Aurora kinase A activates the vacuolar H\(^+\)-ATPase (V-ATPase) in kidney carcinoma cells

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Aurora kinase A activates the vacuolar H\(^+\)-ATPase (V-ATPase) in kidney carcinoma cells. Am J Physiol Renal Physiol 310: F1216–F1228, 2016. First published February 24, 2016; doi:10.1152/ajprenal.00061.2016.—Extracellular proton-secreting transport systems that contribute to extracellular pH include the vacuolar H\(^+\)-ATPase (V-ATPase). This pump, which mediates ATP-driven transport of H\(^+\) across membranes, is involved in metastasis. We previously showed (Alzamora R, Thali RF, Gong F, Smolak C, Li H, Baty CJ, Bertrand CA, Auchli Y, Brunisholz RA, Neumann D, Hallows KR, Pastor-Soler NM. J Biol Chem 285: 24676–24685, 2010) that V-ATPase A subunit phosphorylation at Ser-175 is important for PKA-induced V-ATPase activity at the membrane of kidney intercalated cells. However, Ser-175 is also located within a larger phosphorylation consensus sequence for Aurora kinases, which are known to phosphorylate proteins that contribute to the pathogenesis of metastatic carcinomas. We thus hypothesized that Aurora kinase A (AURKA), overexpressed in aggressive carcinomas, regulates the V-ATPase in human kidney carcinoma cells (Caki-2) via Ser-175 phosphorylation. We found that AURKA is abnormally expressed in Caki-2 cells, where it binds the V-ATPase A subunit in an AURKA phosphorylation-dependent manner. Treatment with the AURKA activator anacardic acid increased V-ATPase expression and activity at the plasma membrane of Caki-2 cells. In addition, AURKA phosphorylates the V-ATPase A subunit at Ser-175 in vitro and in Caki-2 cells. Immunolabeling revealed that anacardic acid induced marked membrane accumulation of the V-ATPase A subunit in transfected Caki-2 cells. However, anacardic acid failed to induce membrane accumulation of a phosphorylation-deficient Ser-175-to-Ala (S175A) A subunit mutant. Finally, S175A-expressing cells had decreased migration in a wound-healing assay compared with cells expressing wild-type or a phospho-mimetic Ser-175-to-Asp (S175D) mutant A subunit. We conclude that AURKAactivates the V-ATPase in kidney carcinoma cells via phosphorylation of Ser-175. This regulation contributes to kidney carcinoma V-ATPase-mediated extracellular acidification and cell migration.

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human isoforms of Aurora kinases have been identified: Aurora kinase A (AURKA), Aurora kinase B, and Aurora kinase C (11, 23). These kinases associate with the spindle poles and promote centrosome maturation and the initiation of mitosis (11). As expected of many cell cycle-regulatory proteins, AURKA expression is carefully timed in normal cells, where this protein is quickly degraded after mitosis (11, 23). Indeed, this kinase is usually not visualized in nondividing cells (33). Interestingly, activation of AURKA occurs by binding cofactors that contribute to a change in the conformation of the kinase and autophosphorylation of Thr-288 (11, 34).

While defects in the function and expression of Aurora kinases can lead to mitotic arrest, apoptosis, and aneuploidy (42), overexpression of AURKA has been detected in a wide range of human cancers, including renal cell carcinoma (RCC). AURKA gene polymorphisms are also associated with an early onset of cancer and a poor prognosis for the patient (11, 25). In addition, AURKA overexpression correlates with enhanced invasiveness of the cancer and genomic instability, indicating that AURKA function is important for tumor formation and/or progression to metastases (5, 42, 50). It is estimated that there were ~62,000 new cases of kidney cancer in 2015 in the U.S. and ~14,000 deaths from the disease (3a). Chronic hemodialysis patients are at particular risk for renal cell carcinoma (RCC), a malignancy associated with high mortality (38). Primary treatment for RCC is radical nephrectomy, but 20–30% of patients still develop local or metastatic disease recurrence (38). In one study, prominent AURKA immunolabeling was present in ~70% of samples from patients with RCC (26). Another study reported that overexpression of AURKA was significantly associated with advanced tumor stage and poor prognosis of patients with RCC, making AURKA a promising target for early diagnosis and treatment of RCC (29).

However, the mechanisms by which AURKA mediates tumorigenesis and/or metastasis in RCC are still unclear. AURKA has a recognized target phosphorylation site that is highly homologous to the PKA consensus site (Fig. 1). We envisioned that should the tight subcellular regulation of this kinase become disturbed, such as in cancer cells, AURKA could potentially regulate and activate genes that would render the cell more likely to become metastatic, such as the V-ATPase. We decided to investigate the relationship between AURKA and V-ATPase-mediated proton secretion in kidney cancer cells, especially because AURKA is a kinase likely to recognize and phosphorylate the important V-ATPase activating site Ser-175 in the A subunit. We hypothesized that in kidney carcinoma cells such as Caki-2 cells, overabundant cytoplasmic AURKA leads to overphosphorylation of cytoplasmic V-ATPase A subunit at Ser-175, which induces active proton secretion at the plasma membrane of the cell via this H+ pump and in turn increases metastatic potential. Here, we show that AURKA activation increases phosphorylation of the V-ATPase in a Ser-175-dependent manner in vitro and also in Caki-2 cells. Moreover, we find that Ser-175 is required to activate and translocate the V-ATPase to the leading edge of a Caki-2 cell monolayer in a wound-healing assay. Taken together, this study provides a molecular basis for how AURKA may contribute to cancer metastasis, which may occur through modulating the subcellular localization and functional activity of the V-ATPase.

**MATERIALS AND METHODS**

Reagents and chemicals. All chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Pittsburgh, PA) unless otherwise stated. The AURKA activator anacardic acid was obtained from Tocris Bioscience (Bristol, UK). Purified active AURKA protein was obtained from Millipore (Billerica, MA). The AURKA inhibitor III was obtained from EMD Millipore (Darmstadt, Germany).

