Activity of Plasma Membrane V-ATPases Is Critical for the Invasion of MDA-MB231 Breast Cancer Cells*

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Background: The V-ATPase has been proposed to function at the plasma membrane in tumor cell invasion.

Results: Inhibition of plasma membrane V-ATPases prevented invasion of MDA-MB-231 cells.

Conclusion: Activity of plasma membrane V-ATPases is critical for breast cancer cell invasion.

Significance: Plasma membrane V-ATPases are a possible therapeutic target to limit metastasis.

The vacuolar (H+)-ATPases (V-ATPases) are a family of ATP-driven proton pumps that couple ATP hydrolysis with translocation of protons across membranes. Previous studies have implicated V-ATPases in cancer cell invasion. It has been proposed that V-ATPases participate in invasion by localizing to the plasma membrane and causing acidification of the extracellular space. To test this hypothesis, we utilized two separate approaches to specifically inhibit plasma membrane V-ATPases.

First, we stably transfected highly invasive MDA-MB231 cells with a V5-tagged construct of the membrane-embedded c subunit of the V-ATPase, allowing for extracellular expression of the V5 epitope. We evaluated the effect of addition of a monoclonal antibody directed against the V5 epitope on both V-ATPase-mediated proton translocation across the plasma membrane and invasion using an in vitro Matrigel assay. The addition of anti-V5 antibody resulted in acidification of the cytosol and a decrease in V-ATPase-dependent proton flux across the plasma membrane in transfected but not control (untransfected) cells. These results demonstrate that the anti-V5 antibody inhibits activity of plasma membrane V-ATPases in transfected cells. Addition of the anti-V5 antibody also inhibited in vitro invasion of transfected (but not untransfected) cells. Second, we utilized a biotin-conjugated form of the specific V-ATPase inhibitor bafilomycin. When bound to streptavidin, this compound cannot cross the plasma membrane. Addition of this compound to MDA-MB231 cells also inhibited in vitro invasion. These studies suggest that plasma membrane V-ATPases play an important role in invasion of breast cancer cells.

Metastasis is the spread of tumor cells from a primary tumor to other sites in the body and is the leading cause of cancer mortality (1, 2). Multiple steps are required for metastasis to occur, including intravasation of tumor cells from the primary site into the circulatory or lymphatic system and extravasation of cells from the circulation into secondary sites (1). Cancer cells must be able to degrade basement membrane and extracellular matrix for intravasation and extravasation to occur (3). Metastatic cancer cells display a more alkaline cytosolic pH and a more acidic extracellular pH relative to normal cells (4). Several studies have shown that an acidic extracellular pH can enhance tumor cell invasion (5, 6). Thus, determining the mechanisms by which invasive cancer cells generate an acidic extracellular environment may lead to the development of novel anti-metastatic therapeutics.

The vacuolar (H+)-ATPases (V-ATPases)4 are ubiquitously expressed, ATP-dependent proton pumps present in lysosomes, endosomes, secretory vesicles, clathrin-coated vesicles, and the plasma membranes of certain specialized cell types (7–9). The primary role of the V-ATPase is to pump protons from the cytosol into intracellular compartments or the extracellular space. It has been implicated in a number of critical physiological processes, including receptor-mediated endocytosis, proper protein trafficking through endosomal compartments, zymogen activation, and proton-coupled transport of small molecules (7). The V-ATPase contains two functional domains: the peripheral V1 domain, which is composed of eight subunits (A–H) and is responsible for ATP hydrolysis, and the membrane-embedded V0 domain, which, in mammals, is made up of five subunits (a, c, c*, d, and e) and is responsible for proton translocation (7). Subunit a of the V0 domain is a 100-kDa integral membrane protein responsible for localizing the V-ATPase to different subcellular membranes (10, 11). In mammals, subunit a is present in four isoforms (a1–a4), with a3 and a4 responsible for targeting the V-ATPase to the plasma

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4 The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine triphosphatase; Bicine, N,N-bis(2-hydroxyethyl)glycine.
Role of Plasma Membrane V-ATPases in Invasion

First, we have expressed a recombinant form of the V-ATPase containing an epitope tag exposed on the extracellular surface of tumor cells. We have then demonstrated that an antibody against the extracellular tag, added to living cells, inhibits both plasma membrane V-ATPase activity and breast cancer cell invasion. Second, we have utilized a membrane-impermeable form of the V-ATPase inhibitor bafilomycin and found that this compound also inhibits breast cancer cell invasion. The results suggest that plasma membrane V-ATPase activity is important for the invasiveness of at least some tumor cells.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—DMEM, FBS, penicillin–streptomycin, PBS, 0.05% trypsin–EDTA, Lipofectamine 2000, Blasticidin S, the Vivid Colors™ pcDNA™6.2/N-EmGFP-GW/TOPO® mammalian expression vector, the mouse monoclonal antibody recognizing the V5 epitope, the Alexa Fluor® 488-conjugated goat anti-rabbit secondary antibody, the Alexa Fluor® 488-conjugated goat anti-mouse secondary antibody, the Alexa Fluor® 568 phalloidin antibody, the Alexa Fluor® 594 phalloidin antibody, and ProLong® Gold were purchased from Invitrogen. Aprotinin, leupeptin, and pepstatin were purchased from Roche Molecular Biochemicals. Precast polyacrylamide mini-protein Tris-glycine-extended gels, Tween 20, SDS, nitrocellulose membranes, and horseradish peroxidase-conjugated goat anti-mouse IgG were purchased from Bio-Rad. The chemiluminescence substrate for horseradish peroxidase was purchased from General Electric, and the signal was detected using Kodak BioMax Light film. A mouse monoclonal antibody that recognizes the V-ATPase V1 subunit was purchased from Abnova, and mouse monoclonal antibodies against the V-ATPase V0d subunit and the β1 subunit of the (Na+/K+)-ATPase (clone M17-P5-F11) were purchased from Abcam. A mouse monoclonal antibody recognizing α-tubulin was purchased from Genscript. The rabbit polyclonal antibody recognizing the V-ATPase V5 subunit was obtained from Dr. Moshe Reuveni at the Department of Ornamental Horticulture of the Agricultural Research Organization Volcani Center (Bet-Dagan, Israel). SNARF-1 was purchased from Life Science Molecular Probes. Fluoroblok inserts with 8-μm pores were purchased from BD Biosciences, and Matrigel™ was purchased from Corning. Zymolyase 20T was purchased from Seikagaku American, Inc. PMSF, the mouse monoclonal antibody against vinculin, calcine AM, streptavidin, concanamycin A, and all other chemicals were purchased from Sigma.

