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Phosphorylation of Carbonic Anhydrase IX Controls Its Ability to Mediate Extracellular Acidification in Hypoxic Tumors

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Abstract

In the hypoxic regions of a tumor, carbonic anhydrase IX (CA IX) is an important transmembrane component of the pH regulatory machinery that participates in bicarbonate transport. Because tumor pH has implications for growth, invasion, and therapy, determining the basis for the contributions of CA IX to the hypoxic tumor microenvironment could lead to new fundamental and practical insights. Here, we report that Thr443 phosphorylation at the intracellular domain of CA IX by protein kinase A (PKA) is critical for its activation in hypoxic cells, with the fullest activity of CA IX also requiring dephosphorylation of Ser448. PKA is activated by cAMP, which is elevated by hypoxia, and we found that attenuating PKA in cells disrupted CA IX-mediated extracellular acidification. Moreover, following hypoxia induction, CA IX colocalized with the sodium-bicarbonate cotransporter and other PKA substrates in the leading edge membranes of migrating tumor cells, in support of the concept that bicarbonate metabolism is spatially regulated at cell surface sites with high local ion transport and pH control. Using chimeric CA IX proteins containing heterologous catalytic domains derived from related CA enzymes, we showed that CA IX activity was modulated chiefly by the intracellular domain where Thr443 is located. Our findings indicate that CA IX is a pivotal mediator of the hypoxia-cAMP-PKA axis, which regulates pH in the hypoxic tumor microenvironment. *Cancer Res*; 71(24); 7558–67. ©2011 AACR.

Introduction

Carbonic anhydrase IX (CA IX) is a highly active α -carbonic anhydrase isoform expressed in a broad range of solid tumors, in which it protects tumor cells from hypoxia and acidosis in the microenvironment (1, 2). CA IX functions as an important component of a pH-regulating machinery that is activated in response to hypoxia and/or oncogenic alterations (3). It participates in CO₂ diffusion and bicarbonate import as the extracellular constituent of a metabolon—a functional and spatially oriented complex with bicarbonate transporters (4, 5). In the metabolon, CA IX catalyzes hydration of pericellular CO₂ and thus ensures high local production of bicarbonate ions that are available for direct influx by the transporters. This results in intracellular neutralization, which is required for tumor cell

survival. At the same time, protons as by-products of this CO₂ hydration reaction remain in the pericellular space, acidifying the microenvironment, and thereby supporting tumor cell invasiveness.

In line with this concept, CA IX is expressed and activated in cells exposed to chronic hypoxia, which is inherently linked to acidosis. Transcription of the CA IX-encoding gene is strongly activated by the hypoxia-inducible HIF-1 transcription factor that binds an HRE element localized next to the transcription initiation site (6). Hypoxia and/or acidosis also induce the catalytic activity of CA IX thus providing an effective adaptive cellular response to microenvironmental stresses, including pH regulation and cell migration/invasion (7–10). Because hypoxia is associated with poor prognosis and worse response to treatment, CA IX as its surrogate marker and functional component possesses clinical potential as a prognostic/predictive factor and target for anticancer treatment (11).

Functional inhibition of CA IX has been proposed as an attractive option for the therapeutic targeting of various hypoxic tumors (12). In this regard, major efforts are being directed toward pharmacologic inhibition of the catalytic activity of CA IX (13). However, our recent studies have revealed that the catalytic domain is not the only determinant of CA IX activity and that the flanking regions play a role in CA IX performance. Particularly, an intact intracellular (IC) tail has been shown to be critical for the proper functioning of CA IX as mutations in its juxtamembrane region abolish the CA IX-mediated extracellular acidification in hypoxia (14). However, molecular events underlying this inside-out signaling have

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remained unexplored. Here, we show for the first time that phosphorylation of the IC tail is involved in the control of CA IX ability to regulate pH, and that the Thr443 residue and cAMP-dependent protein kinase A (PKA) are key players in this phenomenon.

Materials and Methods

Cell culture and inhibitor treatment

Madin-Darby canine kidney (MDCK), HT-29, A549, HeLa, and HEK293T/17 cells were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum (BioWhittaker). Cell lines were passaged for a maximum of 3 months, after which fresh seed stocks were thawed for experimental use. They were evaluated routinely for morphology and expression of CA IX. The cells (4×10^5 in 1.5 mL of medium) were plated in 3 cm dishes 24 hours prior to 24 to 48 hours incubation in hypoxia (2% O₂, 2% H₂, 5% CO₂, 91% N₂, anaerobic workstation, Ruskinn Technology). Normoxic dishes were maintained in an incubator with 5% CO₂. pH of the culture medium was measured at the end of each experiment and lactic acid was determined with the Lactate Reagent kit (Trinity Biotech). The fluorescent carbonic anhydrase inhibitor [fluorescein isothiocyanate (FITC)-(CAI)] was synthesized and dissolved as described (7), and added to cells at 0.1 mmol/L final concentration.

Cloning and transfection

In vitro mutagenesis of Thr443 and Ser448 to Gly and/or Asp was carried out by inverse PCR using the pSG5C-MN/CA9 expression plasmid as a template (15, GenBank no. X66839). Chimeric CA IX was generated by replacing the original catalytic domain (aa 137-390) with corresponding regions of either CA II (aa 2-259) or CA XII (aa 29-289). pcDNA3.1+dnPKA plasmid encoding the murine dominant-negative mutant of PKA (DN-PKA) regulatory subunit I was obtained by recloning the dnPKA sequence from M7 pdnPKA-GFP (16,716 Addgene plasmid; ref. 16) into pcDNA3.1+ plasmid to remove GFP that would interfere with imaging of the FITC-labeled inhibitor of CA IX. Details of cloning procedures are provided in Supplementary Information and Supplementary Table S1.

MDCK cell lines constitutively expressing the wild-type (wt) CA IX protein or its mutants were obtained by cotransfection of plasmids containing CA9 cDNA or its mutated versions with pSV2neo plasmid in a 10:1 ratio using NeoFectin transfection reagent (Mid-Atlantic Biolabs). The cells cotransfected with empty pSG5C and pSV2neo were used as mock controls. All transfected cell lines were frozen as seed stock at early passage.

