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Intact intracellular tail is critical for proper functioning of the tumor-associated, hypoxia-regulated carbonic anhydrase IX

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ABSTRACT

Carbonic anhydrase IX (CA IX) is a tumor-associated, hypoxia-induced enzyme involved in pH regulation and cell adhesion. Its catalytically active ectodomain (ECD) is linked to a transmembrane region and a short intracellular (IC) tail. Removal of the IC tail causes intracellular localization of CA IX. Mutations of basic amino acids within IC do not perturb the membrane position, but reduce shedding of the CA IX ectodomain as well as CA IX-mediated cell dissociation. Moreover, they abolish the CA IX capacity to acidify extracellular pH (pHe) and bind CA IX-selective sulfonamide inhibitor in hypoxia. These findings provide the first evidence for the critical contribution of the IC tail to the proper functioning of CA IX.

Structured summary:
MINT-7293982: *E-cadherin* (uniprotkb:Q95LE0) and *CA IX* (genbank_protein_gi:223556027) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

1. Introduction

Carbonic anhydrase IX (CA IX) is an integral plasma membrane protein regulated by hypoxia and expressed in a broad spectrum of human tumors, while being absent from majority of normal tissues [1]. It is one of the most efficient members of α carbonic anhydrases (CAs), a family of zinc metalloenzymes that catalyze a reversible conversion of carbon dioxide to bicarbonate ion and a proton [2]. CAs are usually expressed in well differentiated metabolically active cells and tissues, where they play important physiological roles in facilitating ion transport and maintaining pH homeostasis [3]. The tumor-associated CA IX isoenzyme actively contributes to acidification of extracellular pH (pHe) and neutralization of intracellular pH in cells exposed to hypoxia and/or acidosis and thereby functions as a survival factor protecting tumor cells from acidic pericellular microenvironment [4–7]. Based on tumor-related, hypoxia-induced expression and direct functional involvement in tumor physiology, CA IX is intensively studied as an intrinsic marker of hypoxia, prognostic indicator and therapy target for selective inhibitors of catalytic activity and specific monoclonal antibodies [1,8].

The CA IX protein consists of a large extracellular part, containing a unique N-terminal proteoglycan-like region (PG) and a carbonic anhydrase domain (CA) which is highly active for the catalytic function of the enzyme, i.e. CO_2 hydration. This extracellular domain is anchored in the plasma membrane via a single-pass transmembrane region (TM), which is C-terminally extended into a short intracellular tail (IC) [9,10]. The catalytic CA domain is clearly required for the CA IX-mediated pH regulation in hypoxia [4] and the PG domain appears to be involved in cell adhesion-related function of CA IX [11]. However, the role of the IC tail has remained unexplored. Here we demonstrate for the first time that this short C-terminal portion is essential for proper functioning of CA IX.

2. Materials and methods

2.1. Cell culture

MDCK cells and their transfected derivatives were grown in DMEM with 10% FCS and buffered with 22.3 mM bicarbonate [4].

Abbreviations: CA IX, carbonic anhydrase IX; ECD, ectodomain; FITC-CAI, fluorescein-conjugated carbonic anhydrase inhibitor; MAb, monoclonal antibody; pHe, extracellular pH; TACE, TNFα-converting enzyme

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The cells ($0.8–1 \times 10^6$ per 6 cm dish) were plated in 3 ml of medium 24 h before the transfer to hypoxia (2% O_2, 2% H_2, 5% CO_2, 91% N_2) in an anaerobic Workstation (Ruskin Technologies). Parallel normoxic dishes were incubated in air with 5% CO_2. At the end of each experiment, pH of the culture medium was immediately measured, the medium was harvested for the determination of the lactic acid content with the Lactate Reagent kit (Trinity Biotech), the cells were counted and processed either for immunofluorescence or extracted for immunoblotting.

2.2. Sulfonamide treatment of cells

The fluorescent CAI sulfonamide (FITC-CAI) was obtained from homosulfanilamide and fluorescein isothiocyanate as described [4], dissolved in PBS at 100 mM concentration and diluted in a culture medium just before the addition to cells. The cells were incubated for 48 h in hypoxia and normoxia, respectively, and the binding of the FITC-CAI to living cells was viewed by a Nikon E400 epifluorescence microscope.

2.3. Cloning of CA IX mutants and transfection

In vitro mutagenesis of IC tail was performed by PCR using the pSG5C-CA IX plasmid [9] and following primers: ICMUT4S ggaaggcagcacggaggggggaaccggaggggt, ICMUT4A acccccctccggttccc cctccgtgctgccttcc, ICMUT5S gtgcagatgggagggcagcacgga, ICMUT5A tccgtgctgccctcccatctgcac. MDCK cell lines constitutively expressing CA IX protein or its mutants were obtained by co-transfection of pSG5C-CA IX, pSG5C-mut3R1K and pSG5C-mut4R1K plasmids with pSV2neo in a 10:1 ratio using a GenePorter II transfection kit (Genlantis). Transfected cells were selected in 500 μg/ml G418, cloned, tested for CA IX and expanded. At least three clonal cell lines expressing each CA IX form were analyzed to eliminate the effect of clonal variation. The cells cotransfected with empty pSG5C and pSV2 neo were used as mock controls.