Cell Culture—The human breast cancer cell line MDA-MB231 was purchased from American Type Culture Collection. MB231 cells were grown in Falcon™ T-75 flasks in DMEM with phenol red, 25 mM d-glucose, 4 mM L-glutamine, and 1 mM sodium pyruvate supplemented with 10% FBS, 60 μg/ml penicillin, and 125 μg/ml streptomycin. Cells were grown in a 95% air, 5% CO2 humidified environment at 37 °C.

Plasmid Transfection—cDNA encoding the human c subunit was amplified by PCR and cloned into the Vivid Colors™ pcDNA™6.2/N-EmGFP-GW/TOPO® mammalian expression vector to allow for C-terminal expression of the V5 epitope. GFP was removed from the plasmid, and successful deletion of GFP and insertion of the human c subunit cDNA were
verified by sequencing. 15 μg of the plasmid was transfected into MB231 cells using Lipofectamine 2000 in accordance with the manufacturer’s recommendations. Stable transfection was achieved by treatment of cells with 7.5 μg/ml blasticidin S for 21 days, beginning 3 days post-transfection. Cells were subsequently maintained under selective conditions.

Cell Lysis and Western Blotting—Cells were harvested by trypsinization, resuspended in lysis buffer with protease inhibitors (PBS-EDTA containing 137 mM NaCl, 1.2 mM KH₂PO₄, 15.3 mM Na₂HPO₄, 2.7 mM KCl, 2 mM EDTA, pH 7.2, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM PMSF), and lysed by sonication. Cell lysates were centrifuged for 5 min at 4 °C at 500 × g to remove cellular debris. Protein concentrations were determined using the Lowry method (25). SDS sample buffer was added to the lysates, and proteins were separated by SDS-PAGE on 4–15% gradient acrylamide gels. The presence of the V5 epitope, subunit V₁A, subunit V₀d, α-tubulin, or vinculin was detected by Western blotting using mouse monoclonal antibodies followed by a horseradish peroxidase-conjugated secondary antibody. Blots were developed using chemiluminescent detection reagents. Western blots were quantified using ImageJ software with corrections made for loading.

Immunocytochemistry—A mouse monoclonal antibody raised against the V5 epitope was used to localize the V5-tagged subunit c in transfected cells, and a rabbit polyclonal antibody against the V-ATPase subunit V₁E was used to localize the V-ATPase in untransfected and transfected cells. Cells were plated onto 24 × 24-mm coverslips coated with poly-κ-lysine in 6-well plates. Approximately 24 h later, cells were washed, fixed with 4% paraformaldehyde, and either permeabilized with 0.1% Triton-X-100 or incubated in PBS for nonpermeabilized cells. Nonspecific binding was blocked by incubation with 1% bovine serum albumin in PBS for 1 h. Cells were then incubated with the anti-V5 antibody at a 1:5000 dilution or the anti-V₁E antibody at a 1:1000 dilution overnight at 4 °C, washed with PBS, and then incubated with Alexa Fluor® 594 phalloidin or Alexa Fluor® 568 phalloidin to stain F-actin (1:250 dilution) and the Alexa Fluor® 488-conjugated goat anti-mouse secondary antibody (1:1000 dilution) or the Alexa Fluor® 488-conjugated goat anti-rabbit secondary antibody (1:500 dilution). After 2 h of incubation at room temperature, the cells were washed with PBS. The cells were prepared for viewing using ProLong® Gold mounting medium and allowed to cure at room temperature for 24 h. The stained cells were imaged using a Leica TCS SPE confocal microscope. The results from three separate experiments were analyzed. To quantify plasma membrane V-ATPase staining, 90 cells from the three separate experiments were counted, and the percentage of cells showing plasma membrane V-ATPase was determined.

