmegen, The Netherlands; d Contributed equally to the work reported in this manuscript

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Abstract
Background: In the renal collecting duct, vasopressin regulates water permeability by a process that involves stimulation of adenyl cyclase activity, cAMP production and subsequent translocation of water channel aquaporin-2 (AQP2) into the apical plasma membrane. We have previously shown that in cos 1 cells in vitro, both adenyl cyclase activity and cAMP production can be regulated by VACM-1, a cul 5 gene that forms complexes involved in protein ubiquitination and subsequent degradation. Methods: To extend these observations further, the effects of changes in hydration state on the expression of VACM-1 at the mRNA and the protein level were examined in rats deprived of water (WD) for 24 hrs. Results: In the kidney of WD rats Western blot analyses of kidney tissue showed that the decrease in VACM-1 protein concentration was correlated with the increase in the AQP2 protein level. The immunostaining data suggested that VACM-1/cul5 may be decreased in renal collecting duct but increases in the vasculature of the inner medullary region in response to WD. To determine the possible consequences of the WD dependent decrease in VACM-1/cul5, we next examined the effects of VACM-1 expression on AQP2 protein in vitro. Immunocytochemistry and Western blot analyses data indicate that VACM-1/cul5 expression in MDCK line stably expressing AQP2 gene and in cos 1 cells co-transfected with the AQP2 and VACM-1/cul5 cDNAs decreased AQP2 protein concentration when compared to the vector transfected control groups. Conclusion: In summary, our data demonstrate that VACM-1 is involved in the regulation of AQP2 protein concentration and may play a role in regulating water balance.
Introduction

In the renal collecting duct, vasopressin (AVP) regulates water permeability by a process that involves stimulation of adenylyl cyclase activity, cAMP production and subsequent translocation of the water channel aquaporin-2 (AQP2) into the apical plasma membrane [1-8]. These processes can be regulated at the transcriptional, translational and posttranslational levels by factors that include changes in cytosolic free calcium (Ca\(^{2+}\)), mitogen activated protein kinase (MAPK) activity and TonEBP protein induction in response to hypertonicity [7, 8]. The translocation of AQP2 to the membrane and its subsequent endocytosis processes via clathrin coated pits involve reorganization of actin cytoskeleton, interactions with HSP70, and the activation of the Rho protein [3, 9-11]. Ultimately, AQP2 is targeted for ubiquitin-dependent degradation but the underlying mechanism is not well understood [12, 13] and the specific ubiquitin ligases involved in the process have not been identified.

We have previously shown that in cos 1 cells \textit{in vitro} adenylyl cyclase activity, cAMP production and attenuation of MAPK phosphorlylation can be regulated by overexpression of VACM-1 (Vasopressin-Activated Calcium-Mobilizing) cDNA independent of AVP binding [14-17]. VACM-1 is now recognized as a member of the cullin protein family (cul5) that function as a “core scaffold” for a subclass of ubiquitin E3 ligases (Cullin-Ring Ligases, CRLs) involved in protein ubiquitination, and ultimately, degradation [18, 19]. The best characterized substrate for the cul5-specific CRL activity is the APOBEC3G protein essential in the prevention of HIV infectivity [20]. To date, VACM-1/cul5-CRLs have also been implicated in the degradation of non-receptor tyrosine kinase Src, E-cadherins, iNOS and the cytokine signaling proteins [20-22]. The cul5-based CRLs use elongins B/C as adaptor proteins and their numerous substrate recognition proteins include Dab1, ASB2, XRAB40, Hsp90 and Hsp70 and the viral proteins vif and E4orf6 [19, 21-24]. Clinically, mutations in cul5 sequence have been associated with the level of HIV infectivity [25] and VACM-1/cul5 expression was induced in patients in response to peptide C used in treatment of diabetic nephropathy [26], implying novel and diverse biological roles for this protein.