Antibodies. The anti-phospho-Thr288-Aurora kinase A (pThr288-AURKA) and mouse anti-AURKA antibodies were purchased from Abcam (Cambridge, MA). The anti-AURKA (also called anti-AIK) antibodies raised in rabbits were obtained from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Burlingame, CA). The nuclear stain TO-PRO-3 and the secondary goat anti-chicken antibody conjugated to Alexa 488 were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA), while the secondary HRP-conjugated anti-rabbit IgG and the anti-mouse IgG antibodies were obtained from GE Healthcare Biosciences (Pittsburgh, PA). An anti-V-ATPase E subunit antibody raised in chickens, the M2 anti-FLAG and anti-FLAG horseradish peroxidase (HRP) antibodies, and the mouse anti-β-actin antibodies were obtained from GeneTex (Irvine, CA). An anti-V-ATPase a4 subunit polyclonal antibody raised in goat was obtained from Santa Cruz Biotechnology (Dallas, TX). Concanavalin A coupled to CY3 was obtained from Vector Laboratories (Burlingame, CA).

Cell culture. Caki-2 human kidney carcinoma cells were obtained from the American Type Culture Collection (www.atcc.org). Cells were used at passages 25–29 and maintained at 37°C in a humidified 5% CO2-95% air incubator in RPMI medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS and penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml). Cells were grown to 90% confluence in 75-cm2 plastic culture flasks and then seeded onto petri dishes or glass coverslips where they were maintained in medium similar to that used to culture the mpkCCD-14 cell line (CCD medium) (4, 14). This CCD medium was composed of equal volumes of DMEM and Ham’s F-12 plus 60 mM sodium selenate, 5 mg/ml transferrin, 2 mM glutamine, 50 mM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 mg/ml insulin, 20 mM d-glucose, 2% (vol/vol) FBS, and 20 mM HEPES, pH 7.4 (reagents from Life Technologies and Sigma-Aldrich). HEK-293 cells were grown and plated in 60-mm dishes (5 × 105 cells/dish) before cell lysis as previously described by our group (2, 3).

**Consensus Phosphorylation Motif**

<table>
<thead>
<tr>
<th>PKA site</th>
<th>RX [S/T]</th>
<th>AURKA site</th>
<th>ATP6V1A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xenopus laevis</td>
<td>170</td>
<td>PRNRTVTYVA</td>
<td>180</td>
</tr>
<tr>
<td>Gallus gallus</td>
<td>170</td>
<td>PRNRTVTYIA</td>
<td>180</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>170</td>
<td>PRNRTSVTYIA</td>
<td>180</td>
</tr>
<tr>
<td>Sus scrofa</td>
<td>170</td>
<td>PRNRTVTYIA</td>
<td>180</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>170</td>
<td>PRNRTVTYIA</td>
<td>180</td>
</tr>
</tbody>
</table>

![Fig. 1. Informatic analysis of ATP6V1A phosphorylation. Top: consensus phosphorylation target motifs for PKA and aurora kinase A (AURKA) are shown, where X represents any residue, S or T is the phosphorylated residue, and Φ represents a hydrophobic residue at the P + 1 position. Bottom: sequence alignment of residues near ATP6V1A at position 175 across various species (frog, chicken, mouse, pig, and human).](http://ajprenal.physiology.org/doi/10.1152/ajprenal.00061.2016)
Immunolabeling of Caki-2 cells. Cells were seeded onto coverslips at 2.5 × 10^4 cells/cm² and maintained in culture for 4–5 days to form a confluent monolayer. The cells were then fixed in 2% paraformaldehyde in PBS buffer for 30 min and permeabilized by the addition of a buffer containing 1% PBS, 1% BSA, and 0.1% Triton X-100 for 10 min at 37°C as described previously (3). After an additional wash, coverslips were immunolabeled with a primary antibody raised in chickens against the V-ATPase E subunit (1:100 dilution) along with rabbit anti-AURKA (1:100 dilution) for 75 min. Coverslips were then incubated with secondary goat anti-chicken antibody conjugated with Alexa 488 (1:800 dilution) and secondary goat anti-rabbit antibody conjugated with CY3 (1:800 dilution), and then TO-PRO-3 (1:400 dilution, Invitrogen) to stain the nuclei for 5 min. All antibodies were diluted in Dako diluent (Dako Laboratories, Carpinteria, CA). Coverslips were mounted in ProLong Gold antifade reagent (Invitrogen). The coverslips were imaged in a Leica confocal microscope using a ×100 oil-immersion objective with identical laser, acquisition, and X-Z reconstruction settings for all samples.

Immunoblotting of HEK-293 and Caki-2 cells. SDS-PAGE was performed on cell lysate samples on a 4–12% gradient gel (Nu-PAGE, Invitrogen), followed by transfer to nitrocellulose membranes and immunoblotting as previously described (2, 3) for expression of active phosphorylated AURKA using a rabbit anti-pThr288-AURKA antibody and then for β-actin.

Immunoprecipitation studies to evaluate endogenous AURKA complex formation with V-ATPase subunits. HEK-293 and Caki-2 cells were grown under standard conditions. AURKA was immunoprecipitated from cell lysates using the AURKA mouse monoclonal antibody. Immunoblotting was performed for the expression of the V-ATPase a4 subunit (AP6V0A4). Then, the membrane was stripped and reblotted using rabbit anti-AURKA raised in rabbits. Controls for the immunoprecipitation reaction were performed in the absence of the immunoprecipitating antibody and in the absence of lysate for both cell types.