Cytosolic pH Measurements—Cells were grown on rectangular coverslips (8 × 22 mm) and treated overnight with or without the anti-V5 antibody. Cells were then loaded with SNARF-1 (5- [and-6] carboxy-SNARF-1-AM) to measure cytosolic pH. Cells were transferred to a cell perfusion system and were continuously perfused at 3.0 ml/min at 37 °C with cell superfusion buffer containing 1.3 mM CaCl₂, 1 mM MgSO₄, 5.4 mM KCl, 0.44 mM KH₂PO₄, 110 mM NaCl, 0.35 mM NaH₂PO₄, 5 mM glucose, 2 mM glutamate, and 20 mM HEPES at pH 7.4. The emission spectra of SNARF-1 (644/584 nm) were acquired using 534 nm as the excitation wavelength. The conversion of ratio values to cytosolic pH (pHₜₐ) was performed as previously described (18). The fluorescence was monitored with a SLM-8100/DMX spectrophotometer (Spectronics Instruments, Rochester, NY) equipped for sample perfusion. The sample temperature was maintained at 37 °C by keeping both the water jacket and perfusion media at 37 °C using an iso-temperature immersion circulator water bath (Lauda model RM20; Brinkmann Instruments, Westbury, NY). All measurements were performed using 4-nm-bandpass slits and an external rhodamine standard as a reference. Fluorescence data were converted to ASCII format for analyses.

In Situ Calibration of SNARF-1—In situ calibration curves were generated as described previously (18). Briefly, the cells attached to coverslips were perfused with High K⁺ buffer (10 mM NaCl, 146 mM KCl, 10 mM HEPES, 10 mM MES, 10 mM Bicine, 2 μM valinomycin, 6.8 μM nigericin, 5 mM glucose, 2 mM glutamate, pH 5.5–8.0 adjusted with NaOH). The buffer contains high K⁺ to approximate the intracellular K⁺ concentration. Nigericin is an ionophore that exchanges H⁺ and K⁺ across the membrane, rendering the pHₜ equal to the extracellular pH (pHₑₓ). Valinomycin is an ionophore that moves K⁺ across the plasma membrane and, together with nigericin, helps to equilibrate pHₑₓ and pHₜ. The pH of the buffers was determined using a Beckman pH meter with a glass electrode (Corning Inc., Horseheads, NY) calibrated at 37 °C with commercially available standard solutions (VWR Scientific, San Francisco, CA). The ratios (r = 644/584) of SNARF-1 were converted to pH using a modified Henderson–HasSELbalch equation (26). The equation was solved using nonlinear least squares analysis with Sigma Plot to obtain the values of pKₐ, Rₘᵦₜ, and Rₘᵦ for SNARF-1 in these cells.

Plasma Membrane Proton Flux Measurements—The initial rate of pHₑₓ recovery from an acid load induced by K⁺-acetate was measured as the dpH/dt, which is the slope of the linear regression curve relating time and pHₑₓ. Cells loaded with SNARF-1 were perfused with cell superfusion buffer until the steady-state pHₑₓ was reached. The cells were then perfused with 50 mM K⁺-acetate in HCO₃⁻ and Na⁺-free cell superfusion buffer to eliminate the contribution of potential HCO₃⁻ transporters and Na⁺/H⁺ exchanger. The perfusion with K⁺-acetate will cause a rapid intracellular acidification followed by a subsequent pHₑₓ recovery. We then evaluated the pHₑₓ recovery from this acidification within the first 3 min. The individual pHₑₓ data points were then used to plot a linear regression curve relating time and ΔpHₑₓ. To quantify the pHₑₓ recovery, we expressed the recoveries as proton flux (Jₑₓ), which is given by the apparent H⁺ buffering (Bₑₓ) capacity multiplied by the dpH/dt (27).

Cell Fractionation and Analysis of V-ATPase Assembly—Cell fractionation was performed as previously described (28). Cells were harvested by trypsinization, washed in cold PBS, and resuspended in homogenization buffer (250 mM sucrose, 1 mM EDTA, 10 mM HEPES, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 1 mM PMSF). Cells were then homogenized using a ball-bearing homogenizer that allows 10 μm of
clearance. Cells were passed through the homogenizer 20 times, and the lysate was centrifuged at 500 × g for 10 min at 4 °C to pellet nuclei and cell debris. The supernatant was then centrifuged at 100,000 × g for 30 min at 4 °C to separate the membrane and cytosolic fractions. The cytosolic fraction was concentrated using an Amicon Ultra filter with a 10-kDa cutoff, and the membrane pellet was resuspended in homogenization buffer. Protein concentration was determined by the Lowry method (25). Protein from each fraction was separated by SDS-PAGE, and a Western blot was performed as described above. The anti-V$_d$ antibody and the anti-vinculin antibody were used as loading controls for the membrane fraction and the cytosolic fraction, respectively, and the anti-gV$_A$ antibody was used to assess the relative amounts of the V$_1$ domain in the cytosolic and membrane fractions. Western blots were quantitated using ImageJ software with corrections made for loading. The ratio of the amounts of the V$_1$A subunit in the membrane fraction versus the cytosolic fraction was calculated and served as a measurement of V-ATPase assembly. The ratio for each experimental condition tested was divided by the ratio observed for untreated MB231 cells to normalize the results. To examine whether treatment with the anti-V5 antibody altered V-ATPase assembly, cells were treated with or without anti-V5 at a concentration of 1:500 for 8 h prior to harvesting.