Since \textit{in vivo} VACM-1/cul5 protein localizes to the vascular endothelial cells and to the collecting tubule cells in the kidney [27], structures recognized for the control of body water homeostasis [1-6], we hypothesized that VACM-1/cul5-based E3 ligases may be involved in the control of the degradation of proteins involved in the maintenance of water balance. A recent report by Lee et al. [28] suggests that \textit{in vivo} the expression of cul5 may indeed be controlled by hydration state. Further, our recent work implicated VACM-1/cul5 in the control of AQP1 concentrations both \textit{in vivo} and \textit{in vitro} [29]. Specifically, we demonstrated that overexpression of VACM-1/cul5 cDNA \textit{in vitro} attenuated the expression of AQP1 and \textit{in vivo} water deprivation increased vascular expression of VACM-1/cul5 and affected its posttranslational modification by ubiquitin-like protein, Nedd8 [29]. Although water deprivation did not affect AQP1, AQP1 concentration was inversely correlated with the ratio of VACM-1 to Nedd8 modified VACM-1 in vascular tissue.

Consequently, in this study we examined whether changes in hydration state affect expression of VACM-1 in the kidney \textit{in vivo}. In addition, we investigated the effect of VACM-1 protein expression on AQP2 levels \textit{in vitro}. Our data demonstrate that in kidneys isolated from water deprived rats, changes in VACM-1 protein levels may be region-specific and are inversely correlated with AQP2 protein levels. Immunocytochemistry and Western blot analyses data indicate that VACM-1 expression in cos 1 cells co-transfected with the AQP2 cDNA and in MDCK line stably expressing AQP2 gene decreased AQP2 protein concentration when compared to the vector transfected control groups. The VACM-1 receptor, therefore, may represent a unique protein involved in the regulation of AQP2 dependent cellular permeability.
Materials and Methods

Materials
All tissue culture media and reagents were purchased from Gibco (Grand Island, NY). 32P-ATP was purchased from DuPont-New England Nuclear (Boston, MA). Restriction enzymes were purchased from Promega Co. (Madison, WI). The AQP2 cDNA obtained by RT-PCR from rat kidney mRNA was subcloned into the Eco RI/Sma I site in the pSV-Sport1 vector and its sequence was confirmed (Sequi-net, Colorado State Univ, Ft. Collins, Co).

Tissue Culture
AQP2-expressing MDCK cells [30] and cos 1 cells transfected with AQP2 were grown in DMEM and high glucose DMEM media, respectively, supplemented with 10% FBS at 37°C under a water-saturated 5% CO2 atmosphere as described previously [15]. Cells were subcultured for 24 hr before transfection at a density of 6 x 10^5 cells per 100 mm plate. Both, cos 1 and MDCK cells were transfected with VACM-1 cDNA [15] subcloned into the Sal I/Not I restriction site in the polylinker region of pBK-CMV vector (Stratagene Co., La Jolla, Ca) at 0.1 - 5 µg DNA per 100 mm culture dish using the FuGENE 6 (Roche Diagnostics Inc., Indianapolis, IN) and/or with AQP2 cDNA. When appropriate, cells were treated with 10 µM Forskolin for 15 min and lysates collected as described below.

Total Cell Lysate Preparation
Cells were grown to 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 mM 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).

Northern blot /Slot blot analysis
Poly A+ mRNA was isolated by Fast Track™ method as described previously [14]. For Northern blot analysis, 10 µg of polyA+ mRNA were separated on a 1% agarose gel containing 16% formaldehyde, transferred to a nitrocellulose membrane, and fixed by baking under vacuum at 80 °C for 1 hr. For slot blot analyses, 10 µg of polyA+ mRNA was loaded per slot under vacuum and fixed by baking under vacuum at 80 °C for 1 hr. All mRNA blots were prehybridized for 1 hour at 42 °C in a solution containing 50% formamide, 2X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5x Denhardt’s solution, 0.1% SDS and 100 µg/ml of denatured salmon sperm DNA/ml. The hybridization was carried out for 24 hours at 42 °C in 50% formamide, 2X SSC, 1x Denhardt’s and 100 µg of denatured salmon DNA/ml and 2 x 10^6 cpm per ml of the 32P-labeled Eco RI/Bcl I fragment of VACM-1 cDNA (nucleotides 355-1245). Following hybridization, the blot was washed twice in 1X SSC, 0.1% SDS at room temperature and twice in 0.1X SSC, 0.1% SDS at 45 °C (30 min each) and then examined by autoradiography. The blot was then stripped and reprobed with GAPDH cDNA fragment to confirm the concentration of mRNA in each lane.

