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T47D breast cancer cell growth is inhibited by expression of VACM-1, a cul-5 gene☆

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Abstract

Vasopressin-activated calcium-mobilizing (VACM-1), a cul-5 gene, is localized on chromosome 11q22-23 close to the gene for Ataxia Telangiectasia in a region associated with a loss of heterozygosity in breast cancer tumor samples. To examine the biological role of VACM-1, we studied the effect of VACM-1 expression on cellular growth and gene expression in T47D breast cancer cells. Immunocytochemistry studies demonstrated that VACM-1 was expressed in 0.6–6% of the T47D cells and localized to the nucleus of mitotic cells. Overexpressing VACM-1 significantly attenuated cellular proliferation and MAPK phosphorylation when compared to the control cells. In addition, VACM-1 decreased egr-1 and increased Fas-L mRNA levels. Further, egr-1 protein levels were significantly lower in the nuclear fraction from VACM-1 transfected cells when compared to controls. These data indicate that VACM-1 is involved in the regulation of cellular growth.
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Vasopressin-activated calcium-mobilizing (VACM-1) receptor was originally identified as a cell membrane protein that binds arginine vasopressin (AVP) [1], regulates Ca^{2+}, and attenuates cAMP-dependent signaling [1,2]. VACM-1, however, does not share sequence homology with the traditional AVP receptors [1]. Rather, it is homologous in sequence with cullins, a highly conserved multigene family of intracellular proteins identified in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Homo sapiens*. These proteins appear to have a rather ubiquitous tissue distribution and diverse biological roles [3–5]. It is now apparent that VACM-1 is the mammalian cul-5, as they share 96% sequence homology at the carboxy termini (157/163 aa) [3,4].

The cullins' gene products associate with distinct ubiquitin ligase complexes involved in the regulation of diverse cellular functions. For example, the cul-1 gene product and its yeast homolog, cdc53p, are involved in the ubiquitin–proteosome complex mediated degradation of cell cycle control proteins [6–10]. The cul-2 gene product associates with a cellular protein complex composed of the VHL gene product and elongins B and C (VBC), which prevents elongins B and C from associating with elongin A, and thus inhibits activation of transcription and, ultimately, tumor growth [11–13]. The biological role of recently cloned Hs-cul-3 and Hs-cul-4 genes expressed in cultured colon cancer cells [14] and primary breast cancer cells [15], respectively, is not clear, but cul-4A may be important for early embryonic development [16].

Little is known about cul-5, which only recently has been shown to form complexes with ligases involved in

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IF2 and p53 protein ubiquitination and degradation [17,18], and which may be biologically distinct from the other cullins [3,19]. Interestingly, the human VACM-1 gene localizes to a region close to the gene for ataxia telangiectasia (ATM) on chromosome 11q22-23 that has been previously associated with a loss of heterozygosity in breast cancer tumor samples [3]. VACM-1 gene proximity to this region, and its sequence homology to cell cycle regulatory cullins, suggests that it may be a possible tumor suppressor gene [3].

Our work characterizing VACM-1 protein clearly distinguishes VACM-1 from other AVP receptors and from cullins characterized to date [1,2,20,21]. Unlike cul-1, which appears to be rather ubiquitous [4,5], VACM-1 protein expression in vivo localizes exclusively to the vascular endothelial cells and to the renal collecting tubule segment responsible for the AVP-dependent regulation of water transport [20]. Furthermore, whereas cullins remain stable throughout the cell cycle, VACM-1 protein expression in vitro is cell cycle dependent [21]. Also unlike cullins, which are either nuclear and/or cytosolic, VACM-1 localizes to either the cell membrane or to the nucleus. In nonproliferating and in arrested (G0) endothelial cells (rat adrenal medullary endothelial cells, RAMEC), VACM-1 expression localizes largely to the cell membrane, and is undetectable during the S phase. As cells enter mitosis, nuclear localization becomes apparent again and at the completion of cytokinesis it translocates to the cell membrane [21]. Neither the mechanism nor the significance of the VACM-1 cell cycle-dependent localization is clear. However, only when localized to the cell membrane does VACM-1 regulate AVP-dependent changes in Ca^{2+} [21]. Finally, when overexpressed in cos-1 cells, VACM-1 expression attenuates growth, inhibits cAMP production, prevents MAPK phosphorylation, and induces expression of p53 [22], pathways well recognized for their role in the regulation of cellular growth [23–25].

We reasoned therefore that if VACM-1 regulates cell cycle transitions and cellular signaling pathways involved in growth regulation, its expression and/or activity would likely be reduced or absent in rapidly proliferating cells. Consequently, we examined the expression and biological effects of VACM-1 in breast cancer derived cell line T47D [26]. In addition, we screened a human Path Finder-1 GEArray system, using cRNA probes from T47D cells stably transfected with a vector or VACM-1 cDNA, respectively. The data reported here indicate that only a small fraction (0.6–6%) of asynchronous T47D cells express endogenous VACM-1 protein. VACM-1 protein localization in T47D cells appears to be transient and cell cycle dependent. Overexpression of VACM-1 in T47D cells significantly attenuates growth and MAPK phosphorylation. In addition, VACM-1 attenuated expression of egr-1 and induced that of Fas-L mRNAs. Western blot analyses demonstrated that egr-1 protein levels were significantly lower in the nuclear fraction from VACM-1 transfected cells when compared to the controls. These data indicate that in T47D cancer cells, VACM-1 is involved in the regulation of cellular growth by a mechanism that controls MAPK phosphorylation and egr-1 gene expression.