**Invasion and Migration Assays—**Assays for in vitro invasion and migration were performed as described previously (17–19). Fluoroblok inserts with an 8-μm pore size membrane were placed into the wells of a 24-well plate (29). To evaluate invasion, Matrigel™ was diluted to a final concentration of 0.2 μg/μl, and a total of 11 μg was coated onto the membrane in each well. The membrane was allowed to dry overnight under vacuum at room temperature. Matrigel™-coated membranes were rehydrated with 60 μl of DMEM plus phenol red, 25 mM d-glucose, 4 mM l-glutamine, and 1 mM sodium pyruvate (termed “Medium”) for at least 2 h. For evaluation of the effects of the anti-V5 antibody on in vitro invasion and migration, cells were harvested by trypsinization and brought to a concentration of 1.75 × 10^5 cells/ml in Medium containing DMSO, 100 nM biotin-conjugated bafilomycin (received as a gift from Dr. Markus Huss, Universität Osnabrück in Osnabrück, Germany) and 750 nM streptavidin were allowed to bind by rotating the compounds together for 1 h at 4 °C. Invasion and migration assays were performed as described above, with cells being brought to a concentration of 1.75 × 10^5 cells/ml in Medium containing DMSO with 750 nM streptavidin, 100 nM concanamycin A, or 1500 nM biotin-conjugated bafilomycin with 750 nM streptavidin and incubated for 15 min at 37 °C before being seeded onto the coated or uncoated membranes.

**Yeast Culture Conditions and Isolation of Vacuolar Membrane Vesicles—**The yeast strain YPH500 (MATa, ura3-52, lys2-801amber, ade2-101ochre, trp1-Δ63, his3-Δ200, leu2-ΔI) (30) was maintained in YPD (1% yeast extract, 2% bacto-peptone, 2% glucose) buffered to pH 5.5. Vacuolar membranes were isolated as previously described (31). Briefly, YPH500 was grown overnight to A$_{600}$ of 1.2–1.5 in 1 liter of YPD, pH 5.5. The cells were then pelleted, washed with water, and resuspended in 100 ml of 100 mM Tris–HCl, pH 9.4, with 10 mM dithiothreitol. The cells were then incubated for 20 min at 30 °C at 100 rpm before washing in 50 ml of YPD medium containing 0.7 mM sorbitol, 2 mM dithiothreitol, and 100 mM MES-Tris, pH 7.5. After resuspension in 50 ml of YPD containing 0.7 mM sorbitol, 2 mM dithiothreitol, 100 mM MES-Tris, pH 7.5, and 20 mg of Zymolase 20T, yeast were incubated for 1 h at 30 °C at 100 rpm. The spheroplasts were osmotically lysed, and vacuoles were isolated by consecutive Ficoll gradients and diluted in transport buffer (15 mM MES-Tris, pH 7.0, 4.8% glycerol), and protein content was measured using the Lowry method (25).

**Assessment of ATP-dependent Proton Transport Activity in Yeast—**ATP-dependent proton transport was measured as described previously (31, 32). Fluorescence quenching of the fluorescence probe 9-amino-6-chloro-2-methoxy-acridine in transport buffer (25 mM MES-Tris, pH 7.2, 5 mM MgCl$_2$) was measured in the presence or absence of DMSO with 0.5 μM streptavidin, 1 μM biotin-conjugated bafilomycin, or 1 μM biotin-conjugated bafilomycin bound to 0.5 μM streptavidin. Biotin-conjugated bafilomycin and streptavidin were allowed to bind by rotating the compounds together for 1 h at 4 °C before starting experiments.

**Statistical Analysis—**The graphed data are averages from the indicated number of independent experiments. A Student’s *t* test was used to calculate significance. *p* < 0.05 was considered significant.

**RESULTS**

**MDA-MB231 Cells Stably Transfected with a V5-tagged Construct of Subunit c of the V-ATPase Express the V5 Epitope at the Plasma Membrane—**To determine whether plasma membrane V-ATPases are critical to the invasiveness of MDA-MB231 cells, cells were first stably transfected with a construct expressing the proteolipid c subunit of the V-ATPase tagged with the V5 epitope at the C terminus (termed c-V5). The C terminus of subunit c has previously been shown to be present on the luminal surface of the protein such that, for a V-ATPase complex present at the plasma membrane, this epitope would be exposed on the extracellular surface of the membrane (33). Further investigation revealed that antibody binding to an extracellular epitope does not by itself affect invasiveness, we performed an overnight in vitro Matrigel™ invasion assay with MB231 cells treated with or without the anti-V5 antibody and the anti-vinculin antibody were used as loading controls for the membrane fraction and the cytosolic fraction, respectively, and the anti-gV$_A$ antibody was used to assess the relative amounts of the V$_1$ domain in the cytosolic and membrane fractions. Western blots were quantitated using ImageJ software with corrections made for loading. The ratio of the amounts of the V$_1$A subunit in the membrane fraction versus the cytosolic fraction was calculated and served as a measurement of V-ATPase assembly. The ratio for each experimental condition tested was divided by the ratio observed for untreated MB231 cells to normalize the results.
thermoe, because the c subunit is present in several copies per V-ATPase, selection of this subunit for epitope tagging enhances the likelihood that the epitope will be incorporated into most, if not all, of the V-ATPases in the cell (7, 34). Expression of the c-V5 construct in the MB231 transfected cells was verified by Western blotting of whole cell lysates (Fig. 1A). We next wanted to determine whether c-V5-containing V-ATPases were present at the plasma membrane. To address this, immunocytochemistry was conducted using an anti-V5 antibody in cells that either were or were not permeabilized by treatment with 0.1% Triton X-100. As shown in Fig. 1B, permeabilized cells were readily stained with phalloidin, which stains the intracellular marker F-actin, whereas nonpermeabilized cells showed no phalloidin staining, confirming that cells remained impermeable to antibodies under the conditions of fixation employed. Staining with the anti-V5 antibody was observed in both permeabilized and nonpermeabilized c-V5-expressing cells. In permeabilized cells, both diffuse and punctate staining for V5 was observed, with significant perinuclear staining, consistent with localization to intracellular compartments such as the Golgi and lysosomes. By contrast, in nonpermeabilized cells, only diffuse staining with the anti-V5 antibody was observed, indicating the presence of V5-tagged V-ATPase complexes diffusely distributed over the surface of cells (Fig. 1B). No staining with the V5 antibody was observed in untransfected MB231 cells (data not shown), indicating that the antibody specifically targets the V5 epitope.