Immunostaining
Kidney issue sections were fixed in 3% paraformaldehyde (in 1xPBS, pH 7.4), washed in PBS, permeabilized with 5% Tween-20 solution for 20 min, washed with PBS/2% BSA, and incubated with antibody B (Ab-B, see below) diluted in PBS containing 0.1% BSA (PBS/BSA) for 1 hr as described previously [14, 27]. Affinity purified polyclonal antibodies directed against the amino (Ab-A) and the carboxy (Ab-B) termini of the rabbit VACM-1 protein and previously characterized were used. To identify collecting duct (CD) cells, tissue sections were co-immunostained with RTCC antibody [31]. Cos 1 and MDCK cells, grown on coverslips, were fixed in 3% paraformaldehyde (in 1xPBS, pH 7.4), washed in PBS, permeabilized with 5% Tween-20 solution for 20 min, washed with PBS/2% BSA and incubated with a specific antibody diluted in PBS containing 0.1% BSA (PBS/BSA) for 1 hr. A 1:200 dilution of anti-AQP2 antibody was used as described by the manufacturer (Alomone Labs, Jerusalem, Israel). Cell cultures were washed in 1X PBS/2% BSA, mounted with Aqua Polymount (Polysciences Inc., Warrington, PA) and viewed by epifluorescence microscopy (BH2, Olympus Corp. Lake Success, NY).
**Western Blot Analysis**

Tissue samples prepared as described previously [14] were resuspended in 2X sample buffer (Biorad Co., Hercules, CA), heated to 95°C for 4 min, and subjected to SDS polyacrylamide gel electrophoresis (PAGE) using a 4-12% running gel. The separated proteins were transferred to nitrocellulose membranes at 30 mV overnight. Nonspecific sites were blocked by incubation of membranes with PBS containing 5% nonfat dry milk and 0.2% Tween-20 at room temperature for 30 min. Membranes were then incubated for 2 hrs at room temperature in ECL solution (20 mM Tris pH 7.5, 135 mM NaCl and 0.05% Tween) containing a specific antibody. A 1:200 dilution of AQP2 antibody was used as recommended by the manufacturer. Affinity purified polyclonal antibodies directed against the amino (Ab-A) and the carboxy (Ab-B) termini of the rabbit VACM-1 protein characterized previously [14-16] were used at 1:200 dilution. To ascertain antibodies specificity against rat tissue, rat VACM-1 cDNA sequence was first determined by RT-PCR. Our results indicate that both the amino and the carboxy termini of VACM-1 are identical in sequence to the rabbit VACM-1 sequence (unpublished observation). Our antibody was further confirmed using rat tissue samples [29]. After incubation with the antibodies, the membranes were washed in the ECL solution for 15 min and twice for 5 min and then incubated for 2 hours with a 1:2,000 or 1:10,000 dilution of a horseradish peroxidase (HRP) conjugated anti-rabbit antibody, depending on date of purchase (Cell Signaling Technology Inc., Beverly, MA). The nitrocellulose membranes were washed as described above, exposed to the luminal detection reagents (Cell Signaling Technology Inc., Beverly, MA) for one minute, and then exposed to the X-ray film.

**Animals**

All animals were housed individually in stainless steel cages in an animal facility at an ambient temperature of 24°C and with lights on from 0700 to 1900 hours. Animals were allowed standard commercial chow and water ad libitum except during water deprivation periods when water, but not food, was withheld.

Male Sprague Dawley rats (n = 16, weight range 198-252 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The rats were divided into a control group of 8 rats and a group of 8 rats deprived of water for 24 hours prior to sacrifice. Rats were anesthetized with methoxyflurane, a 6 ml blood sample was taken by cardiac puncture, following which they were euthanized by an intracardiac injection of Socumb solution (0.5 ml). Kidneys were removed and rinsed in an ice-cold phosphate buffer. The samples for RNA and protein analysis were frozen in liquid N₂. The samples for immunocytochemistry were placed in Tissuetek® and frozen in n-butanol chilled to -70°C over a dry ice/isopentanol bath. All tissues were stored at -80°C. The experiments were approved by the Hope College Animal Care and Use Committee.