Immunolabeling and wound-healing assays for imaging of the leading edge of untransfected Caki-2 cells. Untransfected Caki-2 cells were plated as above to form confluent cell monolayers (12, 39, 43). The monolayers were scraped using a sterile 10-μl pipette tip, washed twice to remove nonadherent cells, and incubated in either vehicle (DMSO), anacardic acid (25 μM; 4 h), or in one experiment AURKA inhibitor III (50 nM; 4 h). Cells were then incubated with concanavalin A coupled to CY3 for 5 min before fixation to label the plasma membrane (1:100 dilution in PBS containing Mg^{2+} and Ca^{2+}). Under these conditions, there was minimal internalization of this lectin. This incubation was followed by a brief wash in PBS containing Mg^{2+} and Ca^{2+} before immunolabeling with an antibody against the V-ATPase E subunit as described above. Images from these coverslips were acquired using a Leica confocal microscope with identical laser confocal microscope settings across all conditions as per our previously published protocol in other cell lines (1). The images were saved in TIF format, and the V-ATPase E subunit accumulation at the leading edge was quantified on X-Z reconstructions using NIH ImageJ software. Analysis of the images was done by a macro program that measures fluorescence intensities as a function of distance from the leading edge of cells as described previously (41). The fluorescence intensity profile starting from the center of the nucleus and extending 2 μm outside the cell was traced, and the mean gray value was measured (i.e., the sum of the gray values of all the pixels in the selection divided by the number of pixels). Three different profiles were traced randomly in each cell of a total of >45 cells per condition, and at least 3 different experiments (coverslips) were performed per condition. For quantification, the ratio of fluorescence intensity of the leading edge (at 1 μm) and of the cytoplasm region (half the distance between leading edge and nucleus) was calculated.

Evaluation of AURKA phosphorylation status changes in response to anacardic acid in Caki-2 cells. Caki-2 cells were seeded onto 60-mm dishes and grown in CCD media for 5 days, as above. After anacardic acid treatment (25 μM; 4 h), cells were lysed, and the AURKA was immunoprecipitated using the AURKA mouse monoclonal antibody. After SDS-PAGE and transfer to nitrocellulose membranes were conducted, immunoblotting for expression of active phosphorylated AURKA was performed using the anti-pThr288-AURKA antibody raised in rabbits. Then, the membrane was stripped and reblotted using the anti-AURKA antibody raised in rabbits. Quantification of the relative signal of phosphorylated AURKA was performed as previously described (3).

Cell surface biotinylation assays. Caki-2 cells were grown on Transwell filters in Caki-2 media for 5 days at 37°C and 5% CO₂, 95% air. Cells were incubated in Caki-2 media in the absence or presence of anacardic acid (25 μM; 4 h). Apical cell surface biotinylation assays were then conducted as previously described (28). Briefly, cells were first washed with ice-cold PBS containing Mg^{2+} and Ca^{2+} four times for 5 min. Then, the apical membrane was biotinylated using 1 mg/ml EZ link sulfo-NHS-SS-biotin (Thermo Fisher Scientific), and free NHS-biotin was then quenched, followed by several ice-cold PBS washes. Protein concentration was determined using the Bradford technique, and 1 mg of protein was incubated with 30 μl of streptavidin-agarose beads (50%, Thermo Fisher Scientific) in a total volume of 1 ml overnight at 4°C, while kept in constant rotation. After washing, biotinylated proteins were eluted from the beads by boiling samples in 2× Laemmli sample buffer containing 20% DTT. The protein samples were then separated by SDS-PAGE on a 4–12% gradient gel (Nu-PAGE) and subjected to immunoblot analysis using anti-V-ATPase a4 and anti-β-actin antibodies.

Measurements of changes in V-ATPase-dependent extracellular acidification rates. Caki-2 cells were seeded onto 24-well plates (2.5 × 10^5 cells/well) at 90% confluence for 5 days. On the day of experimentation, the cells were incubated in the absence or presence of anacardic acid (25 μM for 4 h). The medium was then removed, and the cells were washed with a NaCl-free, low-buffering capacity solution, as previously described (3). Then, pH measurements were performed in cells after each of two additional 20-min incubation periods in the same buffer solution. In the first incubation period, the cells were treated with control (buffer) or vehicle (DMSO) alone in the absence or presence of anacardic acid. During the second incubation period, bafilomycin (1 μM; Sigma-Aldrich), a specific V-ATPase inhibitor, was added to all wells before pH measurements. The samples were maintained at 37°C throughout the experiment in a water bath. The V-ATPase-dependent rate of pH acidification was defined as the difference in the measured acidification rates in the absence vs. presence of bafilomycin. Following the pH measurements, the number of viable cells at the end of the experiment was assessed by trypan blue exclusion assay (48). In some experiments (such as in Fig. 10), Caki-2 cells were first transfected using Lipofectamine 2000 (Invitrogen, Life Technologies) with pTracer [green fluorescent protein (GFP)-expressing] plasmids encoding either FLAG-tagged wild-type (WT) A subunit or the FLAG-tagged Ser-175-to-Ala mutant (S175A; phosphorylation-deficient) characterized in a previous study (3). The following day, transfection efficiency was evaluated by estimating the percentage of GFP-expressing cells and found to be similar between transfection conditions. The transfected cells were then plated onto a 24-well plate as described above, followed by treatment with or without anacardic acid for each of the plasmids and then pH measurements.

V-ATPase A subunit in vitro phosphorylation assays. In vitro phosphorylation assays were carried out according to standard published protocols in our laboratory (3, 20). HEK-293 cells were transiently transfected to express either the FLAG-tagged WT, S175A, or Ser-175-to-Asp (S175D; phosphomimetic) mutant A subunit constructs (3). FLAG-labeled V-ATPase A subunits were immunoprecipitated from precleared lysates using the M2 anti-FLAG monoclonal antibody as shown previously (2, 3). In vitro phosphorylation was performed using commercially available purified active AURKA (Millipore, Billerica, MA) with [γ-32P]ATP labeling, as
described previously (2, 3). The immunoprecipitated samples were subjected to immunoblotting using an anti-FLAG antibody coupled to HRP (1:100,000) and quantified using a Versa-Doc Imager with Quantity One software (Bio-Rad, Hercules, CA). Phosphorylated bands on the membrane were identified by exposure of the membrane to a phosphoscreen, and bands were quantitated using a Bio-Rad PhosphorImager with Quantity One software. The intensity of each phosphoscreen band was corrected by subtracting out the local background in the same lane and further normalized to the immunoblot signal for the corresponding condition (2, 3).