Materials and methods

Materials. All tissue culture media and reagents were purchased from Gibco (Grand Island, NY). [^{32}P]ATP was purchased from DuPont-New England Nuclear (Boston, MA). The restriction enzymes were purchased from Promega (Madison, WI). pBK-CMV vector (Stratagene, La Jolla, CA) was used to subclone VACM-1 cDNA for stable transfections.

Cell culture. T47D cancer cells [26] were grown in RPMI with L-glutamine, 10% or 0.2% FBS, 7.32 μg/ml insulin, and 0.1 μg/ml penicillin/streptomycin at 37 °C in a water-saturated 5% CO_2 atmosphere. Cells were subcultured for 24 h before transfection at a density of $6–8 \times 10^5$ cells/100 mm plate. Cells were transfected with 5 μg VACM-1 cDNA/100 mm culture dish using FuGENE 6 (Roche Diagnostics, Indianapolis, IN). 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/*Not*I restriction site in the polylinker region of pBK-CMV vector. After 24 h, cells were split and incubated with fresh medium containing 600 μg/ml G418 (Gibco-BRL, Gaithersburg, MD). The medium was changed every 3 days. Two weeks after transfection, G418 resistant cells were harvested and transferred into six-well plates containing selective medium (250 μg/ml G418). Twelve clones were propagated, but several failed to progress beyond the first division. Ultimately, seven clones were isolated and characterized. T47D cells were synchronized using thymidine (2 mM) arrest, and RAMEC were arrested in the G1/G0 phase using aphidicolin (1.25 μg/ml) [27]. For the immunostaining studies, RAMEC were grown on untreated glass coverslips placed in six-well plates as described previously [21].

Northern blot analysis. Poly(A)$^+$ mRNA was isolated by the C Fast Track method. Varying concentrations of poly(A)$^+$ 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 h. The mRNA blot was prehybridized for 1 h at 42 °C in a solution containing 50% formamide, 2× SSC, 5× Denhardt's solution, 0.1% SDS, and 100 μg/ml of denatured salmon sperm DNA/ml. The hybridization was performed for 24 h at 42 °C in 50% formamide, 2× SSC, 1× Denhardt's, and 100 μg of denatured salmon DNA/ml and 2×10^6 counts/ml of the ^{32}P-labeled *Eco*RI/*Bcl*I fragment of VACM-1 cDNA (nucleotides 355–1245). Following hybridization, the Northern blot was washed twice in 1× SSC and 0.1% SDS at room temperature and twice in 0.1× SSC and 0.1% SDS at 45 °C (30 min each) and then examined by autoradiography. Blots were reprobed with GAPDH to standardize for equal RNA loading [1].

Cell membrane and nuclear fraction preparation. T47D cells were grown to approximately 70% confluency, washed with ice-cold PBS, and transferred into 5 ml PBS containing 1 μg aprotinin/ml. Cells were centrifuged at 1500 RPM for 5 min and the pellet was resuspended in 0.5 ml NP40 buffer, 1 mM Pefabloc SC (Boehringer–Mannheim, Germany), and 1 μg/ml aprotinin (Sigma, St. Louis). The suspension was vortexed gently, incubated on ice for 5 min, and centrifuged at 1100–1200 RPM for 4 min. The resulting nuclear pellet was resuspended in NP40 buffer, centrifuged at 1100–1200 RPM for 4 min at 4 °C and resuspended in 100 μl NP40 buffer. The supernatant was homogenized with a polytron homogenizer, the membranes were centrifuged at 45,000 RPM at 4 °C for 30 min, and the cellular membrane pellet was

resuspended in 100 μl of membrane buffer. Protein concentration was determined by the Bradford method (Bio-Rad).

Western blot analysis. Total cell lysates, cellular membranes, and nuclear fractions prepared as described above were resuspended in 2× sample buffer (Bio-Rad), 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 nitrocellulose membranes at 30 mV overnight. Nonspecific sites were blocked by membrane incubation for 30 min at room temperature with PBS containing 5% nonfat dried 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 either the N-terminus (Ab-A) or the C-terminus (Ab-B) of VACM-1 protein [1]. Antibody against egr-1 protein was used at 1:500 dilution. Next, the nitrocellulose membranes were washed in the same buffer for 15 min and twice for 5 min and then incubated for 2 h with a 1:10,000 dilution of a horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Amhersham, Piscataway, NJ). The nitrocellulose membranes were washed again as described above, exposed to the luminol detection reagents (Cell Signaling, Beverly, MA) for 1 min, and then exposed to the X-ray film (Amhersham). To correct for equal protein loading, blots were reprobed with anti-α-actin antibody (Sigma, St. Louis, MO) [1].

Immunostaining. Affinity purified polyclonal antibodies directed against either the N-terminus (Ab-A) or the C-terminus (Ab-B) of VACM-1 protein, characterized previously [1,2], were used to stain cells grown on coverslips by indirect immunofluorescence. Cells grown on coverslips were fixed in 3% paraformaldehyde (in 1× PBS, pH 7.4), 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, in PBS containing 0.1% BSA (PBS/BSA) for 1 h. Cells stained with Ab-B were first permeabilized with 5% Tween 20 solution for 20 min, washed with PBS/2% BSA, and exposed to Ab-B as described for Ab-A. The primary antibodies were detected by incubating the sections in the presence of 1:80 dilution of FITC-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) in 1× PBS/2% BSA for 1 h. The nuclear staining was achieved by DAPI found in the Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and viewed by epifluorescence microscopy (BH2, Olympus, Lake Success, NY).