**Data Analysis**

A Kodak Digital Science 1D LE 3.0 was used to scan the Western blot results and NIH-Scion Imaging program was used for analysis (http://rsb.info.nih.gov/ij/index.html). As appropriate, either ANOVA followed by Tukey’s test for multiple mean comparisons or a paired Student’s t test was used for data analysis. Significance was set at p<0.05.

**Results**

To identify physiological factors that may regulate the expression of VACM-1/cul5 in the kidney, we examined the effect of water deprivation on VACM-1/cul5 concentrations. Rat kidney samples were examined by Northern blot, Slot-blot and Western blot analyses. Northern blot analysis of rat kidney mRNAs probed with a 1.7 kb fragment of VACM cDNA identified a 6.4 kb species of mRNA in rat kidney (Fig. 1A), which corresponds in size to VACM-1 mRNA identified in rabbit and in human [14, 18]. To demonstrate the presence of mRNA in the samples, the blot was reprobed with a GAPDH cDNA probe (Fig. 1A, lower panel). Subsequently, a slot blot approach was used to analyze kidney mRNA levels. Again, to correct for uneven mRNA loading, all blots were stripped and reprobed with anti-GAPDH mRNA probe, and data are expressed as a ratio of VACM-1 mRNA to GAPDH mRNA. As shown in Fig. 1B, kidney VACM-1 mRNA levels significantly (p<0.05) decreased in WD rats when compared to the control group. To examine if changes in VACM-1 mRNA concentration...
following WD could be confirmed at the protein level, we performed Western blot analysis of the kidney tissue samples. Our results demonstrated lower VACM-1 protein concentrations in WD rats when compared to controls (Fig. 1C-upper panel). Since kidney AQP2 mRNA levels are regulated by the hydration state of the organism [1-10], we reprobed our blot with anti-AQP2-specific Ab. As anticipated, 24 hr WD significantly (p<0.05) increased AQP2 concentration (Fig. 1D). The ratio of AQP2 to VACM-1 protein level was significantly increased in the WD group when compared to control. Further, the increases in AQP2 levels were inversely correlated with VACM-1 concentrations in both control and WD groups (Fig. 1E, r=0.96, n=4 each group, P<0.005, all data). The immunocytochemistry results suggested that VACM-1 protein expression in the collecting tubules, identified by co-immunostaining with RTCC antibody that is specific against those cells [31], was decreased in the WD group when compared to controls (Fig. 1F). Results from the immunostaining studies also suggested that in a WD state VACM-1 protein expression may be increased in the medullary vascular structures and the glomeruli (Fig. 1G-H).
To explore the effect of VACM-1 on AQP2 further, we examined whether VACM-1 expression had a similar effect in a MDCK line expressing exogenous AQP2 and the V2 receptor \[30\]. Transfection of MDCK cells with VACM-1 cDNA resulted in significantly (p<0.05) decreased AQP2 concentrations when compared to the CMV-vector transfected cells (Fig 2A-B). Subsequent immunostaining results, shown in Fig. 2C, indicate that transfection of MDCK cells with 2 µg VACM-1 cDNA reduced AQP2 protein expression. These immunostaining results also suggest that in VACM-1 cDNA transfected MDCK cells, AQP2 levels and its localization to the cell membrane in forskolin treated cells (10 µM for 15 min) were also attenuated (Fig. 2C). Interestingly, transfection of MDCK cells with a mutated VACM-1 cDNA (S730A VACM-1 cDNA) previously shown to reverse cos 1 and rat endothelial cell phenotype \[15, 16\] did not affect AQP2 levels in either control or Forskolin treated groups.