V-ATPase A subunit phosphorylation assays in Caki-2 cells. Cells were seeded onto 60-mm petri dishes (5 × 10⁶ cells/dish), grown to 90% confluence, and then transiently transfected with plasmids expressing either FLAG-tagged WT or S175A A subunit constructs (6 μg of plasmid DNA) (2, 3). Two days after transfection, [³²P]orthophosphate labeling in intact Caki-2 cells was performed as previously described by our group (2, 3, 20) in the presence or absence of an anacardic acid (25 μM; 4 h). After labeling was completed, the A subunit WT and S175A proteins were immunoprecipitated from precleared lysates using the M2 anti-FLAG monoclonal antibody, and immunoblotting was performed with an anti-FLAG antibody raised in mouse directly coupled to HRP (1:100,000 dilution). The probed protein and phosphorylated proteins were then visualized and quantified as described in the previous section (3).

Immunolabeling of wound-healing assays of transfected Caki-2 cells. Caki-2 cells were transfected as above using the WT or S175A A subunit constructs, plated on coverslips, and then the monolayers were subjected to a wounding assay as described above, in the absence or presence of an anacardic acid. After 4 h, the monolayers were immunolabeled with concanavalin A-CY3 for 3 min before fixation to label the plasma membrane, followed by a brief wash in PBS containing Mg²⁺ and Ca²⁺. Cells were then fixed and immunolabeled with the anti-FLAG antibody, imaged, and quantification was performed as described above (41).

Extracellular acidification assays of transfected cells. Caki-2 cells were transiently transfected using the X-tremeGENE transfection reagent with either FLAG-tagged WT A subunit or the S175A mutant (8 μg of plasmid DNA). One day after transfection, cells were seeded onto 24-well plates for another day. The cells were then incubated in medium containing vehicle or anacardic acid. pH₄ experiments were normalized to the value determined for control cells (indicating transfection with and expression of the A subunit mutants) under untreated conditions by comparing the images obtained at 3 and 5 h for each plate. They then evaluated the migration of GFP-expressing cells between the 5- and the 7-h postwounding images (after 2 h of treatment with anacardic acid). The evaluators scored a 2 if there was significant movement of the majority of the cells from the edge of the wound to the denuded area at time interval 3–5 or 5–7 h postwounding. The evaluators gave a score of 1 if there were approximately equal numbers of GFP-expressing cells that had significant movement vs. no significant movement. A score of 0 was given if there was no significant movement of the majority of GFP-expressing cells. Two to three images per plate per time point were evaluated. The scores of all evaluators for each plate and time point were averaged. At least three plates for each A subunit mutant were evaluated.

RESULTS

Aurora kinase A and the V-ATPase are coexpressed in the Caki-2 kidney carcinoma cell line. In most nondoning normal epithelial cells, AURKA is associated with the centrosome (10, 11), a cellular compartment that is not known to express the V-ATPase. Due to the potential importance of the V-ATPase in the process of metastasis, we investigated whether the A subunit was expressed in close proximity to AURKA in kidney carcinoma cells by performing immunofluorescence labeling of Caki-2 cells grown on coverslips (Fig. 2). Confocal images revealed that both AURKA (red) and the E subunit of the V-ATPase (green) are abundant in this cell line of kidney carcinoma origin. Confocal image stacks revealed that AURKA is expressed in the Caki-2 cell cytoplasm (Fig. 2A, left), while the V-ATPase E subunit is present in both the cytoplasm and outlining the plasma membrane of these cells, which do not appear to have clear apical vs. basolateral domains (Fig. 2A, middle), and partially overlaps the distribution of AURKA (Fig. 2A, right). Similarly, AURKA (Fig. 2B, left) is coexpressed in Caki-2 cells along with the V-ATPase A subunit (Fig. 2B, middle), and both proteins colocalize in some intracellular compartments by immunofluorescence labeling (Fig. 2B, right). Immunoblots of cell lysates were also performed, and all three phosphorylated Aurora kinase isoforms were identified in HEK-293 cells, yet only bands that likely represent phospho-AURKA and -Aurora kinase B were consistently detected in Caki-2 cells (Fig. 2C, top 2 arrows). There are more AURKA isoform-related reagents, such as an activator, which are not available for Aurora kinases B or C, so we have concentrated on studying the effects of AURKA on the V-ATPase in the human kidney carcinoma cell line Caki-2. As there was sig-
significant colocalization of V-ATPase subunits with AURKA in Caki-2 cells, we next performed immunoprecipitation of AURKA from both HEK-293 cells and Caki-2 cells (Fig. 2D) followed by immunoblotting with an antibody against the V-ATPase V0 sector a4 subunit (ATP6V0A4). We used HEK-293 cells as an additional cell line to confirm our findings as these cells are highly undifferentiated. We detected coinmumoprecipitation of the V0 a4 subunit with AURKA in both cell lines. We then proceeded to evaluate whether activating AURKA had an effect on the subcellular distribution of the V-ATPase.