Treatment of c-V5-expressing Cells with an Anti-V5 Antibody Causes Acidification of the Cytoplasm—To investigate the effect of addition of the anti-V5 antibody on cytosolic pH (pHcyt) in cells expressing the c-V5 construct, the fluorescence pH indicator SNARF-1 was employed. Transfected and untransfected MB231 cells were loaded with SNARF-1, and cytosolic pH was determined by measuring the ratio of fluorescence intensity at 644 and 584 nm upon excitation at 534 nm, as described under “Experimental Procedures.” As shown in Fig. 2, c-V5-expressing cells had a more alkaline cytosolic pH than untransfected cells. Addition of the anti-V5 antibody decreased the pHcyt of c-V5-expressing cells but did not affect the pHcyt of untransfected cells.

To determine whether the higher cytosolic pH observed in c-V5-expressing cells was due to an increase in total V-ATPase expression, we examined the protein levels of subunit A (part of the V1 domain) and subunit d (part of the V0 domain) by Western blotting of whole cell lysates using anti-A and anti-d subunit antibodies. As shown in Fig. 3A, no difference in expression levels of subunit A or subunit d was observed between untransfected and transfected MB231 cells, indicating that expression of the c-V5 protein did not alter total V-ATPase expression. Next, we assessed whether the more alkaline cytosolic pH observed in c-V5-expressing cells was due to an alteration in V-ATPase assembly. To test this, untransfected and transfected cells were homogenized, and the cytosolic and membrane fractions were isolated by high speed centrifugation followed by Western blotting using antibodies against subunits A and d (28). A change in assembly would be reflected as a shift in subunit A between the cytosolic and membrane fractions. No difference in assembly was observed between untransfected and transfected cells (Fig. 3B). Finally, we assessed whether the more alkaline pH in transfected cells was a result of increased V-ATPase expression at the plasma membrane. To address this, immunocytochemistry was performed using an anti-V5 antibody on permeabilized cells. This subunit is present in all of the V-ATPases in the cell (7). Phalloidin was used to stain actin and outline the cell periphery. Colocalization of subunit E and phalloidin at the cell periphery was used to identify plasma membrane V-ATPases, as previously described (18, 19). As shown in Fig. 3C, untransfected and transfected cells exhibited a similar localization of V-ATPases, and a similar fraction of

FIGURE 1. c-V5-transfected cells express V5 both intracellularly and at the plasma membrane. A, cell lysates were prepared from untransfected and transfected cells and cell lysates were separated by SDS-PAGE using 4–15% gradient acrylamide gels, and proteins were transferred to nitrocellulose. Immunoblotting was conducted using monoclonal antibodies against V5 and α-tubulin as described under “Experimental Procedures.” The blot displayed is representative of data obtained from two separate experiments. B, c-V5-transfected cells were grown as a monolayer on coverslips in 6-well plates. Permeabilized cells (Perm) were treated with 0.1% Triton X-100 for 3 min, whereas nonpermeabilized cells (Non-Perm) were not. Cells were immunostained using a monoclonal antibody against V5 and Alexa Fluor® 594 phalloidin to stain actin followed by incubation with secondary antibodies as described under “Experimental Procedures.” Images were taken with identical exposure times and antibody concentrations. The top panels show permeabilized cells, whereas the bottom panels show nonpermeabilized cells. The left panels display phalloidin staining, the middle panels display V5 staining, and the right panels display the merged images. The results shown are representative of three independent trials.
cells showed staining for V-ATPases at the cell periphery. Although it remains unclear why c-V5-transfected cells experience a more alkaline cytosolic pH, our results suggest that expression of c-V5 does not alter V-ATPase expression, assembly, or localization in MB231 cells.

Treatment of c-V5-expressing Cells with an Anti-V5 Antibody Inhibits Proton Flux across the Plasma Membrane—To assess whether addition of the anti-V5 antibody inhibits proton flux across the plasma membrane of c-V5-expressing cells, the rate of pHcyt recovery following an acute acid load induced by K+/acetate was monitored as described under “Experimental Procedures.” Cells loaded with SNARF-1 were first perfused with buffer in the absence of HCO3\(^{-}\) and Na\(^+\) to eliminate the contributions of HCO3\(^{-}\) transporters and Na\(^+\)/H\(^+\) exchangers to proton flux. Cells were subsequently equilibrated with 50 mM K+/acetate, resulting in a rapid intracellular acidification followed by a subsequent pHcyt recovery. The rate of proton extrusion (\(J_{H^+}\)) during the first 3 min of pHcyt recovery was measured. As shown in Fig. 4A, no difference in proton flux across the plasma membrane was observed between untransfected and transfected cells. Upon addition of the anti-V5 antibody, proton flux was significantly reduced in c-V5-expressing cells but not in untransfected cells. These results suggest that the anti-V5 antibody inhibits proton flux across the plasma membrane of c-V5-transfected cells by blocking the activity of V-ATPases.