Cellular proliferation assays. T47D cells were seeded in 24-well plates at equal densities (2×10^4 cells/ml) in 10% FBS. After 24 h, media were changed and cells were allowed to grow for 72 h. 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 the nuclei visualized under epifluorescence microscopy were counted.

Microarray assay. Human PathwayFinder-1 GEArray Kit obtained from SuperArray (Bethesda, MD) was probed with biotin-16-dUTP-labeled VACM-1 cRNA fragment derived from control and VACM-1 transfected cell mRNA. Blots were exposed to the AP-streptavidin chemiluminescent detection system as described in the manufacturer's instructions.

Statistical analysis. Data are expressed as means ± 1 standard error (SE) of the mean. Student's t test was used for data analysis. Significance was set at $p < 0.05$.

Results

An approximately 6.4 kb mRNA species was identified in T47D (Fig. 1A) by Northern transfer blot analysis using a probe derived from VACM-1 cDNA (*Eco*RI–*Bcl*I restriction fragment containing nucleotides 355–1245). A protein of approximate M_r 81 kDa size

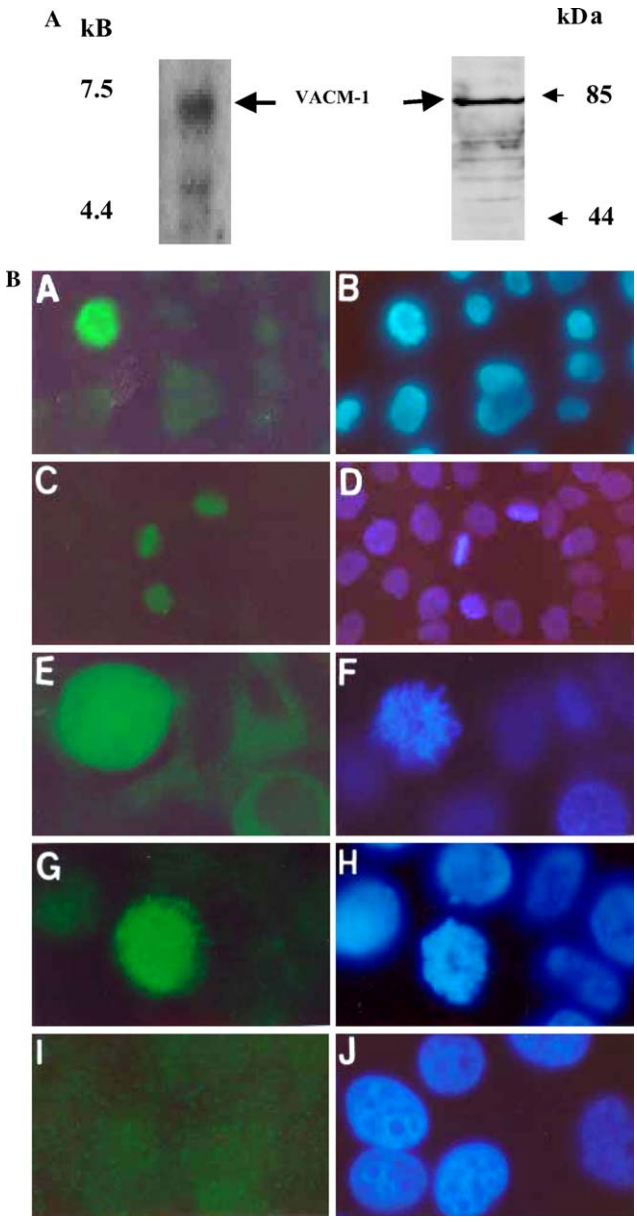


Fig. 1. (A) Northern and Western blot analyses of VACM-1 expression in T47D cells. (A) Poly(A)$^+$ mRNA from T47D cells was isolated and separated for Northern blot analysis as described in the Materials and methods. Blots were hybridized with the ^{32}P-labeled *Eco*RI/*Bcl*I fragment from VACM-1 cDNA. A 300 μg protein sample of total cell lysate was run on SDS–PAGE, transferred to nitrocellulose membrane, and probed with antibodies against the amino (Ab A) terminus of VACM-1. Blots were visualized by the chemiluminescent reagent following incubation with HRP-linked secondary antibody. VACM-1 is indicated. (B) Cellular localization of VACM-1 protein expression by immunohistochemical staining of T47D cells with anti-VACM-1 specific antibodies. (A,E) Immunostaining of T47D cultures with an antibody generated against the carboxy terminus of VACM-1. (B,F) DAPI nuclear staining of cells shown in (A,E), respectively. (C,G) Immunostaining of cell cultures with an antibody generated against the amino terminus of VACM-1. (D,H) DAPI nuclear staining of cells shown in (C,G), respectively. (I) No VACM-1 expression can be observed in cells immunostained with the antibody preincubated with 10 μg peptide. (J) DAPI nuclear staining of cells shown in (I). Cells were grown on coverslips, fixed, permeabilized, and immunostained with anti-VACM-1 Ab as described in Materials and methods. (A–D) 10× and (E–J) 100×.