**AURKA modulates V-ATPase expression at the leading edge of Caki-2 cells.** We hypothesized that AURKA phosphorylation of the V-ATPase decreases pHo and thus increases the metastatic potential of Caki-2 cells. The V-ATPase in vertebrate tissues becomes active in intracellular vesicles as the V0 and V1 sectors assemble. An important regulatory mechanism of the V-ATPase is the fusion of active pump-containing vesicles to the plasma membrane (9). To characterize changes in endogenous V-ATPase subcellular localization in Caki-2 cells in response to AURKA activity modulation, cell monolayers grown on glass coverslips were wounded (see MATERIALS AND METHODS) and incubated in the absence or presence of the AURKA activator anacardic acid (25 μM). This wound assay procedure allowed for the formation of a leading edge in the cells around the original scraping of the monolayer. The cells were then fixed after the different treatments and labeled with an antibody against the E subunit of the V-ATPase, a marker for active V-ATPase at the membrane (7, 30, 47) (Fig. 3A, left). To quantitate the fluorescence as a function of distance from the leading edge, the fluorescence intensity profile was determined using a previously described method (41). Immunofluorescence profile analysis showed an approximately equal distribution of V-ATPase immunostaining at the leading edge vs. in the cytoplasm in cell monolayers incubated with vehicle (Fig. 3A, top, and B). However, cell monolayers incubated with the AURKA activator had significantly increased V-ATPase-associated fluorescence at the leading edge relative to the cytosol with a ratio ~1.8:1 (Fig. 3A, top, and B). We also observed that under control conditions some of the Caki-2 cells growing at the edge of the monolayer had cell projections that were immunolabeled with the E subunit of the V-ATPase (Fig. 3C). When these cells were treated with Aurora kinase inhibitor III, these projections were consistently absent from the cells growing at the monolayer edge (Fig. 3C, arrow).

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**Fig. 2. AURKA and the vacuolar (V)-ATPase in the Caki-2 kidney cancer cell line are coexpressed in cytoplasm and form a complex.** A: confocal stacks and X-Z and Y-Z reconstructions of Caki-2 cells immunolabeled for AURKA (red) and the V-ATPase E subunit (green). B: epifluorescence images of immunolabeling for AURKA (green) and the V-ATPase A subunit (red) show extensive colocalization of V-ATPase subunits with mislocalized cytosolic AURKA with V1 sector subunits of the V-ATPase. Scale bar = 10 μm. C: immunoblot of HEK-293 and Caki-2 cell lysate using the pThr288-Aurora A antibody (top) and β-actin (bottom). The bands in the top blot (arrows) likely represent the 3 phosphorylated Aurora kinase isoforms, as determined by their known molecular weights. D: representative immunoprecipitation (IP) experiments from HEK-293 cells and Caki-2 cells, using mouse anti-AURKA antibody. The samples were then immunoblotted for the V-ATPase V0 sector a4 subunit (ATP6V0A4; top) and AURKA (bottom) using antibodies raised in rabbits. These results are consistent with the existence of protein complexes containing AURKA and V-ATPase V0 and V1 sector subunits in cell lines of kidney origin.
We then confirmed that treatment with anacardic acid activated AURKA in this cell line by monitoring levels of the activated form of AURKA phosphorylated at Thr-288 (pThr288-AURKA; Fig. 4) (52). Because the anti-pThr288 antibody also recognized other Aurora kinase isoforms, AURKA was first immunoprecipitated from cells incubated in the absence and presence of anacardic acid, and those samples were then blotted with the pThr288-AURKA (Fig. 4A, top). This signal was then normalized to total immunoprecipitated AURKA (Fig. 4A, bottom), revealing that anacardic acid treatment increased active kinase levels (Fig. 4B).

AURKA activation increases cell surface expression of V-ATPase in Caki-2 cells. As we had detected the presence of the cytoplasmic V-ATPase E subunit at the cell edge in the presence of AURKA activation (Fig. 3), we then performed cell surface biotinylation assays to detect whether one of the V-ATPase subunits from the V₀ membrane sector (the “a4” subunit or ATP6V0A4) that has an extracellular domain was also more abundant at the plasma membrane with AURKA activation. Indeed, anacardic acid increased expression levels of V-ATPase a4 subunit at the wound edge in Caki-2 cells treated with AURKA activator, while it is more diffuse in the cytoplasm in cells incubated in vehicle (DMSO). *P < 0.05 relative to vehicle control. C: immunolabeling against the V-ATPase E subunit (green) in cells treated with the Aurora kinase inhibitor III (50 nM) vs. vehicle control revealed that the inhibitor qualitatively decreased the number of cellular projections (arrow) 4 h after a scratch assay.
The V-ATPase-dependent acidification rate \([\text{final buffer pH} - \text{initial buffer pH}] / \Delta t\) was measured before and after the addition of the V-ATPase inhibitor bafilomycin during a 20-min incubation period. The experiments were performed in the absence or presence of the AURKA activator anacardic acid in Caki-2 cells incubated in CCD media in the absence or presence of AURKA activator anacardic acid (25 μM, 4 h). An anacardic acid treatment induced a significant increase in phosphorlated pThr288-Aurora A compared with untreated cells. *\(P < 0.05\) relative to vehicle control; \(n = 3\).

**Fig. 4.** The AURKA activator anacardic acid increases AURKA phosphorylation in Caki-2 cells. **A:** AURKA activity as measured by immunoblotting of pThr288-aurora A for IP AURKA (top) relative to AURKA as a loading control (bottom) in Caki-2 cells incubated in CCD media in the absence or presence of AURKA activator anacardic acid (25 μM, 4 h). **B:** anacardic acid treatment induced a significant increase in phosphorlated pThr288-Aurora A compared with untreated cells. *\(P < 0.05\) relative to vehicle control; \(n = 3\).

**Fig. 5.** AURKA activation increases cell surface expression of the V-ATPase in Caki-2 cells. **A:** representative immunoblots of V-ATPase a4 subunit (ATP6V0A4 in the V-ATPase V0 integral membrane domain) in whole cell lysates and cell surface biotinylation protein samples from Caki-2 cell monolayers grown under either control conditions or treated with anacardic acid for 60 min. **B:** relative V-ATPase band intensity measured in the biotinylated fraction and normalized to cell lysates revealed that the presence of the AURKA activator more than doubled V-ATPase surface expression relative to vehicle control. *\(P < 0.05\); \(n = 3\).