Next, we determined whether the anti-V5 antibody inhibits V-ATPase activity in c-V5-expressing cells by reducing assembly of the two functional domains. We incubated untransfected and transfected cells in the presence or absence of the anti-V5 antibody for 8 h and assessed V-ATPase assembly as described above. As shown in Fig. 4B, treatment of transfected cells with the anti-V5 antibody did not cause a decrease in V-ATPase assembly. The small—and not significant—increase in assembly observed after antibody treatment may be the result of reduced disassembly of the V-ATPase complex caused by inhibition of activity, because dissociation of the V-ATPase has been shown to be activity-dependent (35).

Treatment of c-V5-expressing MB231 Cells with an Anti-V5 Antibody Inhibits In Vitro Invasion and Migration—We next determined what effect addition of the anti-V5 antibody would have on the invasiveness of the c-V5-expressing cells. To address this, invasion was measured using an in vitro Matrigel™ assay. Cells were incubated in the presence or absence of the anti-V5 antibody, plated on Matrigel™-coated wells and induced to invade using a chemoattractant (fetal bovine serum) on the trans-side of the well. After 8 h, cells on the trans-side were stained with calcein AM and counted as described under “Experimental Procedures.” As shown in Fig. 5A, transfected cells displayed a similar degree of invasiveness as untransfected cells, indicating that expression of the c-V5 construct has no effect on MB231 cell invasion. Both cell lines show reduced invasion after treatment with the membrane-permeable, V-ATPase-specific inhibitor concanamycin A, as previously described (17–19). By contrast, whereas addition of the anti-V5 antibody had no effect on invasion of untransfected cells, invasion by the c-V5-expressing cells was significantly inhibited after antibody treatment (Fig. 5A). To confirm that the binding of an antibody to an extracellular epitope does not by itself reduce invasiveness, we treated MB231 cells with an antibody against an extracellular epitope of the β1 subunit of the (Na\(^+\),K\(^+\))-ATPase, which is found on the plasma membrane of all cells. Anti-β1 treatment did not alter MB231 cell invasiveness compared with untreated cells (data not shown). This confirms that the reduction in invasiveness observed upon treatment of cells with the anti-V5 antibody is a specific effect. Overall, our results suggest that inhibition of plasma membrane V-ATPases in c-V5-expressing MB231 cells is sufficient to block their invasion.

Because invasiveness of cells also requires cell migration, we assessed the effect of the anti-V5 antibody on the ability of the c-V5-expressing cells to migrate using an in vitro migration assay. As shown in Fig. 5B, both transfected and untransfected cells display reduced migration after treatment with a V-ATPase inhibitor, as previously observed (17). However, treatment with the anti-V5 antibody reduced migration of only the c-V5-expressing cells.
FIGURE 3. The more alkaline cytosolic pH of c-V5-transfected cells is not a result of enhanced V-ATPase subunit expression, an alteration in pump assembly, or plasma membrane localization. A, cell lysates from untransfected control cells and cells transfected with c-V5 were prepared. 10 μg of protein for each sample was separated by SDS-PAGE using 4–15% gradient acrylamide gels, and proteins were transferred to nitrocellulose. Immunoblotting was conducted using monoclonal antibodies against subunit A, subunit d1, and vinculin. The blot displayed is representative of data obtained from three separate experiments. Western blots from each experiment were quantitated, and the ratio of the expression of subunits A and d in transfected cells versus untransfected cells was calculated. B, untransfected control and c-V5-transfected cells were harvested, and cytosolic (lanes C) and membrane (lanes M) fractions were prepared as explained under “Experimental Procedures.” 19 μg of protein from each fraction was then separated by SDS-PAGE using 4–15% gradient acrylamide gels, and proteins were transferred to nitrocellulose. Immunoblotting was conducted using monoclonal antibodies against subunit A, subunit d1, and vinculin (not pictured). The blot displayed is representative from three separate experiments. The ratio of subunit A in the membrane versus the cytosolic fraction was calculated as a measure of V-ATPase assembly. The graphed data represent the average degrees of assembly for transfected cells relative to untransfected cells. C, untransfected control and c-V5-transfected cells were grown as a monolayer on coverslips in 6-well plates. Permeabilized cells were immunostained using an antibodies against the V1E subunit of the V-ATPase and Alexa Fluor® 568 phalloidin to stain actin followed by incubation with secondary antibodies as described under “Experimental Procedures.” Images were taken with identical exposure times and antibody concentrations. The three panels show representative staining for phalloidin (left panel), V1E (middle panel), and the merged images (right panel) for both untransfected and transfected cells. White arrows indicate plasma membrane V-ATPase expression. To quantitate plasma membrane V-ATPase expression, 90 cells from thee separate experiments were counted, and the number of cells showing plasma membrane V-ATPase localization was determined. The graphed data represent the percentage of untransfected cells and c-V5-transfected cells that exhibit plasma membrane V-ATPase localization. All error bars indicate S.E.
between streptavidin and the $V_1$ domain would be unable to inhibit the pump from the cytoplasmic surface. We isolated vacuoles from the yeast strain YPH500 and measured ATP-dependent proton transport by 9-amino-6-chloro-2-methoxyacridine fluorescence quenching, as described previously (31, 32), in the presence of biotin bafilomycin or streptavidin-bound biotin bafilomycin. As shown in Fig. 6A, the addition of streptavidin alone had no significant effect on proton transport activity relative to vacuoles treated with DMSO. Treatment with $1 \mu$M biotin-bafilomycin completely ablated ATP-dependent proton transport activity, consistent with V-ATPase inhibition. When biotin-bafilomycin was bound to streptavidin, its ability to inhibit proton transport was prevented. This suggests that biotin bafilomycin is membrane-impermeable when bound to streptavidin.