Table 1
VACM-1 expression in T 47D breast cancer cells at the protein level

Days after passage	2	3	4	7
Number of cells counted	155	289	322	1674
Number of cells expressing VACM-1 protein	9	11	2	40
% cells expressing VACM-1	5.8	3.8	0.6	2.4

was identified in Western transfer blot of T47D lysates probed with an antibody generated against the amino terminal of VACM-1 (Ab A) (Fig. 1A). However, high protein concentration (300 μg) had to be loaded to detect the signal.

The immunocytochemistry studies revealed that only 0.6–6% of T47D cells express VACM-1. (Table 1 and Fig. 1B). In those cells, VACM-1 expression detected with antibodies generated against the amino (Figs. 1B—A and E) or carboxy terminals of VACM-1 (Figs. 1B—C and G) appeared to be limited to cells undergoing mitosis (identified with DAPI staining, Figs. 1B—B, D, F, and H), and seemed to be specific to the metaphase–anaphase transition (Figs. 1B—E–H, magnification 100×). The immunostaining with anti-VACM-1 protein Ab was no longer visible when Ab B was preincubated with 10 μM peptide identical in sequence to the carboxy terminus of VACM-1 and used to generate Ab B (Figs. 1B—I and J).

We have shown previously that the expression and cellular localization of VACM-1 protein in endothelial cells is cell cycle dependent [21]. Consequently, we wanted to examine whether VACM-1 expression in rapidly proliferating T47D cells is also transient. T47D cells grown to about 50% confluency were arrested with 2 mM thymidine [24] and immunostained with anti-VACM-1 Ab A. For comparison, VACM-1 expression in RAMEC arrested with aphidicolin (1.25 μg/ml), as described previously [21], was also examined. The immunocytochemistry data indicate that the cellular distribution of VACM-1 protein in T47D is cell cycle dependent (Fig. 2, left panels). No apparent VACM-1 expression could be detected in thymidine synchronized T47D cells (Figs. 2A and B), or at 4 h after the release from thymidine arrest, as cells enter the S phase (Figs. 2C and D). By 8 h after the release from the treatment, the immunostaining signal appears to be nuclear (Figs. 2E and F). After 10 h, VACM-1 again becomes absent from the T47D cells (Figs. 2G and H). This pattern of expression differs from that observed in RAMEC, summarized in Fig. 2 (right panels). A membrane specific VACM-1 expression is clearly observed in arrested RAMEC (Figs. 2I and J). VACM-1 expression largely disappears during the S phase (Figs. 2K and L). At 8 h after the release from the arrest, nuclear/cytosolic staining becomes evident again (Figs. 2M and N). As RAMEC enter the G1/G0 phase, VACM-1 expression localizes largely to the cell membrane and cytosol (Figs. 2O and P).

To test the hypothesis that VACM-1 affects growth, we established a number of T47D stable cell lines overexpressing VACM-1 (Fig. 3A). Bright field microscopy, shown in Fig. 3B, illustrates that T47D derived cell lines overexpressing VACM-1 grow more slowly than the sham transfected controls originally seeded at the same density. Cells shown in Fig. 3B—A represent the fourth passage of the vector transfected control T47D cells. Cells shown in Fig. 3B—B represent clone 1 cells also passaged four times. Cells shown in Figs. 3B—C and D are from clones 5 and 8, respectively, and were only passaged twice. Finally, cells shown in Figs. 3B—E and F represent clones 9 and 2, respectively. Whereas clone 9 cells continued to divide, clone 2 cells could never be passaged. In fact, out of 12 lines selected at random, four were arrested at the stage shown for clone 2 and were ultimately lost. Western blot analysis confirmed VACM-1 protein expression in all cell lines but clone 1 (Fig. 3A). The significance of the lower molecular weight band in cell lines 9 and 11, but not in cell lines 5 or 8, is not clear but may indicate a further processing of VACM-1.

Immunostaining of the representative cell line expressing VACM-1 protein is shown in Fig. 3C. Unlike in the control T47D cells where VACM-1 was observed only in the mitotic cells (Fig. 3C—A), in asynchronous clone 5 cells, VACM-1 was expressed in all cells and localized to the nucleus (Fig. 3C—B). A similar immunostaining pattern was observed in clone 8 cells (data not shown). The immunostaining of the slowly proliferating clones 9 (Figs. 3C—C and D) and 2 (Figs. 3C—E and F) suggested nuclear/cytosolic localization of VACM-1. In clone 2, which failed to propagate successfully (Fig. 3C—F), nuclear staining with DAPI suggested failure of cells to undergo cytokinesis (Figs. 3C—E and F). To further quantitate the effect of VACM-1 expression on T47D growth, clone 1, clone 5 and clone 8 were seeded at the same density and grown for 5 days and their growth rates were quantitated. Our results (Fig. 3D) indicate that VACM-1 overexpression significantly attenuates cellular growth of both clone 5 and clone 8 when compared to controls ($n = 6$, $p < 0.05$). Interestingly, the growth of clone 1 cells, with undetectable levels of VACM-1 protein (Fig. 3A), was not significantly different from the vector transfected control T47D cells.

To examine whether MAPK phosphorylation was compromised by VACM-1 protein, Western blot analyses of cell lysates from control and VACM-1 transfected T47D cells were performed using anti-phospho p44/p42 MAPK specific antibodies. Our results indicate that VACM-1 protein expression reduced MAPK phosphorylation when compared to the control cells (Fig. 3B, $n = 4$, $p < 0.05$).