HEK293T/17 cells were cotransfected with various combinations of the following plasmids: pCalpha-EV-PKA encoding CS-PKA (pCα, catalytic subunit C alpha, 15,310 Addgene plasmid; ref. 17), pcDNA3.1+dnPKA encoding DN-PKA (pdn), pSG5C-MN/CA9 encoding CA IX (pCA9) and respective empty plasmids by NeoFectin in the ratio 10:1, 4 μg of plasmid DNA in total.

ELISA

Transfected cells were plated in parallel in 2 microplates at a density of 10^4 cells per well, in quadruplicates. The first plate

was fixed in methanol, the second plate was assayed without fixation. The cells were then incubated at 37°C for 30 minutes with a biotinylated M75 MAb diluted 1:5,000 (200 ng/mL) in PBS. The amount of bound antibody was determined with the peroxidase-conjugated streptavidin (Pierce).

Immunoblotting and immunoprecipitation

The cells were harvested in ice-cold radioimmunoprecipitation assay buffer with inhibitors of proteases (Complete, Roche) and phosphatases (Phosphatase inhibitor cocktail 1 and 2, Sigma-Aldrich). The proteins (50 μg/lane) were resolved in 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Active PKA was detected by Phospho-PKA C-(Thr197) antibody, total PKA by PKA C-Alpha antibody, and PKA phosphorylated proteins were detected by Phospho-PKA Substrate (RRXS*/T*) antibody (all from Cell Signaling Technology, at 1:1000 dilution). Total CA IX was detected by M75 antibody (15), CA IX phosphorylated at Thr443 by P-T443 CA IX antibody (1:5,000; GenScript) and actin was visualized by Actin (C11) antibody (Santa Cruz Biotechnology).

For immunoprecipitation, cell lysates were precleared with Protein A Sepharose CL-4B (GE Healthcare Bio-Sciences AB) and incubated overnight at 4°C with 1 μg of phospho-Thr443 antibody bound onto 50 μL of beads per sample. Immuno-complexes were separated by SDS-PAGE and blotted. Phosphorylated CA IX was detected by a peroxidase-conjugated M75 MAb.

In vitro phosphorylation and dephosphorylation

A portion of CA9 cDNA representing the IC tail (nt 1,345–1,419) was cloned into pGEX-4T1 plasmid (GE Healthcare Bio-Sciences AB) from pSG5C-MN/CA9 or its mutated variant pSG5C-T → A (Thr443 substituted with Ala). The fusion proteins (1 μg per reaction) were bound onto Glutathione Sepharose 4B (GE Healthcare Bio-Sciences AB). The beads were washed with kinase buffer (20 mmol/L magnesium acetate, 40 mmol/L Tris-HCl, pH 7.4) and the reaction was started by addition of the kinase buffer with 1 mmol/L DTT, 1 μL of CS-PKA (Promega), 0.2 mmol/L ATP (Sigma-Aldrich) and 1 μL of [γ -³²P]ATP. The samples were incubated at 30°C for 30 minutes and reaction was stopped by 2× Laemmli sample buffer. Finally, the ³²P signal was assessed by SDS-PAGE and autoradiography.

For *in vitro* dephosphorylation assay, purified protein phosphatase 2 (PP2A) was used in A/C heterodimer (Upstate) or A/C/B55 heterotrimer form (prepared in-house). The assay was carried out in phosphatase buffer (150 mmol/L NaCl, 0.25% Nonidet P-40, proteases inhibitors, 50 mmol/L Tris-HCl) containing 0.2 unit of PP2A dimer or trimer and 1 μg *in vitro* phosphorylated glutathione S-transferase (GST)-IC wt protein at 30°C for 30 minutes. Termination of the dephosphorylation reaction and assessment of the ³²P signal were carried out analogously to the phosphorylation assay.

cAMP direct immunoassay and 8-Br-cAMP treatment

MDCK-CA IX cells were incubated in normoxia or hypoxia for 32 hours and were lysed in 0.1 mol/L HCl. The assay was carried out with 200 μg of proteins per sample using the cAMP

Direct Immunoassay Kit (Biovision Research Products). To stimulate PKA activity in normoxia, MDCK-CA IX cells were grown for 48 hours in normoxia, treated with 0.5 mmol/L 8-Br-cAMP (Sigma) for 1 hour, lysed and analyzed for CA IX Thr443 phosphorylation by immunoprecipitation and immunoblotting as described above.

Immunofluorescence and confocal microscopy

A549 cells grown on glass coverslips were exposed to hypoxia for 24 hours and fixed in ice-cold methanol at -20°C for 5 minutes. To colocalize sodium-bicarbonate ($\text{Na}^+/\text{HCO}_3^-$) cotransporter 1 (NBC1, SLC4A4) with CA IX, the cells were first incubated with rabbit anti-NBC1 polyclonal antibody raised against aa 338–391 (1:100, AB 3212 Millipore) and then with M75 MAb. Similar procedure was used to colocalize CA IX with phospho-PKA substrates. Anti-mouse and anti-rabbit antibodies conjugated with Alexa Fluor 594 or 488 (1:2,000; Invitrogen) were used as secondary antibodies. Nuclei were stained with DAPI (1:36,000, Sigma-Aldrich). All samples were analyzed by Zeiss LSM 510 Meta confocal microscope. The images were processed in ImageJ using colocalization highlighter plugins.

Migration assays

MDCK cells transfected with pSG5C-MN/CA9, pSG5C-T $\rightarrow$ D/S $\rightarrow$ G, pSG5C-T $\rightarrow$ G/S $\rightarrow$ D plasmids were grown to confluent monolayers, wounded with a pipette tip, and allowed to migrate in presence of hepatocyte growth factor (HGF, 20 ng/mL) in serum-free medium. The wound was photographed at the start of the healing process and after 10 hours. The diameter of the wound was measured and the closure was calculated in %.

For the scatter assay, cell clusters were obtained by rotation of suspended MDCK transfectants for 16 hours. The cell clusters were allowed to attach and spread for 24 hours. The colonies (at least 20 for each cell type) were photographed at the beginning and at the end of the spreading period. Their size was statistically analyzed using Student *t* test.

Results

Mutation of Thr443 modulates CA IX-mediated extracellular acidification

As described earlier, mutations of basic amino acids within the 436 to 444 region of the IC tail abolish CA IX ability to acidify extracellular pH (pHe) and bind the CA IX-selective sulfonamide inhibitor in hypoxia (14). To shed light on the underlying mechanism(s), we investigated the role of phosphorylation. Cytoplasmic portion of CA IX contains 3 possible phosphorylation sites: Thr443, Ser448, and Tyr449. Phosphorylated Tyr449 signals to the Akt/PI3K pathway (18), but no information is available on Thr443 and Ser448.