We next treated MB231 cells with the membrane-impermeable, streptavidin-bound biotin-bafilomycin to determine whether inhibition of plasma membrane V-ATPases reduces invasion. After confirming that treatment with biotin-bafilomycin does not induce MB231 cell death (data not shown), we performed in vitro invasion assays and allowed MB231 cells to invade in the presence or absence of streptavidin-bound biotin-bafilomycin for 8 h. As shown in Fig. 6B, we found that the in vitro invasiveness of these cells is significantly reduced after treatment with this compound. This reduction in invasiveness is similar to what is observed after treatment with a membrane-permeable inhibitor. The in vitro migratory capability of MB231 cells was also decreased following treatment with streptavidin-biotin-bafilomycin (Fig. 6C). These results thus further support the role of plasma membrane V-ATPases in the invasiveness of breast cancer cells.

**DISCUSSION**

Although a number of studies have implicated the V-ATPase in tumor cell invasion, the mechanisms by which the pump is involved in this process have not been elucidated (17–24). Several reports have identified a correlation between cancer cell invasiveness and V-ATPase localization to the plasma mem-
brane (17–21, 26). Moreover, siRNA-mediated knockdown of subunit a isoforms known to localize V-ATPases to the plasma membrane has been shown to reduce invasion (18, 19). One limitation of previous studies has been the inability to specifically inhibit V-ATPases localized to the plasma membrane, because the available V-ATPase inhibitors, although highly specific, are membrane-permeant and thus inhibit all the V-ATPases in the cell. This is important because of the possible role of intracellular V-ATPases in the secretion of proinvasive factors, such as proteases and growth factors (21, 24). The purpose of the present study was to determine whether V-ATPase activity at the plasma membrane plays a role in cancer cell invasion by specifically inhibiting activity of plasma membrane V-ATPases.

To address this question, we have employed two methods to specifically ablate plasma membrane V-ATPase activity. First, we have tagged the proteolipid c subunit of the V-ATPase with a V5 epitope tag at the C terminus. The topology of the c subunit ensures that the V5 tag in this construct will be exposed on the luminal, or, for plasma membrane localized V-ATPases, the extracellular surface of the protein (33). An added benefit of epitope tagging subunit c is that it is present in multiple copies per V-ATPase, making it likely that the V5 tag is present in nearly every V-ATPase in the cell (7, 34). The tagged construct

![FIGURE 5. In vitro invasion and migration of c-V5-transfected cells is reduced after treatment with an anti-V5 antibody. A and B, untransfected control cells or c-V5-transfected cells were treated in the presence or absence of the V-ATPase specific inhibitor concanamycin A (ConA) or a monoclonal antibody against V5 and then plated on Matrigel\textsuperscript{TM}-coated FluoroBlok\textsuperscript{TM} inserts (A, invasion) or uncoated FluoroBlok\textsuperscript{TM} inserts (B, migration) and allowed to migrate toward a chemoattractant on the trans-side of the well for 8 h. Cells were then stained with calcein AM, and the number of cells that had invaded or migrated to the trans-side was counted, with three wells analyzed per sample and an average of 10 images analyzed per well. The values for both assays are the means of at least three independent experiments expressed relative to untransfected, untreated cells. All error bars indicate S.E. *, \(p < 0.05\) compared with untransfected, untreated cells.](image)
cytosolic pH upon addition of the anti-V5 antibody in cells expressing the c-V5 construct but not in untransfected cells (Fig. 2). Moreover, addition of the anti-V5 antibody inhibited proton flux across the plasma membrane in c-V5-transfected MB231 cells but not untransfected cells (Fig. 4A). These results suggest that the anti-V5 antibody is able to inhibit proton transport across the plasma membrane mediated by the V-ATPase.

The mechanism by which the anti-V5 antibody inhibits V-ATPase activity is uncertain. We showed that this inhibition is not due to decreased assembly of the complex, because antibody treatment did not reduce the degree of membrane association of the V₁ domain (Fig. 4B). The slight increase in assembly after antibody treatment may be the result of increased plasma membrane V-ATPase assembly caused by inhibition of activity. It has previously been shown that inhibition of V-ATPase activity prevents dissociation of the V₁ and V₀ domains and favors the assembled state of the pump (35). It is possible that binding of the antibody to the c subunit sterically inhibits rotation of the proteolipid ring, which is required for ATP-driven proton transport (37, 38), because each proteolipid ring contains multiple copies of the c subunit (7, 34). The presence of multiple V5 antibodies bound to each V-ATPase complex may set up interactions with the stator portion of the complex that obstruct rotation. Additional studies will be required to address this question.

Interestingly, we found that cells expressing the c-V5 construct had a more alkaline cytosol than untransfected cells (Fig. 2). Because transfected cells express endogenous subunit c in addition to the V5-tagged subunit c, it is possible that increased expression of proteolipid subunits leads to an alteration in the number of or assembly status of V-ATPase complexes. The fact that the levels of subunit A and subunit d do not change following transfection (Fig. 3A) indicates there is no change in V-ATPase expression levels in transfected cells. Furthermore, no difference in V-ATPase assembly or plasma membrane localization was observed between transfected and untransfected cells (Fig. 3, B and C). Additionally, the fact that c-V5 expression does not alter proton flux across the plasma membrane (Fig. 4A) suggests there is no difference in the total V-ATPase activity at the plasma membrane. It remains possible that a more alkaline pH is observed in transfected cells because of increased proton transport into intracellular compartments.