To further examine this inhibitory effect of VACM-1 expression on AQP2 protein levels, we examined the effects of VACM-1/cul5 on AQP2 expression in cos 1 cells transfected with AQP2 cDNA. Rat AQP2 cDNA was generated by RT-PCR from kidney mRNA, subcloned into an expression vector (pSV-Sport1) and its sequence was confirmed (data not shown). The effect of VACM-1 cDNA co-expression on AQP2 protein levels was then examined using immunocytochemistry and Western blot approaches. The data from these experiments, shown in Fig. 3, suggest that transient expression of VACM-1 at 2 µg cDNA/plate almost completely inhibited AQP2 protein expression (Fig. 3A, C). When signal intensities from these experiments were quantitated, there was a significant (p<0.05) decrease in the intensity of the signal in the AQP2 cDNA transfected cells when compared to the controls, whereas no change in the cell number was observed (Fig. 3B, DAPI stain). The effect of VACM-1 expression on AQP2 protein level was confirmed by the Western blot analyses (Fig. 4 A-B). As in the MDCK cells, in cos 1 cells transfected with AQP2, the expression of VACM-1/cul5 following
transfection with 2 µg VACM-1 cDNA decreased AQP2 protein levels (Fig. 4A) while this effect was not seen following transfection with 2 µg S730A/VACM-1 cDNA. Quantification of the Western blots (Fig. 4 B) demonstrated that the reduction of AQP2 induced by VACM-1 expression was significant (p<0.05). Immunostaining of the cos 1 cells for AQP2 confirmed the findings from the Western blot experiments (Fig. 4C). In addition, the increase in AQP2 in response to forskolin treatment was attenuated in AQP2 and VACM-1/cul5 cDNAs co-transfected cells, but not in cells co-transfected with AQP2 and S730A/VACM-1 cDNAs.
Discussion

To ascertain the involvement of VACM-1/cul5 in the control of water and solute reabsorption, we first examined whether water deprivation, which is known to regulate AQP2 levels [1-10], would affect VACM-1/cul5 expression in the kidney. We observed a significant decrease in VACM-1 mRNA and protein levels in the kidneys from the WD rats when compared to control rats (Fig. 1A-B). In agreement with previous reports [1-6], the expression of AQP2 was significantly increased in the kidneys from WD rats when compared to controls (Fig. 1C-D). Interestingly, there was a significant, inverse correlation between VACM-1/cul5 and AQP2 levels in kidney samples from both control and WD rats (Fig. 1E).

The immunocytochemistry data presented here suggest that the expression of VACM-1/cul5 in the collecting tubules may decrease whereas its levels may increase in the vasculature of the glomeruli and the inner medullary region (Fig. 1F-H). Unlike in other tissues, where VACM-1/cul5 protein expression is largely endothelial, in the kidney VACM-1/cul5 protein is expressed in the glomeruli, vascular endothelial cells and in collecting tubule cells [27].

Our recent work suggests that in rats, 24 hr water deprivation increased vascular VACM-1/cul5 concentration in the mesenteric arteries and in skeletal and heart ventricle tissues [29]. Whereas AQP1 concentrations in rat vasculature were not affected by water deprivation, we saw an inverse correlation between AQP1 concentration and the ratio of VACM-1 to Nedd8 modified VACM-1. It is possible therefore, that regulation of VACM-1/cul5 expression in vivo is cell type specific and in the water deprived state VACM-1/cul5 expression may be increased in renal vasculature and decreased in the collecting tubule cells. A recent report by Lee et al. [28], also suggests regional difference in VACM-1/cul5 protein expression may be increased in the glomeruli, vascular endothelial cells and in collecting tubule cells [27].

The observation that expression of VACM-1/cul5 protein may be regulated differently in the collecting tubule and in the vascular structures is not unique to this system. The expression of proteins thought to play a role in water balance, often altered by water deprivation, is “regionally” specific [33-36]. When different kidney regions of the rat were examined, it was found that progressive water deprivation decreased V₂ mRNA in the cortex and in both the inner and outer medulla [33]. The V₂ protein levels, however, decreased in the cortex and the outer medulla but did not change in the inner medulla. Further, water deprivation reduced vasopressin binding and V₂ mRNA levels in rat kidneys [33]. In contrast, water deprivation increased the expression of the angiotensinogen gene and the binding of both angiotensin II and atrial natriuretic peptide in the brains and other organs of rats [35, 36].