To examine the role of these putative phosphorylation sites, we generated CA IX mutants with Thr443 or Ser448 individually substituted by glycine, a nonphosphorylatable residue. The corresponding T443 $\rightarrow$ G and S448 $\rightarrow$ G mutants were transfected into MDCK cells, an established model for CA IX-mediated extracellular acidification in

hypoxia (7) and examined for oligomerization and plasma membrane localization. The mutations had no effect on these properties (Fig. 1A–C). However, the T443 $\rightarrow$ G mutant was impaired in its ability to acidify pHe in hypoxia. In contrast, the S448 $\rightarrow$ G mutation resulted in acidosis comparable with the wt protein (Fig. 1D). These results were corroborated by the accumulation of the FITC-labeled CA inhibitor (Fig. 1E), which in this cellular context binds only to the active enzyme (7). This data indicated that Thr443 is instructive to the activation of CA IX.

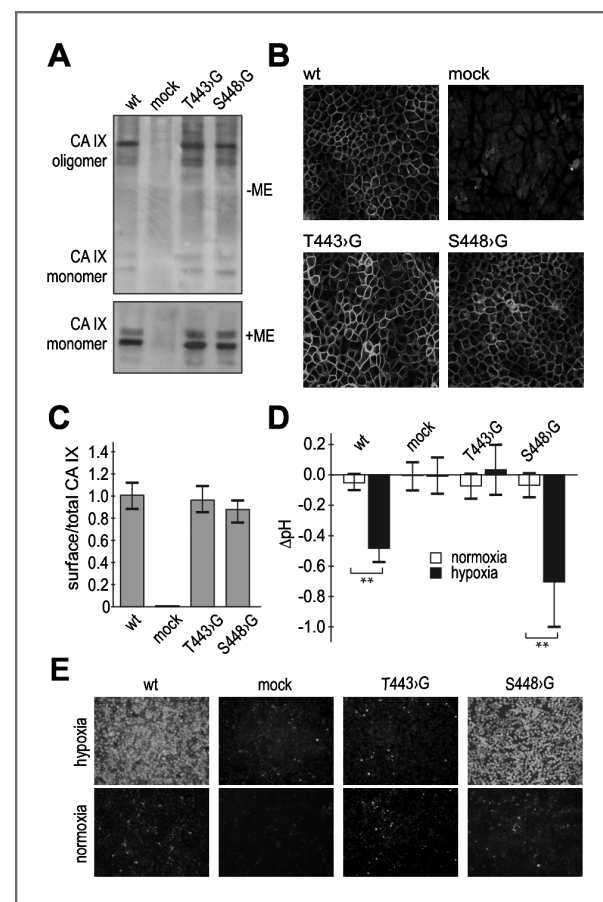


Figure 1. Analysis of CA IX variants with single inactivating mutations in phosphorylation sites Thr443 (T443 $\rightarrow$ G) and Ser448 (S448 $\rightarrow$ G). **A**, immunoblotting analysis of the mutants separated under nonreducing conditions (without β 2-mercaptoethanol, –ME) and reducing conditions (+ME), and visualized with M75 Mab shows their correct molecular weight and oligomerization. The figure shows a representative blot of 3 independent experiments. **B**, immunofluorescence analysis with FITC-conjugated M75 MAb shows plasma membrane localization of both mutants. **C**, cell surface fraction of CA IX assessed by ELISA was calculated as a ratio of absorbance values of live cells (corresponding to cell surface CA IX) and fixed cells (corresponding to both surface and intracellular CA IX). **D**, effect of mutations on pHe was measured in transfected MDCK cells incubated in normoxia and hypoxia for 48 hours. The graph shows differences in pHe values (ΔpH). Data correspond to mean values from 10 independent experiments (**, $P < 0.01$), bars represent SD. **E**, accumulation of FITC-CAI occurred in hypoxic cells expressing wt CA IX and the S448 $\rightarrow$ G mutant, whereas it was diminished in mock-transfectants and in cells expressing the T443 $\rightarrow$ G mutant.

Cross-talk between phosphorylation of Thr443 and Ser448 regulates CA IX function

We then generated a series of CA IX double mutants at positions Thr443 and Ser448, either mimicking phosphorylation (by substitution with Asp) or dephosphorylation (by substitution with Gly; Fig. 2A). None of the double mutants showed altered oligomerization ability or cell surface localization (not shown).