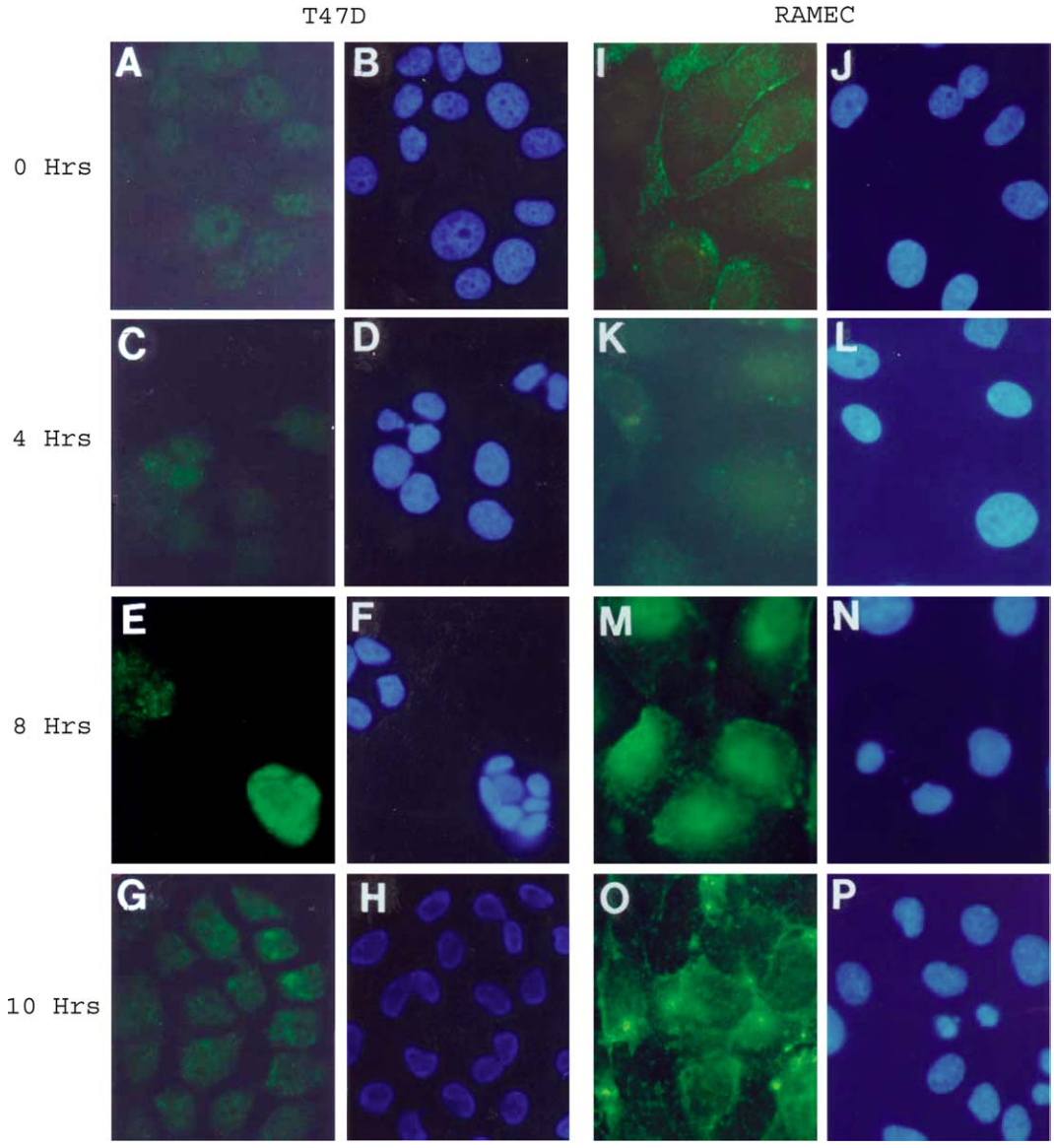


Fig. 2. Immunocytochemistry of T47D (A–H) and RAMEC (I–P) progressing through the cell cycle after release from the thymidine and aphidicolin arrest, respectively. Cells grown on coverslips were arrested as described in Materials and methods and prepared for immunostaining at the time points indicated. Cells were fixed, permeabilized, and immunostained with anti-VACM-1 Ab as described in Materials and methods. The nuclear compartment was visualized with DAPI present in the mounting medium. Time points after the release from arrest are 0 h for (A, B, I and J), 4 h for (C, D, K, and L), 8 h for (E, F, M, and N), and 10 h for (G, H, O, and P). Magnification 40×.

To identify steps/pathways in the cellular signaling systems that may be implicated in the VACM-1-dependent signaling in T47D cells, we performed an analysis with a Human PathwayFinder-1 GEArray Kit. Our data are summarized in Figs. 4A and B and indicate that out of the 24 genes present on the blot, four were affected by VACM-1 overexpression (discounting the small decreases in the two heat shock genes). These changes were observed only in clone 5 cells expressing high levels of VACM-1 protein (Fig. 3B) but not in clone 1 cells where VACM-1 expression could not be detected (Fig. 3A). VACM-1 expression in T47D cells attenuated egr-1 gene expression and increased Fas-L, IL2, and iNOS gene expression. At present, only egr-1 expression has been followed at the protein level. Western blot analyses of total cell lysates ($n = 3$) for expression of egr-1 were inconclusive, as numerous bands were detected and only small changes were observed (data not shown). Consequently, samples from several subsequent experiments were pooled and nuclear and cytosolic fractions were separated as described in Materials and methods ($n = 4$). These results, summarized in Figs. 4C and D, demonstrate a significant decrease in the 84 kDa size nuclear egr-1 when compared to the control samples. Interestingly, the 84 kDa egr-1 was largely absent from the cytosolic fractions where only the smaller, 54 kDa species could be detected.