**Fig. 6.** AURKA regulates V-ATPase activity at the plasma membrane in cultured Caki-2 cells. The rate of extracellular acidification in each set of treatments was measured in a low-buffering capacity solution in the extracellular buffer of cells preincubated in the absence or presence of anacardic acid. The mean ± SE rate of extracellular acidification ([final buffer pH − initial buffer pH]/\(\Delta t\)) was obtained in the absence and presence of bafilomycin, a specific V-ATPase inhibitor, for each treatment condition. There was negligible cell death after these experiments as determined by a trypan blue exclusion assay across conditions (not shown). *\(P = 0.05\); \(n = 3\).

**Fig. 7.** AURKA-dependent phosphorylation of the V-ATPase A subunit in vitro requires Ser-175. We have previously shown that the V-ATPase A subunit is directly phosphorylated by PKA at Ser-175 (3), a residue located within a larger phosphorylation consensus sequence for Aurora kinases (Fig. 1). To test whether V-ATPase phosphorylation could be induced by AURKA, we expressed either FLAG-tagged WT, S175A (phosphorylation-deficient), or S175D (phospho-mimetic) A subunit constructs in HEK-293 cells. We then immunoprecipitated the different A subunits using an anti-FLAG antibody (2, 3). The in vitro \(\gamma\text{-}32\text{P}\text{-ATP}\) labeling of WT, S175A, and S175D A subunits treated with purified active recombinant AURKA protein were assessed in the presence of [\(\gamma\text{-}32\text{P}\text{-ATP}\)] (Fig. 7A, top). After normalizing to the respective FLAG-tagged A subunit expression signal on the immunoblot from the same membrane (Fig. 7A, bottom), both mutants (S175A and S175D) showed a significant reduction in phosphorylation by ~70% compared with the WT-A subunit (Fig. 7B), confirming AURKA-dependent phosphorylation at this residue in vitro. Furthermore, we detected apparent binding of AURKA to the V-ATPase A subunit, as autophosphorylated kinase (\(~50\text{kDa}\)) coprecipitated with the immunoprecipitated A subunit (Fig. 7A, top). No other significant phosphorylated bands were observed in the phosphoscreen. These results also support our findings reported above that active AURKA and the V-ATPase A subunit form a complex in Caki-2 cells.

**AURKA-dependent phosphorylation of the V-ATPase A subunit occurs at Ser-175 in intact Caki-2 cells.** To determine whether V-ATPase A subunit Ser-175 is a target for AURKA-dependent phosphorylation in intact Caki-2 cells, we compared
orthophosphate labeling of the FLAG-tagged WT and the S175A mutant A subunits expressed into Caki-2 cells under control conditions or following treatment with the AURKA activator anacardic acid (25 μM) (Fig. 8A). The phosphate labeling signal on the phosphoscreen that corresponds to the molecular weight of the A subunit (Fig. 8A, arrow, top) was normalized to the respective FLAG-tagged A subunit expression signal on the immunoblot (Fig. 8A, bottom) from the same membrane. AURKA activation significantly increased the phosphorylation level of the WT-A subunit to about two times that of vehicle control-treated cells, indicating that AURKA activation contributes to A subunit phosphorylation in kidney carcinoma cells (Fig. 8B). In addition to the A subunit, anacardic acid stimulated the phosphorylation of several other proteins that coimmunoprecipitated with the V-ATPase A subunit, as demonstrated by the labeling of additional bands on the phosphoscreen (Fig. 8A, top). Vehicle control-treated cells expressing the A subunit S175A mutant had comparable levels of phosphorylation to vehicle control-treated cells expressing the WT-A subunit (Fig. 8B, lanes 1 and 3). This result indicates that under the conditions of the assay, AURKA is not significantly activated unless an activator, anacardic acid, is applied. Moreover, in contrast to the AURKA-induced increase in phosphorylation observed in WT-A subunit-expressing cells, no change in phosphorylation of the S175 mutant A subunit was observed in anacardic acid-treated cells (Fig. 8B, lane 4), confirming that Ser-175 is likely a target of phosphorylation by AURKA in Caki-2 cells.

AURKA regulates V-ATPase A subunit expression at the leading edge of Caki-2 cells via Ser-175. To examine the role of A subunit residue Ser-175 in the subcellular localization of the V-ATPase in response to AURKA modulation, we transfected Caki-2 cells with either the FLAG-tagged WT V-ATPase A subunit or the S175A A subunit mutant. Two days later, cell monolayers grown on glass coverslips were wounded as described above and incubated in CCD media in the absence or presence of anacardic acid. We then performed immunofluorescence labeling of the A subunit using the anti-FLAG antibody followed by confocal fluorescence microscopy (Fig. 9A). In cells transfected with the WT A subunit, AURKA activation significantly increased the V-ATPase-associated fluorescence at the leading edge (colabeled with concanavalin A-CY3, in red) compared with untreated cells. However, cells transfected with the S175A mutant A subunit displayed a more predominant cytosolic distribution under both untreated and AURKA activator-treated conditions (Fig. 9, A and B). Together, these results suggest that A subunit residue Ser-175 plays an important role...
functional role in the AURKA-mediated cell membrane accumulation of the V-ATPase in kidney cancer cells.

Ser-175 in the A subunit is required for bafilomycin-sensitive V-ATPase-dependent extracellular acidification with AURKA activation in Caki-2 cells. To more directly assess the role of a subunit residue Ser-175 in the activity of the V-ATPase at the plasma membrane, we monitored pHo changes under acute treatment conditions (Fig. 10). Moreover, there was a trend toward decreased proton secretion in cells expressing the phosphorylation-deficient mutant compared with WT expressing cells (Fig. 10, **). Of note, there was comparable expression of each FLAG-A subunit in different conditions by immunoblotting (not shown). Taken together, the results presented so far suggest that AURKA-mediated phosphorylation of the A subunit at Ser-175 in Caki-2 cells is necessary for it to promote V-ATPase activity at the plasma membrane.