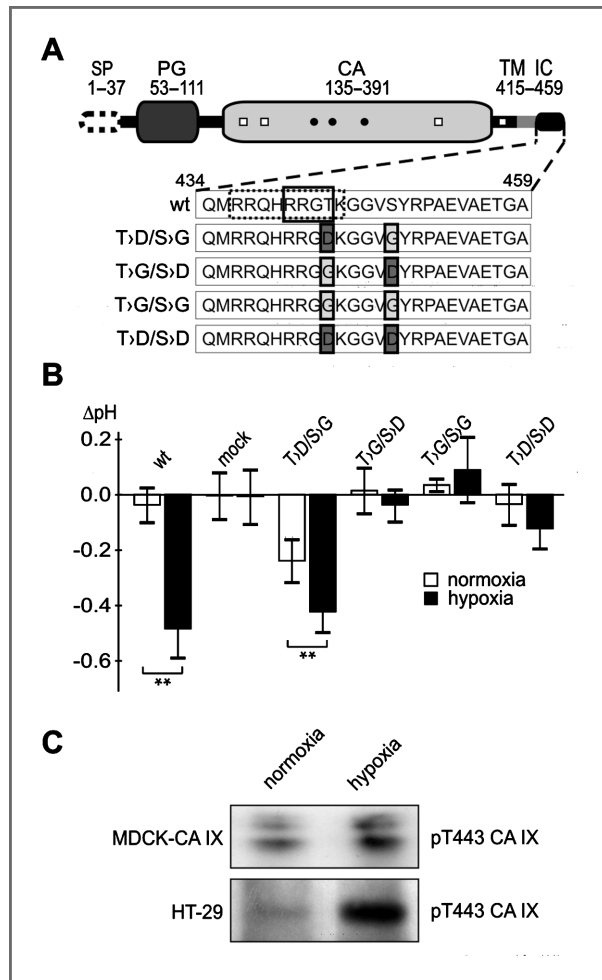


Figure 2. Effects of double mutations of CA IX at Thr443 and Ser448, and phosphorylation of Thr443 in hypoxic cells. **A**, domain composition of CA IX: SP–signal peptide, PG region, CA domain, TM–transmembrane domain, IC tail. Changes in IC amino acid sequence are indicated by boxes (dark gray for pseudophosphorylated residues and light gray for dephosphorylated residues). Empty box with solid line frame encloses the consensus-binding site for PKA, empty box with dotted line frame encloses the basic amino acids important for the function of CA IX (14). **B**, effect of double mutations on pH in transfected MDCK cells incubated for 48 hours in normoxia or hypoxia. The graph shows differences between mean pH values (Δ pH) $\pm$ SD from 10 independent experiments (**, $P < 0.01$). **C**, phosphorylated CA IX was immunoprecipitated from cell extracts using the pT443 antibody, blotted, and visualized using the peroxidase-conjugated M75. CA IX phosphorylation was increased in hypoxia compared with normoxia. The figure represents 1 of 5 independent experiments with similar results.

As expected, mimicking phosphorylation at Thr443 while simultaneously preventing phosphorylation at Ser448 (T $\rightarrow$ D/S $\rightarrow$ G mutant) did not significantly alter enzymatic activity of CA IX in hypoxia (Fig. 2B). However, this mutant led to acidification of pHe also in normoxia. This may indicate that phosphorylation at Ser448 negatively controls enzymatic activation in normoxia. The T $\rightarrow$ G/S $\rightarrow$ D mutant, mimicking phosphorylation at Ser448 and dephosphorylation at Thr448 had completely lost its acidification capacity in hypoxia. Similar observations were made with the T $\rightarrow$ G/S $\rightarrow$ G mutant nonphosphorylatable at both sites, suggesting that the release of the negative control provided by Ser448 is insufficient for hypoxic CA IX activation. Finally, the T $\rightarrow$ D/S $\rightarrow$ D mutant mimicking phosphorylation at both sites showed lower acidification activity in hypoxia than the wt CA IX. Altogether, these data suggested that phosphorylation of each of these residues may have opposing effects on the functional status of CA IX.

We then investigated whether Thr443 was indeed phosphorylated *in vivo*. CA IX was immunoprecipitated from both MDCK-CA IX and HT-29 cells, cultured in normoxia or hypoxia, and analyzed by immunoblotting using an antibody specific to phosphorylated Thr443 (p-T443). As shown in Fig. 2C, phosphorylation of Thr443 was increased under hypoxic conditions in both cell types. This observation supported the view that phosphorylation of Thr443 might participate in the activation of CA IX in hypoxia and prompted us to search for the responsible kinase.

Thr443 is a substrate of c-AMP-dependent PKA

The sequence around Thr443 perfectly matches the canonical recognition motif of c-AMP-dependent PKA, that is, RRXS^{*}/T^{*} (19). To test whether Thr443 is a bona fide phosphorylation site for PKA, we generated GST-fusion proteins of the 25-aa intracellular (IC) fragment of wt or T443 $\rightarrow$ A mutant CA IX. These fusion proteins were subjected to an *in vitro* kinase assay in the presence of recombinant catalytic subunit of PKA (CS-PKA) and γ [³²P]-ATP (Fig. 3A). The IC domain of wt CA IX was very efficiently phosphorylated by CS-PKA (Fig. 3A). In contrast, the T443 $\rightarrow$ A mutant was very poorly phosphorylated under the same conditions thus identifying Thr443 as the major PKA site in the IC domain of CA IX.

PP2A is the primary PKA-opposing phosphatase (20). We thus tested whether it could dephosphorylate PKA-phosphorylated Thr443 using an *in vitro* dephosphorylation assay of GST-CA IX with recombinant PP2A. As shown in Fig. 3B, an active PP2A core enzyme efficiently dephosphorylated phospho-Thr443 of CA IX *in vitro*, suggesting that it might be involved in balancing the PKA-mediated CA IX.

In the next step, we used a "cold" PKA kinase assay followed by immunoblotting with the p-T443 antibody to confirm that the antibody recognized the PKA-modified phospho-Thr443 in CA IX (Fig. 3C). The relationship between CS-PKA and phosphorylation of CA IX at Thr443 was shown via cotransfection of plasmids encoding wt-CA IX (pCA9) and either CS-PKA (pC α) or the dominant-negative mutant DN-PKA (p α n) followed by immunoblotting (Fig. 3D). The results revealed a clear Thr443-related phosphorylation signal only in cells cotransfected with pCA9 in combination with pC α , but not