2.4. Immunofluorescence and confocal microscopy

Cells grown on glass coverslips were fixed in ice-cold methanol at −20 °C for 10 min and stained with CA IX-specific FITC-labeled monoclonal antibody (MAb) M75 directed to the PG domain [12]. To colocalize CA IX with E-cadherin, the fixed cells were first incubated with anti-E-cadherin MAb (1:500, BD Transduction Laboratories) and anti-mouse antibody conjugated with Alexa Fluor 594 (1:2000, Invitrogen), and then with FITC-conjugated M75. Nuclei were stained with DAPI (1:36 000, Sigma). Samples were analyzed by Zeiss LSM 510 Meta confocal microscope, scanned in multitrack mode and deconvoluted by Huygens software (Scientific Volume Imaging). ΔIC mutant was colocalized with endoplasmic reticulum using protein disulfide isomerase (PDI)-specific goat polyclonal antibody (1:50, Santa Cruz) and with Golgi apparatus using Golgin-97 mouse MAb (1:100, Invitrogen) followed by Alexa Fluor 594 and 633-conjugated secondary antibodies (Invitrogen). Consequent staining with FITC-M75 and analysis were performed as above.

2.5. Immunoblotting and ELISA

The cells were rinsed with PBS and extracted in RIPA buffer for 30 min on ice. Protein concentrations were quantified using the BCA kit (Pierce). The proteins (50 μg/lane) were resolved in 10% SDS–PAGE under reducing and non-reducing conditions, respectively, transferred to PVDF membrane and CA IX was detected with the M75 as described [4].

CA IX ectodomain shed to culture medium was determined by sandwich ELISA using capture V/10 MAb and detector M75 MAbs as described earlier [13].

2.6. Cell dissociation assay

MDCK cells were grown for 3 days to form a highly dense monolayer. After washing twice with PBS containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$, the cells were detached using a cell scraper, passed 30 times through the Pasteur pipete and counted using Coulter Counter (Beckmann Coulter). The extent of dissociation was expressed as a ratio of Np/Nc, i.e. number of disrupted particles per total number of cells obtained by counting the cells from the parallel monolayer fully dissociated in PBS. Resulting values of dissociation were compared by t test and $p < 0.05$ was considered significant.

3. Results

3.1. Removal of IC tail leads to loss of plasma membrane localization of CA IX

The domain-deletion strategy employed in our earlier experiments was helpful in elucidating the functional significance of the extracellular portion of CA IX. Therefore, we decided to clarify the role of IC tail using similar approach. MDCK cells were transfected with pSG5C-ΔIC plasmid encoding the CA IX protein lacking 24 amino acids from the C-terminus. However, confocal microscopic analysis revealed that this C-terminally truncated protein is not present at the cell surface, but is localized intracellularly, overlapping with the marker of endoplasmic reticulum (Fig. 1A–C). Cell surface fraction of ΔIC determined by ELISA represented less than 5% of the total cellular content of ΔIC, in contrast to wt CA IX that was almost completely localized in the plasma membrane (Fig. 1D). Aberrant localization of ΔIC indicated that TM region preserved in this deletion variant is not sufficient for the correct transport and/or anchorage of CA IX in the plasma membrane and that the intracellular tail of CA IX is needed for these processes. This idea strongly implicated that IC tail might be involved in intracellular protein–protein interactions that drive CA IX transport and stabilize its cell surface localization.

3.2. Mutations of basic amino acids in IC tail perturb neither localization nor oligomerization of CA IX

Intracellular position of ΔIC precluded further functional analyses of truncated CA IX, since both cell adhesion- and pH regulation-related roles require presence of CA IX at the cell surface. We thus performed a hydrophobic cluster analysis (HCA) of the IC sequence to identify amino acids that could mediate binding of CA IX with intracellular proteins. HCA revealed that the basic amino acids, namely arginines and lysine localized just next to hydrophobic TM region, form a distinct cluster (Fig. 2A). Since diverse proteomic and bioinformatic approaches propose that basic, positively charged amino acids are exposed at the protein–protein interfaces, we assumed that these residues might be involved in CA IX interaction(s) with other molecules. On this basis, we generated two variants of CA IX, the first one with three arginine → glycine and one lysine → glycine substitutions (named mut3R1K → G) and the second one with four arginine → glycine and one lysine → glycine substitutions (named mut4R1K → G), see Fig. 2B. Both mutants were correctly transported to plasma membrane and were also able to form oligomers similar to wt CA IX (Fig. 2C and D).