In Caki-2 cells, AURKA activation increases coinmunoprecipitation of AURKA to the V-ATPase A subunit in a Ser-175-dependent manner. To more directly assess the role of AURKA in regulation of the V-ATPase, we then tested whether AURKA interaction with the V-ATPase A subunit is modulated by AURKA phosphorylation of the A subunit at Ser-175. We used a specific antibody that recognizes AURKA to probe immunoblots of immunoprecipitated FLAG-tagged A subunits (WT and S175A) (Fig. 11A). In the presence of an AURKA activator, there was more coinmunoprecipitated AURKA with the WT-A subunit of V-ATPase. On the other hand, the phosphorylation-deficient S175A mutant A subunit showed no significant changes in the coinmunoprecipitation levels with AURKA whether the cells had been incubated in the presence or absence of the AURKA activator. These levels of coinmunoprecipitation of the S175A subunit with AURKA were not different from those observed in untreated cells expressing the WT-A (Fig. 11B). These results suggest that AURKA complexes with the V-ATPase A subunit in an AURKA phosphorylation-dependent manner.

Ser-175 in the V-ATPase A subunit is important for Caki-2 cell motility. To test the role of the V-ATPase and residue Ser-175 in the A subunit in Caki-2 cell motility, which may correlate with metastatic potential, we performed wound-healing scratch assays on untransfected Caki-2 cells and on Caki-2

Fig. 9. AURKA regulates V-ATPase A subunit expression at the leading edge of Caki-2 cells via Ser-175. A: representative confocal images of wound assays performed on Caki-2 cells transiently transfected with either the WT or S175A mutant A subunit in the absence or presence of anacardic acid. After the different treatments, the cell monolayers were incubated with concanavalin A (red), fixed, and then labeled with an anti-FLAG antibody (green) followed by TOPRO-3 to stain the nuclei (blue). Fluorescence intensity profiles (see Fig. 3) were determined for the V-ATPase A subunit as the mean gray value. All confocal images shown were acquired using identical laser setting in cells immunolabeled on the same day and under the same conditions. B: ratio of the mean gray value corresponding to the leading edge and the cytoplasm areas were calculated for >45 cells/condition and expressed as the ratio of the fluorescence intensity measured in the leading edge vs. that in the cytoplasm for the V-ATPase A subunit. V-ATPase concentrates at the wound edge in Caki-2 cells treated with the AURKA activator, while it is more diffuse in the cytoplasm in cells incubated in vehicle control, *P < 0.05 relative to vehicle control.

Fig. 10. The phosphorylation-deficient A subunit S175A mutant decreases V-ATPase-dependent extracellular acidification with AURKA activation in Caki-2 cells. A: the mean ± SE rate of extracellular acidification [(final buffer pH – initial buffer pH)/Δt] was obtained in the absence and presence of bafilomycin for each treatment condition with expression of WT vs. S175A mutant FLAG-tagged A subunit. There was negligible cell death after these experiments, as determined by a trypan blue exclusion assay across conditions (data not shown). Data were obtained from 4 separate experiments/condition. Immunoblots of cell lysates from these experiments showed no significant differences in FLAG-A subunit expression across the 4 conditions (not shown). *P < 0.05. **P = 0.06; n = 4.
motility and extracellular acidification, further AURKA activation, although Ser-175 phosphorylation is important for Caki-2 cell monolayer closure (not shown). These results likely indicate the role of AURKA in the migration of kidney cancer cells. We then observed that the phosphorylation-deficient S175A mutant A subunit significantly inhibited cell migration in Caki-2 cells compared with the phospho-mimetic S175D mutant or the WT A subunit (Fig. 12, C and D). This finding is consistent with the requirement of an active V-ATPase at the membrane of Caki-2 cells for cell migration and thus, potentially, metastasis. However, when we added anacardic acid to the media of the transfected cells recovering from the wound, the motility scores increased substantially and were not significantly different among the three transfection conditions (WT, S175A, or S175D; not shown). Together, these results indicate that although Ser-175 phosphorylation is important for Caki-2 cell motility and extracellular acidification, further AURKA activation likely engages additional V-ATPase-independent pathways that promote motility of these cancer cells.

DISCUSSION

The V-ATPase is an important and ubiquitous membrane transport protein that has been linked to cancer progression and metastasis, especially in breast malignancies (13, 18). An acidic extracellular milieu contributes to the activation of extracellular cathepsins that in turn promote invasion of malignant cells through the extracellular matrix with metastasis from one location to a more distant one.

Our group previously identified Ser-175 in the V-ATPase A subunit as a key phosphorylation site required for accumulation of an active proton pump at the membrane of kidney cells (3). Initially, this phosphorylation was characterized as a PKA target, downstream of an intracellular signaling cascade involving sAC activation and cAMP production. Recently, Ser-175 was also found to be relevant in regulation of the acidification of endosomes containing a G protein-coupled receptor (16). The relevance of this phosphorylation site for V-ATPase regulation is again supported by the work presented in this study. Here, we show that Ser-175 is required for activation of the V-ATPase at the membrane downstream of the activation of aberrantly overexpressed, dysregulated, cytosolic Aurora kinase in kidney cancer cells.

The study of Aurora kinases is important because this family of highly regulated Ser/Thr kinases controls cytokinesis and mitosis (reviewed in Refs. 10 and 11). Dysregulation of Aurora kinases can lead to mitotic arrest, aneuploidy and cell death. During normal cellular processes, for example, AURKA binds to microtubules and associates with the spindle poles. This kinase also participates in centrosome maturation and initiation of mitosis (11). High levels of Aurora kinases in tumor biopsy samples are correlated with increased metastatic potentials of various malignancies and poorer prognoses for patients compared with the same malignancies without increased levels of Aurora kinases (11, 25). Under pathological conditions, increased Aurora kinase activity causes genetic instability and sequential mutations that confer a survival advantage to the cell. All of these changes increase the risk of metastases. The role of Aurora kinases has been studied in kidney cancer cells, where it becomes dysregulated and overexpressed throughout the cell (26). Aurora kinase levels are high in ~70% of RCC samples, suggesting that this kinase may play an important role in the pathogenesis of the disease (26). Here, we used the human kidney cancer cell line Caki-2, which is highly metastatic, and we have shown that AURKA is abundant and overexpressed in the cytosol, rather than in the centrosomal domain. This finding is consistent with a recent study showing that Caki-2 cells have a dysregulated expression of AURKA throughout the cytosol, as opposed to nondividing, noncancorous cells of kidney origin (33).