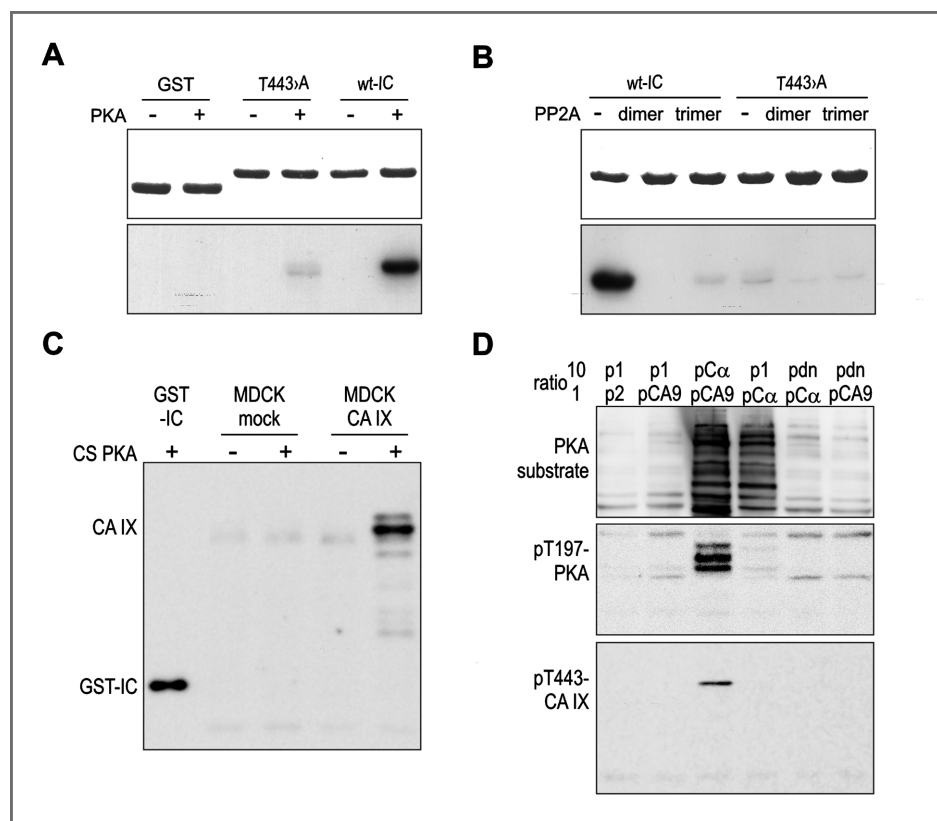


Figure 3. Phosphorylation and dephosphorylation of Thr443. **A**, *in vitro* kinase assay was carried out with the CS-PKA on GST fusion proteins containing the IC domain of CA IX in the wt version and in the mutated version with Thr443 replaced by Ala (T443 → A). The upper part of the figure represents a CBB-stained gel (loading control), the lower part represents autoradiograph of the proteins phosphorylated in the presence of [γ - 32 P]ATP. **B**, *in vitro* phosphatase assay was carried out with heterodimeric and heterotrimeric forms of PP2A on PKA-phosphorylated GST-tagged wt-IC fragment of CA IX. The upper part of the figure represents a gel with CBB-stained substrate proteins (loading control), the lower part represents autoradiograph. The data show that Thr443 is a substrate residue for PKA and PP2A. The experiments (in **A** and **B**) were repeated twice and representative results are shown. **C**, CA IX was immunoprecipitated by the M75 MAAb from cell extracts, *in vitro* phosphorylated by CS-PKA, subjected to PAGE, blotted and incubated with the P-T443 CA IX antibody. *In vitro* phosphorylated GST-tagged wt-IC domain of CA IX protein (GST-IC) was used as a positive control. **D**, all 3 blots contain PAGE-resolved extracts from HEK293T/17 cells transfected with different combinations of the following plasmids: pcDNA3.1 (p1), pSG5C (p2), pSG5C-MN/CA9 (pCα), pcDNA3.1-CSPKA (pCα), pcDNA3.1-dnPKA (pdn). The plasmids were cotransfected in a ratio of 10:1. The blots were incubated with antibodies as indicated on the left side. The data show that phosphorylation of CA IX, activation of PKA and phosphorylation of PKA substrates occur only in the presence of CS-PKA. The experiment was repeated 4 times.

with the pdn plasmid encoding DN-PKA or with the empty plasmid. This corresponded well with the activation status of the CS-PKA protein, which was strongly phosphorylated in cells expressing CS-PKA but not DN-PKA. Weak endogenous CS-PKA phosphorylation that could be seen also in the mock-transfected cells was probably insufficient for the detection threshold of CA IX phosphorylated at Thr443. Accordingly, phosphorylation of the PKA substrates was much stronger in cells transfected with 3.64 μ g of the pCα plasmid compared with 10 times less plasmid and was negligible in the presence of pdn (Fig. 3D).

PKA activation in hypoxia is associated with CA IX-mediated extracellular acidification

To establish a functional link between PKA, hypoxia and CA IX activation, we examined the phosphorylation of Thr197 of CS-PKA, which correlates with the activity of the kinase. Levels

of active CS-PKA were considerably higher in hypoxic A549 and HeLa cells compared with normoxic cells (Fig. 4A). Similar observations were made in MDCK-CA IX cells (Fig. 4A), in which hypoxia-induced PKA activity was linked to significantly higher levels of cAMP in hypoxia versus normoxia (Fig. 4B). We also used a membrane-permeable cAMP analog 8-Br-cAMP to show that activation of PKA can lead to CA IX phosphorylation in normoxia (Fig. 4C).

We then evaluated the role of PKA in CA IX-dependent extracellular acidification. No difference in pH_e values was found between cells transiently transfected with DN-PKA versus mock-transfected cells under normoxia. However, in the presence of DN-PKA, hypoxic cells showed a significantly lower ability to acidify pH_e (Fig. 4D). DN-PKA expression also decreased accumulation of the FITC-conjugated CA inhibitor in hypoxia when compared with cells with an intact endogenous PKA pathway (Fig. 4E and F).