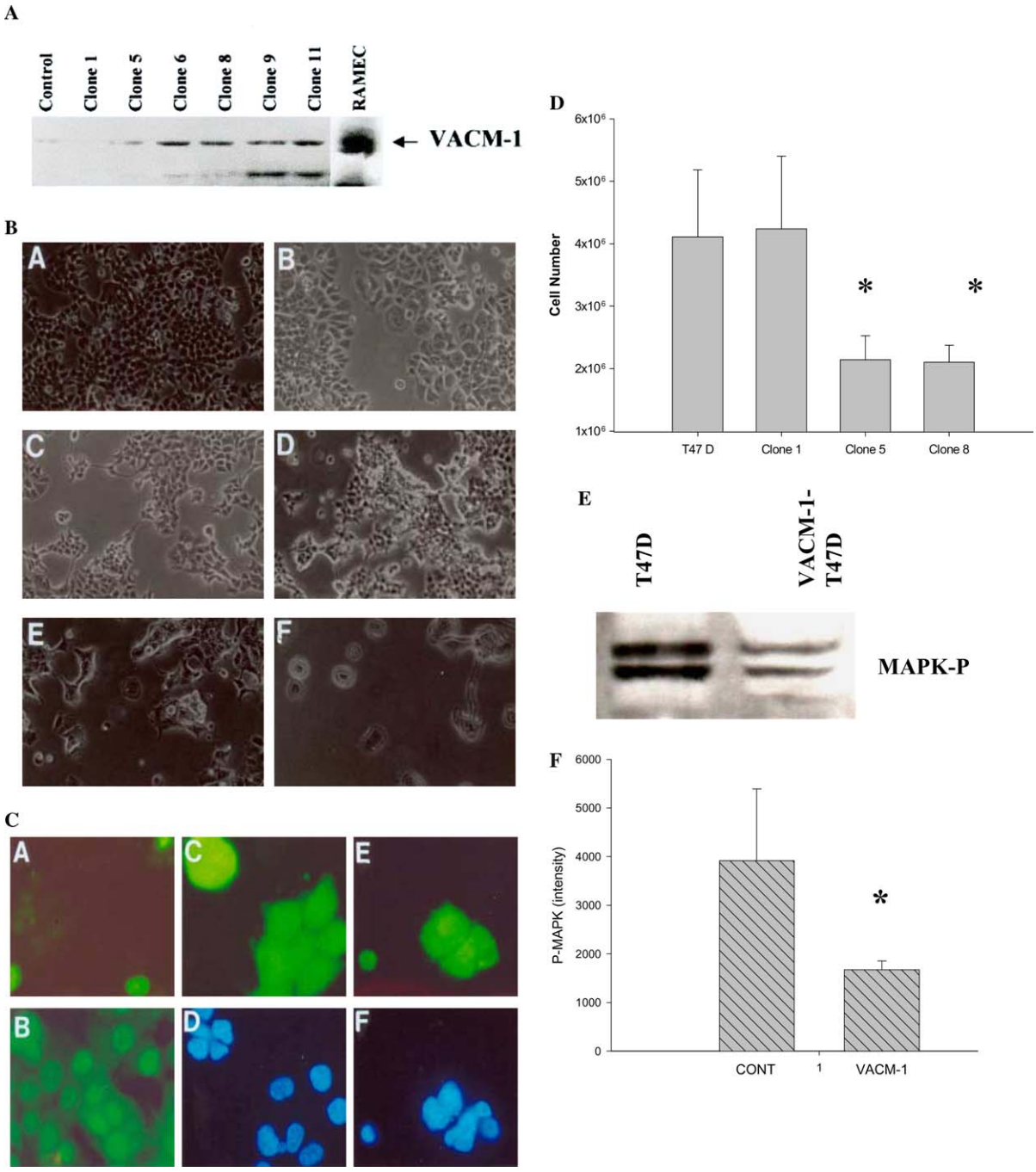


Fig. 3. Effects of stable expression of VACM-1 protein in T47D cells. (A) Western blot analysis of T47D stable cell lines. One hundred micrograms of protein samples of total cell lysate was run on SDS–PAGE, transferred to nitrocellulose membrane, and probed with antibodies against the amino (Ab A) terminus of VACM-1. An aliquot of RAMEC lysate immunoprecipitated with anti-VACM-1 antibody was run as a control. Following incubation with HRP-linked secondary antibody blots were visualized by the chemiluminescence. (B) Light microscopy of representative T47D cell lines transfected with VACM-1. Cells were seeded at equal density and their growth was monitored. A: T47 D cells at day 9, passage four. B: clone 1 cells at day 9, passage 4. C: clone 5 cells at day 9 passage 2. D: clone 8 cells at day 20, passage 2. E: clone 9 cells at day 9, passage 0. F: clone 2 cells at day 36, passage 0. Magnification, 10×. (C) Immunocytochemistry of control T47D breast cancer cells and stable cell lines expressing VACM-1 protein. Representative T47D cells and stable lines expressing VACM-1 protein were immunostained with anti-VACM-1 antibody directed against the amino terminus sequence. A: control T47D cells, passage 4, day 9. B: clone 5, passage 2, day 20. C: clone 9, passage 1, day 25. E: clone 2, passage 0, day 37. Nuclear staining of clone 9 (D) and 2 (F) was achieved by mounting slides in Vectashield containing DAPI. Magnification, 40×. (D) Effects of VACM-1 protein expression on cellular growth in stably transfected T 47D cells. Control T47D (vector