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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 anchoring regions play a role in 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).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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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 \times 10^4 in 1.5 mL of medium) were plated in 3 cm dishes 24 hours prior to 48 to 48 hours incubation in hypoxia (2% O_2, 2% H_2, 5% CO_2, 91% N_2, anaerobic workstation, Ruskin Technology). Normoxic dishes were maintained in an incubator with 5% CO_2. 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 cells (4 \times 10^4 in 1.5 mL of medium) were plated in 3 cm dishes 24 hours prior to 48 to 48 hours incubation in hypoxia (2% O_2, 2% H_2, 5% CO_2, 91% N_2, anaerobic workstation, Ruskin Technology). Normoxic dishes were maintained in an incubator with 5% CO_2.

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 \mu g/lane) were resolved in 10% SDS-PAGE and transferred to polyvinylidene fluoride 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 (BRXS^+^/T^+^) antibody (all from Cell Signaling Technology, at 1:1000 dilution). Total CA IX was detected by M7 antibody (15), CA IX phosphorylated at Thr443 by P-T443 CA IX antibody (1:5,000; GenScript) and actin was visualized by Actin antibody (Santa Cruz Biotechnology).

For immunoprecipitation, cell lysates were precloned with Protein A Sepharose CL-4B (GE Healthcare Bio-Sciences AB) and incubated overnight at 4°C with 1 \mu g of phospho-Thr443 antibody bound onto 50 \mu L of beads per sample. Immunocomplexes were separated by SDS-PAGE and blotted. Phosphorylated CA IX was detected by a peroxidase-conjugated antibody (Santa Cruz Biotechnology).

In vitro phosphorylation and dephosphorylation

A portion of CA IX cDNA representing the IC tail (nt. 1,345–L149) 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 \mu g 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 \mu L of CS-PKA (Promega), 0.2 mmol/L ATP (Sigma-Aldrich) and 1 \mu L of [\gamma^32P]ATP. The samples were incubated at 30°C for 30 minutes and reaction was stopped by 2 \times 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 phosphate 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 \mu g in vitro phosphatase 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 \mu g of proteins per sample using the cAMP

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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 (Na+/HCO3−) 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 → D/S → G, pSG5C-T → G/S → 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. Phosphorylation of Tyr449 signals to the Akt/PI3K pathway (18), but no phosphorylation sites: Thr443, Ser448, and Tyr449. 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 phosphorylation

hypoxia (7) and examined for oligomerization and plasma membrane localization. The mutations had no effect on these properties (Fig. 1A–C). However, the T443 → G mutant was impaired in its ability to acidify pHe in hypoxia. In contrast, the S448 → 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 → G) and Ser448 (S448 → G). A, immunoblotting analysis of the mutants separated under nonreducing conditions (without β-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 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 (ΔpHe). 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 wtCAIX and the S448 → G mutant, whereas it was diminished in mock-transfectants and in cells expressing the T443 → G mutant.

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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).

As expected, mimicking phosphorylation at Thr443 while simultaneously preventing phosphorylation at Ser448 (T→D/S→G mutant) did not significantly alter enzymatic activity of CA IX in hypoxia (Fig. 2B). However, this mutant led to acidification of pH also in normoxia. This may indicate that phosphorylation at Ser448 negatively controls enzymatic activation in normoxia. The T→G/S→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→ G/S→ 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→D/S→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/TT (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 mutant CA IX. These fusion proteins were subjected to an in vitro phosphorylation 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 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) or the dominant-negative mutant DN-PKA (pdn) followed by immunoblotting (Fig. 3D). The results revealed a clear Thr443-related phosphorylation signal only in cells cotransfected with pCA9 in combination with pC, but not...
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 μg of the pCa 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 hypoxia versus normoxia (Fig. 4C).

We then evaluated the role of PKA in CA IX-dependent extracellular acidification. No difference in pHe 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 pHe (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).
The catalytic function of CA IX is linked to its cooperation with bicarbonate transporters, including NBC1 (21, 22). 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 → D/S → G and T → G/S → 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 → D/S → G mutant and decreased motility of cells expressing the T → G/S → 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.
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 NFkB 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 the hypoxic machinery by phosphorylation of HIF-1α, 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\(^*/C^3\)/C3\(^*/C^3\) 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 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...
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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Phosphorylation of Carbonic Anhydrase IX Controls Its Ability to Mediate Extracellular Acidification in Hypoxic Tumors

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