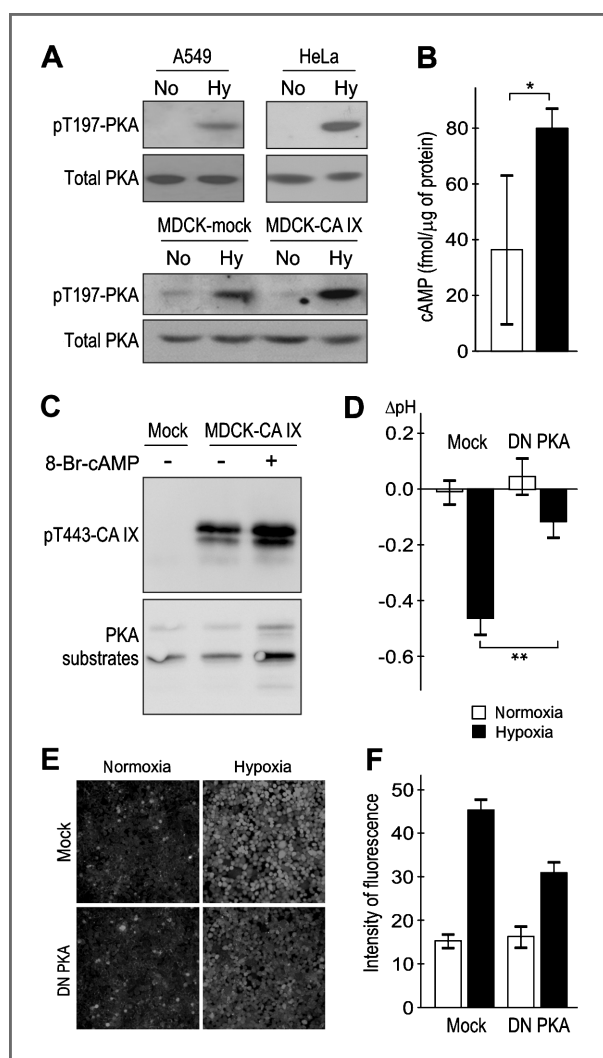


Figure 4. Relationship between hypoxia, cAMP, PKA, CA IX phosphorylation, and pH regulation. **A**, immunoblotting analysis of activation (phosphorylation) of PKA Thr197 in hypoxia. The experiment was repeated 3 times. **B**, intracellular cAMP levels in hypoxic and normoxic MDCK-CA IX cells evaluated using a direct immunoassay. Hypoxia was found to significantly increase cAMP levels in 3 independent experiments (*, $P < 0.05$). **C**, normoxic MDCK cells (mock and CA IX transfected) were treated with 0.5 mmol/L 8-Br-cAMP to activate PKA. PKA activation induced CA IX Thr443 phosphorylation as shown by immunoprecipitation with the pT443-specific antibody, blotting and immunodetection by M75 Mab conjugated with peroxidase. The experiment was repeated twice with similar results. **D**, pH was measured in MDCK-CA IX cells transiently transfected with DN PKA versus mock-transfected control. DN PKA significantly reduced extracellular acidification mediated by CA IX in the hypoxic cell culture (**, $P < 0.01$), as verified in 3 independent experiments. **E**, binding of the FITC-CAI inhibitor to hypoxic MDCK-CA IX cells was reduced after transient transfection of DN PKA as visible from the reduced fluorescent signal. **F**, relative intensity of the fluorescence signal was evaluated using ImageJ software.

The catalytic function of CA IX is linked to its cooperation with bicarbonate transporters, including NBC1 (21, 2). Thus, we examined whether CA IX was spatially associated with NBC1 and with PKA in tumor cells to enable coupling of the

hypoxia-stimulated PKA activation with the NBC1-CA IX metabolon. We therefore used A549 lung carcinoma cells exposed to hypoxia to colocalize these proteins by immunofluorescence. All cells showed diffuse intracellular staining of the studied proteins, which most probably represented their internalized forms resulting from HGF-induced perturbation of cell-cell contacts followed by endocytosis of plasma membrane proteins including CA IX (22, 23). Nevertheless, we could show that CA IX accumulated and colocalized with NBC1 in leading edge membranes of hypoxic A549 cells stimulated by HGF to migration and with phosphorylated PKA substrates that indirectly indicated the positions at which activated PKA was operating (Figs. 5A and B and Supplementary Figs. S1 and S2). These data were compatible with the concept of the PKA-driven phosphorylation-mediated functional activation of CA IX in the context of the bicarbonate metabolon.

Next, we examined whether CA IX phosphorylation could affect cell migration, which critically depends on pH regulation (involving CA IX activity) at protruding cell membranes (23). MDCK cells transfected either with the wt CA IX or with its mutated variants $T \rightarrow D/S \rightarrow G$ and $T \rightarrow G/S \rightarrow D$, were induced to motility in wound healing and scatter assays. In line with the proposed activating role of Thr443 phosphorylation, results of both assays clearly showed a significantly increased motility of cell expressing the $T \rightarrow D/S \rightarrow G$ mutant and decreased motility of cells expressing the $T \rightarrow G/S \rightarrow D$ mutant (Fig. 5C and D). These data suggest that phosphorylation of CA IX is functionally relevant to cell phenotype.

Hypoxia modulates activity of CA II and CA XII inserted into the CA IX backbone

To support the critical role of the intracellular region in the hypoxia-driven activation of the extracellular catalytic domain of CA IX, we generated chimeric CA IX molecules containing heterologous CA domains. Two chimeras were obtained by an insertion of either the CA II or CA XII catalytic domains, in between the N-terminal proteoglycan-like (PG) segment and the C-terminal segment containing the transmembrane and intracellular regions of CA IX (Fig. 6A). Both CA II and CA XII are active CA isoenzymes expressed in many normal tissues and in some tumors, and are known to function and bind sulfonamide inhibitors also under normoxic conditions (24, 13). However, when expressed in the context of the CA IX backbone, they behaved similarly to the original catalytic domain of CA IX. Both constructs were ectopically expressed in MDCK cells and showed cell surface localization (data not shown). Analogously to CA IX, the chimeric proteins were capable of binding the FITC-labeled CA inhibitor only in hypoxia (Fig. 6B). This finding suggests that the enzymatic activity of the chimeric proteins is not determined by the CA domains originating from the different isoforms, but rather by the flanking regions originating from CA IX. Because we previously showed that the deletion of the PG domain has no influence on the hypoxia-related binding of the FITC-labeled CA inhibitor (7), the observed effect can be attributed to the intracellular region.

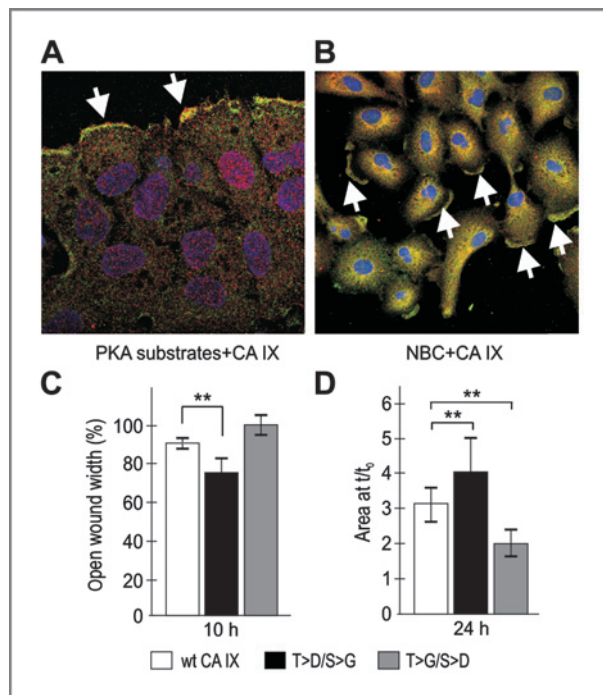


Figure 5. Colocalization of CA IX with NBC1 and PKA substrates and effect of CA IX phosphorylation on cell migration. A and B, A549 cells were incubated in hypoxia for 48 hours to induce expression of CA IX. Then they were treated with 20 ng/mL of HGF for 15 minutes, fixed, the cells were double stained with antibodies specific to CA IX, phospho-PKA substrates and NBC1 and analyzed by confocal microscopy (at original magnification 400 $\times$). Colocalization of CA IX with the examined proteins is clearly visible at the leading edges of cells stimulated to migration. C, wound healing assay on MDCK cells expressing wt-CA IX and double mutants in position 443 and 448. Confluent cell monolayers were starved overnight, then wounded and allowed to migrate as described. Wound width was measured in 16 positions immediately after wounding and 10 hours later, and wound closure in % was calculated. Cells expressing T $\rightarrow$ D/S $\rightarrow$ G mutant showed significantly improved (**, $P < 0.01$) healing capacity. D, cell islands were generated by plating the preformed cell aggregates. Spreading and outward migration were allowed to proceed for 24 hours. Significant increase (**, $P < 0.01$) was observed in areas of MDCK cells transfected with T $\rightarrow$ D/S $\rightarrow$ G mutant.