only), and clone 1, clone 5, and clone 8 were seeded at equal density (2×10^4 cells/plate), grown for 7 days, harvested, and counted. Cells were trypsinized and counted using a hemacytometer. ($n = 6$, $^*p < 0.05$ when compared to control T47D cells or to clone 1 cells). (E) Effects of VACM-1 on MAPK phosphorylation in T47D cells. Fifty micrograms of total cell lysate proteins was run on a SDS–PAGE gel, transferred to a nitrocellulose membrane, and probed with anti-phospho-MAPK antibody as described in the Materials and methods. Blots were reprobed with anti-α-actin to correct for unequal protein loading. (F) The intensity of the signal shown in (E) was quantified using NIH Scion Imaging ($n = 4$, $^*p < 0.05$).

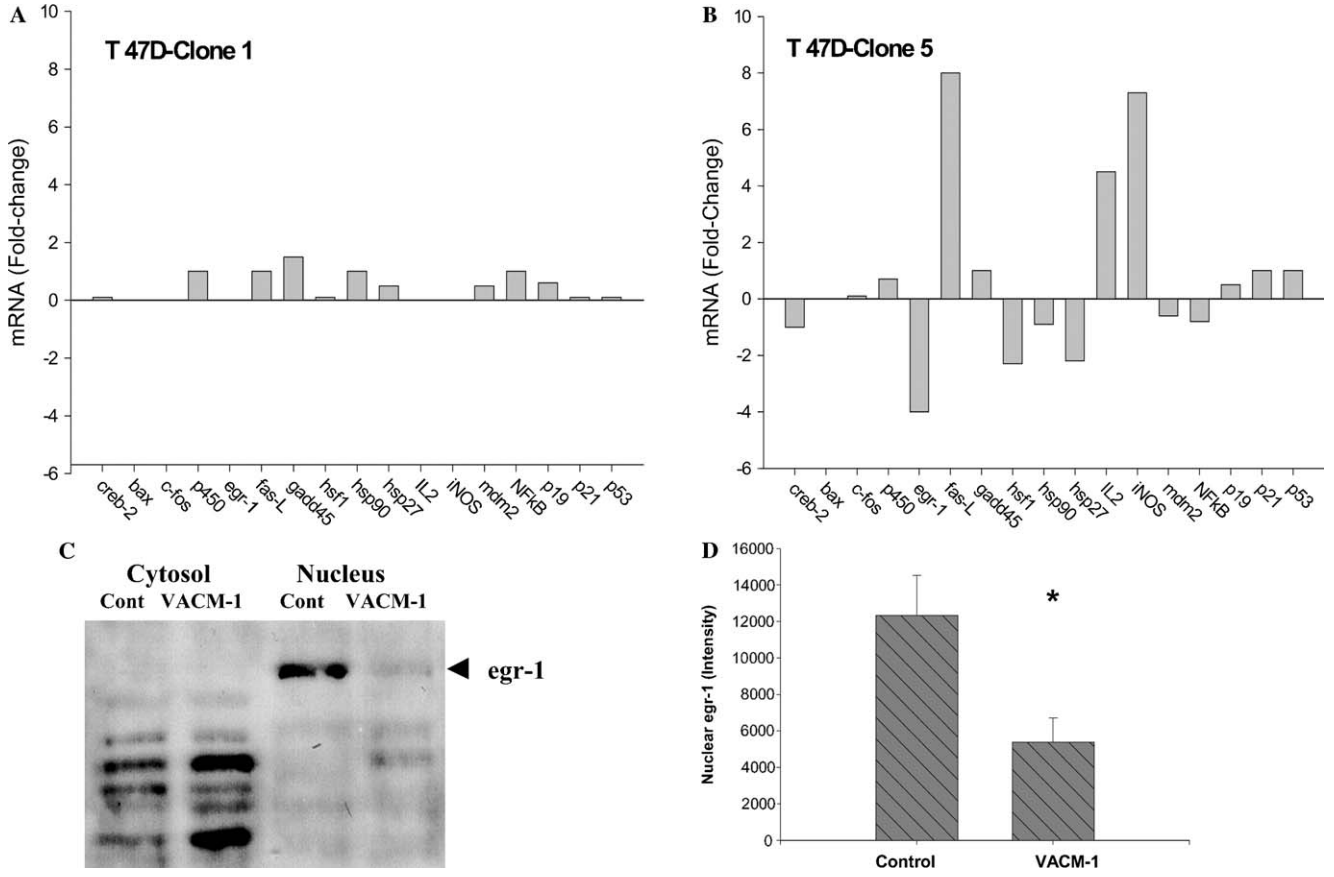


Fig. 4. (A,B) Effects of VACM-1 protein expression on gene induction in T47 D cells. Human PathwayFinder GEArray was probed with cRNA derived from control and VACM-1 transfected T47 D cell lines 1 (no VACM-1 expressed) and 5 (high VACM-1). Data are expressed as a ratio of clones 1 and 5 to control cells. (C) Effect of VACM-1 on egr-1 protein localization in T47D cells. Cytosolic nuclear fraction proteins (30 μg) were run on a SDS–PAGE gel, transferred to a nitrocellulose membrane, and probed with anti-egr-1 antibody as described in the Materials and methods. Equal protein loading was confirmed by Ponceau staining and in some experiments by reprobing blots with anti-tubulin antibody (data not shown). (D) The intensity of the signal shown in the nuclear fractions of (C) was quantified using NIH Scion Imaging ($n = 4$, $^*p < 0.05$).