The regional differences in VACM-1 expression may also explain the observation that the decrease in VACM-1 mRNA levels in kidney from WD rats when compared to controls, contradicts a report that kidney VACM-1 mRNA is increased after 24 hr WD [37]. However, examination of the methods in that paper revealed that the RT-PCR reaction primers used by these investigators targeted the carboxy-terminus of VACM-1 cDNA that encompasses the sequence beginning at nucleotide 2420 of VACM-1. The cRNA probe used in our study is directed against the N-terminus of VACM-1 cDNA sequence. We have observed that a truncated form of VACM-1 protein lacking the N-terminus sequence of VACM-1 protein may be expressed in the kidney [38]. Whether the levels of the truncated form of VACM-1 are
increased in specific regions of the kidney and are controlled by the hydration status remain to be determined.

Using two different approaches (Figs. 2-4), we also obtained data providing the first evidence that VACM-1 protein attenuates basal AQP2 protein concentrations when expressed in vitro. The inhibitory effect of VACM-1/cul5 on AQP2 protein levels was observed both in MDCK cells stably expressing AQP2 and in cos 1 cells transfected with AQP2 gene. In both cell lines, the expression of VACM-1/cul5 may also compromise the forskolin-dependent translocation of AQP2 to the membrane. Interestingly, the expression of S730A VACM-1 cDNA, which reverses the inhibitory effect of VACM-1 on MAPK phosphorylation and cell growth [15] and compromises actin polymerization in rat endothelial cell line in vitro [16], did not affect AQP2 concentration in either the MDCK or cos 1 cells transfected with AQP2.

It is possible that VACM-1/cul5-dependent E3 ligase controls AQP2 indirectly through proteins that regulate its posttranslational modifications, interactions with cellular proteins, subcellular localization and function [4-6, 9-13]. For example, actin microfilaments involved in AVP and cAMP-increased AQP2 translocation to plasma are associated with Rab GTPases, the key regulators of intracellular AQP2 trafficking [4, 9, 10] under control of CRLs [19, 23, 39]. Similarly, during its re-internalization via clathrin-coated pits, AQP2 that had accumulated in membrane after AVP treatment associates with HSP70, which behaves as a client protein for CRL dependent E3 ligases [11, 23, 40]. Together, with our previous work indicating that VACM-1 regulates adenylyl cyclase activity and production of cAMP [15], these results suggest that VACM-1/cul5 dependent CRLs regulate a water-homeostasis pathway at several levels.

Whereas the link between VACM-1/cul5 and the AVP-binding [14] remains unresolved, it is possible that AVP mimics an allosteric regulation where a ligand binds at one site and induces a conformational change at a distant location that can cause changes in the conformation of the active site and thus control protein-protein interactions [41]. For example, vasopressin induces USP10 (ubiquitin specific protease 10) in mouse CCD cells and its interaction with USP10 deubiquitinates sorting nexin 3 [42] and results in stabilization of sodium channel ENaC in the membrane [42, 43]. Importantly, vasopressin can regulate ENaC translocation to the membrane by a mechanism that may control channel ubiquitination [44].

In summary, our data present the first evidence indicating that AQP2 may be under direct control of a system involving VACM-1/cul5 gene product. Since all cullins characterized to date form complexes with the ubiquitin proteasome components involved in protein degradation [19, 32], and the ubiquitination process is responsible for AQP2 degradation [12, 39], the VACM-1 dependent decrease in AQP2 may involve ubiquitin-dependent protein degradation that uses VACM-1/cul5 as a critical component in E3 ligase formation. As mammalian cells may contain hundreds of E3 ligases, identification VACM-1/cul5 as a specific subunit of the system that is expressed in the endothelium and in collecting tubules, structures known for their control of cellular permeability, may have significant implications when designing studies to elucidate the mechanism of water conservation. Thus, the effect of VACM-1 on cAMP signaling and on AQP2 levels in vitro, along with the hydration dependent changes in the expression of VACM-1 in the kidney in vivo, provide further evidence in support for a physiological role of VACM-1 protein in the regulation of water homeostasis.

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