Discussion

PKA has been implicated in various cellular processes, including cell proliferation, survival, differentiation, metabolism, etc. It acts at a crossroad of many signal transduction cascades via communication with other regulatory proteins. Accordingly, PKA has a spectrum of substrates that involves hormone receptors, Raf-1 kinase and RhoA GTP-binding protein, CREB and NF κ B transcription factors, histones, metabolic enzymes, etc. (19). PKA also regulates water and ion transport in various physiologic conditions. Although it is a ubiquitous protein working in virtually all differentiated cell types, its increased expression/activation has been associated with tumors derived from breast, colon, kidney, and other tissues. PKA-regulated processes directly contribute to tumor biology, particularly to invasion, treatment resistance, and adaptation to stress (25). Interestingly, PKA cooperates with

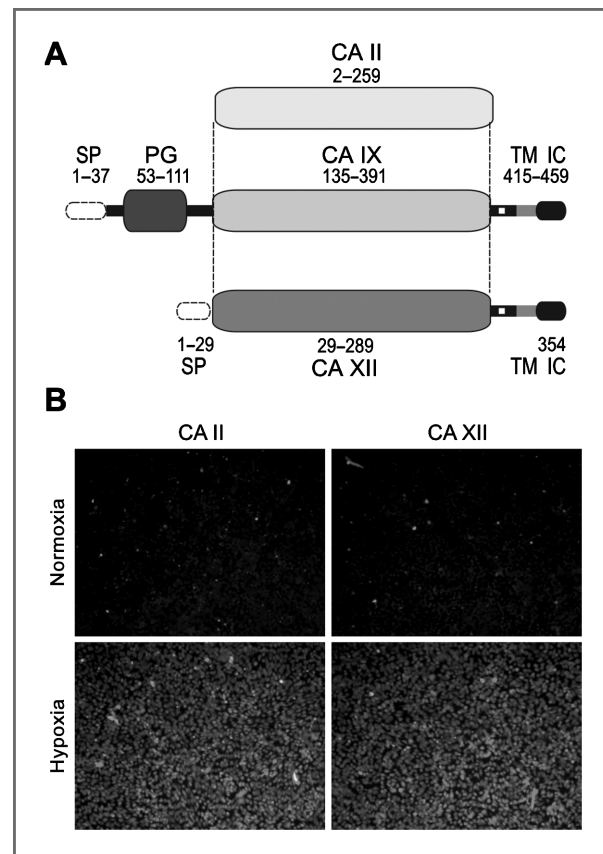


Figure 6. Construction of chimeric CA IX variants and their effects on binding of FITC-CAI to transfected MDCK cells. A, schematic drawing of chimeric constructs with the CA domain of CA IX replaced by CA II and CA XII. Structural domains of CA IX are designated as in Fig. 2A. B, binding of the CA IX-selective FITC-labeled inhibitor (FITC-CAI) to normoxic and hypoxic cells expressing the chimeric proteins.

the hypoxic machinery by phosphorylation of HIF-1 α , a key regulator of molecular responses to hypoxia (26), and thereby influences a panel of molecules induced by hypoxia, some of them also via direct phosphorylation.

Here, we show that PKA controls the CA IX-mediated pH regulation in hypoxic cells via phosphorylation of the Thr443 residue within the PKA recognition site RRGT, which conforms to the RRXS*/T* consensus sequence (19). Mutation of this residue affects the CA IX-related extracellular acidosis in cells exposed to chronic hypoxia. Because the PKA consensus site is embedded in the juxtamembrane basic amino acid motif, our finding offers an explanation for an earlier observation linking mutations of this motif with abolished functioning of CA IX (14). It is conceivable that conversion of the basic motif to a highly acidic amino acid stretch changes the local charge and/or architecture, thus precluding the recognition of Thr443 by PKA. The basic amino acid motif is conserved in the cytoplasmic domains of CA IX proteins expressed in different species (including human, mouse, rat, and dog) and in the other 2 transmembrane CA isoforms CA XII and CA XIV (14). However, the threonine residue is present within this motif only in CA IX, suggesting that it is the only transmembrane

isoform able to respond to physiologic stimuli transmitted via the PKA pathway.

Although we propose that Thr443 plays a dominant role in CA IX regulation, we do not exclude the participation of additional phosphorylation sites. On the basis of the mutagenesis results, phosphorylation of Ser448 may represent a negative regulatory event diminishing the ability of CA IX to acidify pHe. *In vitro* kinase assay indicates that Ser448 is unlikely to be a PKA phosphorylation site. In addition, it is localized within a different amino acid context that does not match the PKA consensus. At this moment, it is not clear whether Ser448 is actually phosphorylated *in vivo* and if so, which kinase modifies this residue. In contrast, Tyr449 phosphorylation was shown to occur in kidney cancer cells and was linked to downstream activation of the PI3K/Akt pathway (18). However, this phenomenon was not reproduced in breast cancer cells (9), indicating that it is cell-type specific. Moreover, information on the pertinent kinase and whether phosphorylation of Tyr449 affects CA IX function is still missing. We can only deduce that the close proximity of Ser448 and Tyr449 possibly prevents their simultaneous modification, but the space constrains and/or induced allosteric changes may also affect the interplay of PKA with other kinases and determine the hierarchy of phosphorylation signaling to the CA IX molecule.