Discussion

In this study, we examined the effect of VACM-1, a cul-5 gene product, on cellular growth and signaling in breast cancer derived T47D cell line. In asynchronous, proliferating T47D cells, VACM-1 protein expression is observed in only 0.6–6% of the cells (Fig. 1 and Table 1) and localizes to the nuclear region in cells undergoing mitosis (Figs. 1B and 2). Further, overexpression of VACM-1 protein leads to a significant inhibition of cellular growth (Figs. 3B–D and [22]). The significance of the inability of VACM-1 protein to translocate to the cell membrane in confluent T47D cells remains unclear. Another finding of this investigation was that VACM-1-dependent inhibition of growth may depend on the regulation of MAPK phosphorylation (Figs. 3E and F). These results are not unexpected as constitutive activation of MAPK signaling contributes to many aspects of human cancers, and the inhibition of MAPK activity triggers apoptosis in cells of breast cancer origin [28]. Further, an inhibitory effect of VACM-1 on cellular growth and on phosphorylation of MAPK has also been observed in cos 1 cells [22].

To identify steps/pathways subsequent to MAPK phosphorylation in the VACM-1-dependent signaling in T47D cells, we performed an analysis with a Human PathwayFinder-1 GEArray Kit. Out of the 24 genes present on the blot, only egr-1 mRNA expression was decreased and that of Fas-L, iNOS, and IL2 was increased in cells overexpressing VACM-1 (clone 5).

We first focused our efforts on the regulation of egr-1 gene expression by VACM-1, which is important in regulating cell growth, differentiation, and development [29–33]. The significantly lower levels of the egr-1 mRNA in VACM-1 transfected line when compared to the vector transfected cells (Fig. 4B) were also observed in clone 8 cells (data not shown) and were further confirmed at the protein level in clone 5 cell line. The 84 kDa species of egr-1 found exclusively in the nucleus, where it presumably regulates transcription [31–34], was significantly lower in cells overexpressing VACM-1 when compared to the vector transfected T47D cells.

Again, these results are not surprising as mitogens, growth factors, and tumor promoters that induce cellular proliferation by transcriptional activation of the egr-1 gene regulate phosphorylation of cytosolic MAPK and nuclear CREB proteins [30–32]. Further, a dominant negative MAPK inhibits egr-1 production [29]. Whether the increased intensity in the 54 kDa band observed in the cytosolic fractions is reflective of an increase in the 84 kDa egr-1 degradation remains to be established.

Whereas in cos 1 cells VACM-1 expression increased p53 levels, we did not detect expression of p53 in T47D cells at the mRNA (Fig. 4A) or protein level (data not shown). This observation is not entirely surprising, as p53 is lost in about half of all human cancers [30–32]. Instead, an 8-fold increase in Fas-L mRNA was observed in VACM-1 transfected cells when compared to a control (clone 5, Fig. 4B). Fas-L mRNA levels were unaffected in cells that had a low VACM-1 protein level and a growth rate similar to that of the control T47D cells (clone 1, Fig. 4A). Although Fas-L expression is typically specific to the activated T lymphocytes and macrophages where its binding to the Fas receptor results in death by apoptosis and is responsible for the immune response termination [35–37], its expression in human melanoma, hepatocellular carcinoma, and metastatic tumors of the colon has also been reported (reviewed in [35]). The presence of Fas-L on tumor cells has been suggested to function in deletion of infiltrating immune cells [32–35]. Maecker et al. [34] suggested that "oncogenic transformation drives tumor cells to lose either p53 or Fas sensitivity as a means of promoting their survival and evade immune surveillance." It is thus possible that in some cells VACM-1 protein expression induces p53, whereas in cells with absent p53, VACM-1 induces Fas-L expression, and thus controls cellular growth.

The role of iNOS in cellular proliferation is also well recognized [38]. Its anti-tumor activity may be dependent on its level of expression and cell type and is cAMP dependent [38]. Whether the increase in iNOS mRNA levels is reflected at the protein level remains to be determined. Similarly, the significance of the increase in IL2 mRNA, observed in the cells overexpressing VACM-1, although intriguing, is not clear and must be confirmed at the protein level. Typically, IL2 is recognized for its role in the proliferative activity of lymphocytes, and it has been used successfully to infiltrate and destroy tumor growth [39]. A model of downregulation of immune response by the Fas/Fas-L system that is linked to the IL2 signaling has been suggested [39]. Whether VACM-1 induced increase in IL2 production at the protein level contributes to the decreased cellular growth in T47D cancer cells will require further study.

In summary, VACM-1 protein expression in T47D cells appears to be transient and cell cycle dependent. Overexpression of VACM-1 in T47D cells significantly attenuates growth, reduces MAPK phosphorylation, and inhibits egr-1 mRNA and protein expression.

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