Finally, we wished to determine whether inhibiting plasma membrane V-ATPase activity blocks invasion of c-V5-expressing cells. Using an in vitro invasion assay, we observed no difference in the invasiveness of transfected and untransfected cells. However, addition of the anti-V5 antibody reduced invasion of c-V5-expressing cells by over 50% relative to untreated cells but had no effect on invasion by untransfected cells (Fig. 5A). This effect is due to plasma membrane V-ATPase inhibition and not a consequence of antibody binding to an irrelevant extracellular epitope, because we confirmed that treatment of MB231 cells with an antibody against an extracellular epitope of the (Na⁺,K⁺)-ATPase did not alter invasiveness. We went on to demonstrate that in vitro migration of transfected (but not untransfected) cells was significantly reduced upon anti-V5 treatment (Fig. 5B). The observed reductions in invasiveness and migration upon antibody treatment were similar to those...
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observed after treatment with the membrane-permeable V-ATPase inhibitor concanamycin A. Overall, these results suggest that inhibition of plasma membrane V-ATPase activity is sufficient to reduce invasion and migration of c-V5-expressing cells.

To confirm the role of plasma membrane V-ATPases in tumor cell invasion and migration, we determined the effect of treatment of cells with the membrane-impermeant V-ATPase inhibitor streptavidin-biotin-bafilomycin. We confirmed that this reagent is membrane-impermeant using isolated yeast vacuoles (Fig. 6A). This suggests that streptavidin-biotin-bafilomycin can be used to selectively inhibit plasma membrane V-ATPases in mammalian cells without inhibiting intracellular V-ATPases. We observed that streptavidin-biotin-bafilomycin was as effective as concanamycin A in inhibiting invasion and migration of MB231 cells (Fig. 6, B and C). These results thus provide further support for the role of plasma membrane V-ATPases in breast cancer cell invasion and migration.

One possible way that plasma membrane V-ATPases could promote invasion is by activating proteases that facilitate degradation of extracellular matrix. Cathepsins are a family of proteases that normally reside in lysosomes and therefore require a low pH for activity and activation (39, 40). Numerous studies have demonstrated that cathepsin secretion and activity are involved in invasion of cancer cells (41–46). Cathepsins may function directly in invasion by degrading extracellular matrix or by activating other proteases (such as matrix metalloproteases) to promote invasion (39, 45). In either case, their activity and activation is enhanced when extracellular pH is acidic. Tumors often exhibit an acidic extracellular pH, and this acidic pH appears to play a role in tumor cell invasion (47, 48). Neutralization of the acidic extracellular pH has been shown to prevent metastasis in mouse models and reduces extracellular matrix metalloprotease activity in tumors (48). We suggest that plasma membrane V-ATPases promote invasion by creating the acidic extracellular pH required for extracellular protease activity that is critical for tumor cell invasion. Indeed, pharmacological or genetic disruption of V-ATPase activity has been shown to reduce matrix metalloprotease activity in pancreatic cancer cells (20). Ongoing studies in our lab are exploring whether plasma membrane V-ATPase activity directly promotes the activation and activity of secreted cathepsins.

A recent report has shown that pharmacological V-ATPase inhibition or siRNA-mediated knockdown of the V_{0} subunits a1 or d1 prevents Rab27-mediated invasion in breast cancer cells by preventing peripheral localization and secretion of Rab27-containing vesicles (24). Furthermore, it has also been observed that the invasiveness of poorly metastatic prostate cells with low plasma membrane V-ATPase expression can be reduced after V-ATPase inhibition (22). The results of these studies suggest that intracellular V-ATPases may play an important role in the trafficking of molecules that participate in invasion. Thus, V-ATPases may participate both in the plasma membrane and within intracellular compartments to promote tumor cell invasiveness.

Our findings also suggest that plasma membrane V-ATPases contribute to migration of breast cancer cells. Although previous studies have demonstrated that inhibition of the V-ATPase with a membrane-permeable inhibitor reduces in vitro migration (17), the current study demonstrates a role for plasma membrane pumps in this process. It is not fully understood how the V-ATPase contributes to cell migration. The pump has been demonstrated to directly interact with actin in yeast, insect, and mammalian cells (49–53), although it is unclear whether this interaction requires ATPase activity. A recent study has found that subunit C interacts with F-actin at the plasma membrane of invasive breast cancer cells and that loss of this subunit disrupts actin arrangement in these cells (54). Moreover, inhibiting V-ATPases with archazolids has been found to impair plasma membrane localization of EGFR and Rac1 in SKBR3 breast cancer cells, a critical process for cell migration (23). It has also been suggested that plasma membrane V-ATPases present in the leading edge of microvascular endothelial cells promote migration through a local increase in cytosolic pH, which facilitates actin polymerization (4, 55). It is possible that a similar phenomenon occurs in invasive breast cancer cells; however, additional studies will be required to resolve this question.

In summary, in this report we present results supporting a role of plasma membrane V-ATPases in in vitro invasion and migration of MB231 breast cancer cells. These results suggest that plasma membrane V-ATPases may be a viable anti-metastatic target. Plasma membrane V-ATPases are only found in a small number of cells in the body, including osteoclasts and renal intercalated cells. It may thus be possible to specifically target plasma membrane V-ATPases in tumor cells to reduce metastasis without causing life threatening side effects. The mechanism by which plasma membrane V-ATPases participate in invasion and migration of tumor cells will require additional study.

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