One important question emerging from this study is how the CA IX phosphorylation via PKA fits in the concept of the bicarbonate metabolon operating in hypoxic cells. To provide a rationale, we have to take into account several facts. (i) The metabolon paradigm involves spatial and functional cooperation of CA IX with bicarbonate transporters, such as the NBC1 and anion exchanger AE2 (4, 5). (ii) NBC1 and AE2 are PKA substrates implicated in pH regulation and migration phenomena in cancer cells and/or regulated by the VHL/HIF pathway (23, 27). (iii) PKA-mediated phosphorylation of NBC1 at Ser982 or Ser1026 permits the negatively charged aspartic acid residues in the C terminus of NBC1 to interact electrostatically with the bicarbonate ion-binding site on the transporter, resulting in a transport stoichiometry favorable for bicarbonate import (28). This mode is compatible with intracellular neutralization activated in cells exposed to hypoxia/acidosis (5). (iv) PKA works in a compartmentalized manner conducive to spatial coordination of its substrates, such as CA IX and NBC1. (v) Hypoxia increases intracellular cAMP concentration and level of activated PKA and thus creates a mechanism feeding the phosphorylation signaling via PKA (Ref. 24; and this work). (vi) Hypoxia increases the catalytic activity of CA IX and its ability to acidify pHe (7, 9).

In favor of the above concept, we found that CA IX colocalized with NBC1 and with phosphorylated PKA substrates (indicating the presence of activated PKA) in the leading edge membranes of hypoxic A549 lung carcinoma cells stimulated to migration by HGF (Fig. 5A and B; Supplementary Figs. S1 and S2). Noteworthy, coexpression of CA IX and PKA substrates is visible also in parallel tissue areas of serial renal cell carcinoma sections (Supplementary Fig. S3), suggesting that the cross-talk between CA IX and PKA might also occur *in vivo*. Thus, it is possible to propose a model, in which hypoxia leads to

increased levels of cAMP, which activates PKA. PKA then phosphorylates both CA IX and NBC1, thereby stimulating import of bicarbonate ions generated by activated CA IX (Fig. 7). On the basis of other examples (such as EGFR; ref. 29), it is imaginable that phosphorylation affects CA IX in terms of folding (e.g., by an allosteric change and opening of the active site) and/or in terms of dimerization (e.g., by releasing the S-S bonds facilitated by the reducing hypoxic microenvironment and reorienting the active sites toward the bicarbonate transporter "entrance"). This could result in an efficient coupling of bicarbonate production and transport. Such scenario is compatible with the definition of metabolons as complexes that form transiently to enhance channeling of constituents (30).

Indeed, CA IX phosphorylation at Thr443 is functionally relevant to cell behavior, because it significantly affects cell migration (Fig. 5). This seems to be directly connected to the regulation of CA IX catalytic activity, which is needed for the intense bicarbonate ion transport and proper pH regulation across the leading edge membranes of moving cells. Such phenomenon is particularly meaningful to hypoxic tumor cells, which tend to escape from the hostile tumor microenvironment to survive and thus gain a migratory phenotype that involves the assembly and activation of the pH-regulatory machinery including the bicarbonate metabolon (23). This view is in agreement with the studies that present CA IX as a dissociation factor destabilizing E-cadherin-mediated cell-cell contacts and a protein regulating cell migration and

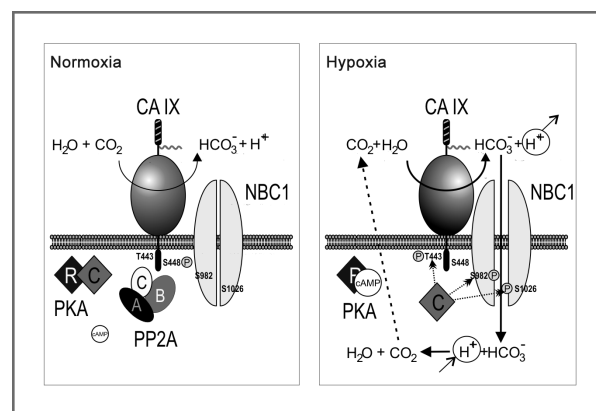


Figure 7. Model of PKA regulation of the CA IX. In normoxia, cAMP levels are relatively low, catalytic subunit of PKA [C] is kept inactive by interaction with the regulatory subunit [R], and PP2A is presumably associated with the IC tail of CA IX. Thus, CA IX remains unphosphorylated at indicated Ser residues and its activity is low. NBC1 is unphosphorylated at indicated Ser residues and the bicarbonate import does not occur. In hypoxia, cAMP levels rise, leading to release of C PKA, which can phosphorylate Thr443 of CA IX and Ser982 and Ser1026 residues of NBC1. This results in a spatially coordinated and functionally coupled activation of CA IX-catalyzed production of bicarbonate ions and in activation of NBC1-mediated bicarbonate transport across the membrane. Intracellular bicarbonate can then consume intracellular protons to form CO₂, which leaves the cells by diffusion. This contributes to intracellular neutralization. On the other hand protons generated at the outer side of the membrane via the CA IX-catalyzed reaction contribute to extracellular acidification. Because this model counts on the presence of CA IX in normoxia, it is mostly relevant to reoxygenated cells, which contain CA IX induced by preceding hypoxia.

invasion (22, 10; Witarski and colleagues; submitted for publication).

These findings strongly imply that the hypoxia-induced activation of PKA leading to phosphorylation of CA IX at Thr443 in the IC tail is an important determinant of the enzymatic activity of CA IX. This is supported by the data obtained with chimeric proteins containing the CA IX backbone and heterologous catalytic domains of CA II and CA XII. Although all 3 CA isoforms differ from each other by their catalytic activity and affinity to inhibitors, they behave very similarly when expressed in the context of a CA IX-derived IC tail. Thus, it seems that the intracellular region of CA IX (the only 1 among other transmembrane CAs, which contains Thr443) is responsible for the functional regulation of the catalytic activity of CA IX.

In conclusion, this work allowed us to make significant progress in elucidating important aspects of the signal transduction that underlies functional regulation of CA IX and its impact on cell phenotype. Moreover, we reinforced our understanding that hypoxia induces CA IX at the level of transcription and at the level of enzymatic activation. This secondary control mechanism might be important for maximizing the catalytic performance of CA IX and also for its dynamic, microenvironmentally driven regulation in the background of

high CA IX protein stability (31). The knowledge obtained here will serve for further investigation toward elucidation of relevant molecular mechanisms and for the development of new approaches aiming at blocking CA IX function from the "inside" and thus reducing survival of cancer cells in therapeutic settings.

Disclosure of Potential Conflicts of Interest

J. Pastorek and S. Pastorekova are consultants for Bayer Pharma AG and both are patent inventors. The other authors disclosed no potential conflicts of interest.

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