In many cancers, AURKA inhibitors have been tested as therapeutic agents (24). However, in the phosphorylation assay in Caki-2 cells (Fig. 8), the baseline level of [32P]orthophosphate incorporation was very low, suggesting that Caki-2 cells under the labeling conditions of the assay did not have significantly active AURKA. This low AURKA activation state is supported by the finding that when we used the Aurora kinase inhibitor III we did not observe any significant changes in subcellular localization or phosphorylation state of the V-ATPase A subunit (not shown). These results likely indicate that the conditions of our assays do not represent the level of activation of AURKA in a metastatic tumor.

The V-ATPase is ubiquitously expressed in eukaryotic organisms, where it participates in the acidification of intracellular organelles and vesicles as well as extracellular domains in, for example, kidney epithelial cells (15). In cancer cells, several types of cellular acid extrusion mechanisms have been identified, such as Na+/H+ exchange and H+ pumping via the V-ATPase. These transport processes are robust and contribute to the maintenance of a pH that is more alkaline than the
extracellular domain in tumors, while nonmalignant cells usually have a pH_i slightly more acidic than in the interstitium. Increased aerobic glycolysis (the Warburg effect) is a hallmark of malignant cancers, yet it is not completely understood why malignant tumors undergo such a metabolic modification (49). It has been proposed that the increase in glycolysis leads to intracellular acidification in cancer cells that in turn activates the V-ATPase (reviewed in Ref. 45). Moreover, this V-ATPase activation leads to an alkaline pH_i in tumors, which in turn activates glycolysis. This model is consistent with the finding that acidosis is the signal for V-ATPase activation in kidney epithelial cells (30). In the future, it will be important to evaluate how dysregulated AURKA is affected by cellular metabolic rate and by changes in pH_i.

The work presented here demonstrates a clear increase in V-ATPase activation at the membrane of Caki-2 cells following treatment with an activator of AURKA, thus uncovering a novel mechanism of proton extrusion from kidney cancer cells. In addition, intracellular expression of a V-ATPase A subunit Ser-175 mutant that cannot undergo phosphorylation by either AURKA or PKA reduces acid secretion from cells as well as cellular motility. Of note, introduction of this mutant did not affect cell viability or response to activation by AURKA of pathways regulating cell motility. Although the evaluation of these pathways is beyond the scope of this study, it is possible that AURKA affects other acid-secreting pathways in kidney cancer cells. For example, in squamous cell carcinoma AURKA overexpression promotes cell migration through activation of the serine/threonine kinase Akt. Conversely, AURKA inhibition abrogated cell migration and induced cell death in other studies (19). In addition, it has been reported that overexpression of AURKA or induction of its activity en-

Fig. 12. AURKA activation and V-ATPase Ser-175 phosphorylation increase Caki-2 cell motility. Brightfield images of untransfected Caki-2 cell confluent monolayers at 0 and 4 h postexposure to AURKA activator (anacardic acid, 25 μM) after wounding of the monolayer are shown. A: wound closure was measured in each image at 3 different locations (white arrows); approximate wound edges (yellow lines). The wound repair was then tracked by obtaining 3 random microscopic images of each wound at each time point with a total of 9 images/condition and time point. B: quantification of wound closure. The data represent the mean distance of cell migration of the area at 4 h after wounding normalized to the control at each time point. Values are means ± SE of 3 independent experiments. *P < 0.05 relative to vehicle control. C: representative images at 3 and 5 h after wounding monolayers of Caki-2 cells transfected with pTracer-S175D (top), -S175A (middle), or -WT (bottom). The top white line in each image demarcates the approximate upper edge of the original scratch at time 0. For illustrative purposes, different colored asterisks (*) were placed in each of the images to demonstrate movement of individual cells between 3 and 5 h. For each condition, 3 typical GFP-expressing cells were identified at the 3-h time point and labeled with a large asterisk (*) of different colors (red, white, and blue). In the 5-h time point panels, the prior 3-h position of each of the cells was marked for reference as well as the current 5-h position of each of the cells, indicated by the small asterisk (*) of the same color. D: means ± SE cell migration scores from 4 blinded observers who scored the overall relative movements of green cells under the 3 conditions as described in MATERIALS AND METHODS. *P ≤ 0.01 relative to WT and S175D mutant; n = 3–4 wound assays/condition.
hances mammary cell migration by activating cofilin, an actin-binding protein (53). Taken together, these data and our findings suggest that, in addition to the V-ATPase pathway, there are additional pathway(s) involved in AURKA-mediated cell migration. Further characterization of the precise roles of the various pathways involved in AURKA-induced cell migration and interactions would be an important goal for future studies. In addition, whether the effects of AURKA activation are dependent on changes in glycolysis or changes in pH, (or both) are important future issues to address.

Aurora B and C may also phosphorylate and regulate the V-ATPase, but the pharmacological tools to evaluate these isoforms are not readily available for further studies at this point. Another interesting point that we have uncovered is that the V-ATPase forms a complex with AURKA, provided that the kinase and the pump are expressed in the same cellular microdomain, which does not normally occur in nonmalignant cells. In summary, our findings suggest that AURKA may participate in the establishment and/or maintenance of an active V-ATPase complex at the plasma membrane in carcinoma cells and thus contribute to the metastatic potential of these cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES