Modulation of cell surface density of carbonic anhydrase IX by shedding of the ectodomain and endocytosis

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Summary. – Carbonic anhydrase IX (CA IX) is a cell surface protein frequently present in human tumors but not in the corresponding normal tissues. Expression of CA IX is primarily determined by the strong transcriptional activation of the gene encoding CA IX by hypoxia via the hypoxia-inducible transcription factor HIF-1. However, the ultimate abundance of the CA IX protein on the cell surface can be affected by additional mechanisms, including ectodomain shedding and endocytosis. In this paper, we summarize the basic knowledge on how these processes modulate CA IX expression at the post-translational level. We propose that the regulation of the CA IX protein residence in the plasma membrane can influence its biological function as well as its clinical exploitation.

Keywords: carbonic anhydrase IX; ADAM17; ectodomain shedding; proteolysis; endocytosis; monoclonal antibody; hypoxia; cancer

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1. Introduction

CA IX, first identified and cloned at the Institute of Virology, Slovak Academy of Sciences, is a transmembrane protein that belongs to the α carbonic anhydrase family of zinc-binding enzymes, which catalyze the reversible hydration of carbon dioxide to bicarbonate ions and protons (Pastorekova *et al.*, 1992; Závada *et al.*, 1993; Pastorek *et al.*, 1994). This reaction is involved in many biological processes that depend on acid-base balance and ion transport (Supuran, 2008). Fifteen human CA isoforms include three inactive proteins and twelve isoenzymes ranging in activity from weak to very strong. Most CA isoforms are expressed in differentiated cells and fulfill specialized roles in various tissues and organs (Pastorekova *et al.*, 2004). CA IX is the only isoenzyme, which is predominantly associated with tumors and only rarely expressed in healthy tissues. Moreover, its presence in tumor cells is often associated with an aggressive tumor phenotype. This is in agreement with the strong regulation of CA IX by hypoxia, a clinically relevant phenomenon characteristic for many solid tumors (Potter and Harris, 2003).

Hypoxia develops as a consequence of poor oxygenation of tumor tissues by aberrant vasculature. Tumor cells adapt to this microenvironmental stress by reshaping their phenotype in terms of the metabolism, cycling, adhesion, migration, invasion and many other attributes that allow them to survive and acquire aggressive properties ultimately leading to metastasis and resistance to therapy (Harris, 2002). These

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Abbreviations: ADAM = a disintegrin and metalloproteinase; ADCC = antibody-dependent cell-mediated cytotoxicity; AE2 = anion exchanger 2; CA IX = carbonic anhydrase IX; CCV = clathrin-coated vesicles; ECD = ectodomain; EGF = epidermal growth factor; HIF = hypoxia-inducible factor; MAb(s) = monoclonal antibody(ies); NBCe1 = sodium bicarbonate cotransporter 1; PRNC = perinuclear compartment; TACE = TNF alpha-converting enzyme

changes are mediated by molecular mechanisms governed by HIF, which transactivates numerous genes involved in the aforementioned phenotypic alteration, including the gene coding for CA IX (Wykoff *et al.*, 2000). Hypoxia also regulates correct splicing of CA IX mRNA, and activates CA IX protein at the functional level through the protein kinase A-mediated phosphorylation of its intracellular tail, which then affects the catalytic performance of the extracellular catalytic domain (Barathova *et al.*, 2008; Ditte *et al.*, 2011).

The role of CA IX in tumor cells is primarily connected with its enzyme activity, although other biological activities (mediated by known or yet undisclosed interactions with various protein partners) can also be involved. The catalytic activity of CA IX participates in two interrelated phenomena contributing to the development of tumor phenotype, namely pH regulation and cell migration/invasion (Pastorekova *et al.*, 2008; Svastova and Pastorekova, 2013). Regulation of pH is essential for the survival of tumor cells in the microenvironment containing regions with hypoxia and also acidosis, which develops due to excess of lactic acid and CO_2 generated by the oncogenic metabolism (Pouyssegur *et al.*, 2006). Moreover, regulation of pH is important for cell migration and invasion that enable the cells to escape from this hostile microenvironment (Stock and Schwab, 2009). CA IX contributes both to neutralization of the intracellular pH and acidification of the extracellular pH by the mechanism that involves a facilitated bicarbonate transport across the plasma membrane (Svastova *et al.*, 2004; Swietach *et al.*, 2008, 2009; Chiche *et al.*, 2009). This is based on the spatial and functional cooperation of CA IX with bicarbonate transporters, including Na^+-coupled HCO_3^- co-transporters (e.g. NBCe1) and Cl^-/HCO_3^- anion exchangers (e.g. AE2). Such cooperative assembly, called bicarbonate transport metabolon, utilizes the CA IX extracellular active site to catalyze the CO_2 conversion to bicarbonate ions, which are directly delivered to bicarbonate transporters that bring them into the cytosol. There the bicarbonate ions take up protons to generate CO_2. The CO_2 leaves the cell by diffusion and further acidifies the pericellular milieu. The same CO_2 hydration reaction catalyzed by CA IX also generates protons, which remain at the outer side of the plasma membrane and contribute to acidosis (Gillies and Gatenby, 2007). Slightly alkaline intracellular pH provides a survival advantage by enabling biosynthetic reactions, whereas acidic extracellular pH induces cell migration and activates proteases degrading the extracellular matrix thereby supporting the invasion of tumor cells into the surrounding tissue. In relation to this it seems obvious that hypoxia, which is associated with acidic metabolism and increased migration/invasion, induces both expression levels and catalytic performance of CA IX (Svastova *et al.*, 2004). Thus, it is imaginable that the processes, which decrease the cell surface abundance of CA IX, can affect its capacity to fully accomplish these tumor-related functions.

In fact, cells often respond to various exogenous stimuli by a rapid decrease in cell surface density of a wide range of diverse regulatory proteins, including receptors and adhesion molecules. The decrease may occur either by the protease-mediated cleavage resulting in ectodomain shedding or by endocytosis, usually in a ligand-dependent fashion. Both of these processes have been demonstrated for CA IX, as described in more detail below.

2. Ectodomain shedding

Extracellular domains of a variety of transmembrane proteins can be released from the cell surface by the enzyme-regulated proteolytic cleavage, a process termed "ectodomain shedding". Shedding is an important regulatory mechanism affecting the abundance of membrane-bound molecules and significantly impacts on the biological functions of these proteins by converting them into soluble molecules that can either be biologically active variants or inactive decoys. They can act in autocrine and/or paracrine manner, either recapitulating or disturbing the functions of their transmembrane "parents". Only about 2% of cell surface proteins are released by ectodomain shedding, indicating that cells selectively shed their protein ectodomains (Hayashida *et al.*, 2010). Shed molecules include receptors (e.g. Notch), growth factors (HB-EGF), adhesion molecules (E-cadherin, CD44), enzymes etc. Shedding is usually low under basal conditions, but can be dramatically induced upon cell activation by phorbol esters, ionophores, growth factors, cytokines, stress factors and other modulators of intracellular signaling. Ectodomain shedding in vivo is tightly controlled by the spatially and temporally coordinated expression of the substrate, sheddase, and extracellular and intracellular regulatory factors.

Most transmembrane proteins are cleaved and shed by proteases that form a large and diverse family of metalloproteinases as well as a disintegrin and metalloproteinase (ADAM) family. The first sheddase was identified as an enzyme that cleaves the transmembrane tumor necrosis factor α (TNF-α) and was named tumor necrosis factor α converting enzyme (TACE/ADAM-17, Doedens, 2003). Subsequently, studies using cells derived from TACE-deficient mice have shown that TACE/ADAM17 can contribute to the shedding of many membrane proteins as reviewed elsewhere (Arribas, 2002). Interestingly, TACE/ADAM17 expression and activity is regulated by severe hypoxia suggesting that it can mediate the effects of hypoxia on the cell surface proteolysis in tumors and thereby affect the spectrum of soluble factors in the extracellular space and in the body fluids of tumor patients (Rzymski *et al.*, 2012).

Ectodomain shedding of transmembrane proteins not only produces extracellular fragments, but can also be a pre-

requisite for regulated intramembrane proteolysis resulting in the release of the soluble cytoplasmic fragments that can accomplish intracellular functions. In this case, ectodomain cleavage generates intramembrane protein stubs, which initially remain in the plasma membrane. Within the hydrophobic environment of a lipid bilayer, they are then subjected to regulated intramembrane proteolysis by the γ-secretase complex, releasing the soluble intracellular fragments that may translocate to the nucleus where they may act as transcriptional regulators (Chalaris, 2010; Wolfe and Kopan, 2004). Thus, it is now evident that ectodomain shedding is not only important as initially thought for the down-regulation of receptor abundance in the cell membrane and intracellular signaling but also for the production of the functional intracellular fragments that some membrane molecules require to carry out particular functions (Montes de Oca, 2010).

One of the important molecules undergoing both extracellular and intracellular cleavage is Notch receptor that mediates signaling essential for cell differentiation during development and beyond (Artavanis-Tsakonas *et al.*, 1999). After Notch is synthesized in the endoplasmic reticulum, it is cleaved in its extracellular domain during the passage through the secretory pathway, and the two resulting pieces remain associated until reaching the cell surface (Logeat *et al.*, 1998). Upon interaction with a cognate ligand, Notch becomes susceptible to a second extracellular proteolysis near the membrane (Brou *et al.*, 2000). The membrane-associated remnant is then cleaved by γ-secretase (De Strooper *et al.*, 1999), releasing the Notch intracellular domain, which translocates to the nucleus and activates transcription after associating with the nuclear partners, including HIF-1α (Gustafsson *et al.*, 2005).

3. Shedding of CA IX ectodomain

Although CA IX is a very stable protein with a half-life corresponding to about 38 hr (as determined in reoxygenated cells), it undergoes a constitutive shedding which affects about 10% of its molecules and is sensitive to metalloproteinase inhibitor batimastat (Rafajova *et al.*, 2004; Zatovicova *et al.*, 2005). Interestingly, moderate hypoxia (2% O_2) maintains the normal rate of basal ectodomain release thus leading to a concomitant increase in the cell-associated and extracellular levels of CA IX. CA IX ectodomain shedding can be induced several fold by treatment with phorbol-12-myristate-13-acetate and pervanadate which both affect phosphorylation signaling. We showed that this activated shedding is mediated by TACE/ADAM17 (Zatovicova *et al.*, 2005). Thus, the cleavage of the CA IX ectodomain appears to be a regulated process that responds to signal transduction-related stimuli and potentially contributes to adaptive changes in the protein composition of tumor cells and of their microenvironment.

CA IX ectodomain shedding can also be activated in response to other stimuli, including the induction of cell death by chemical compounds and chemotherapeutic drugs. This death-related shedding can be blocked by batimastat suggesting that it is also regulated by metalloproteinases (submitted). However, inhibition of CA IX ECD release does not seem to be directly involved in the control of death, but it is rather its consequence with so far no known biological significance.

Interestingly, our preliminary results with γ-secretase inhibitor indicate that CA IX molecule can also undergo proteolysis leading to the release of its cytoplasmic domain (unpublished data). However, further destiny of this intracellular fragment and its potential role still remain unknown.

Thus, CA IX ectodomain shedding appears to be an important regulator of the abundance, distribution and possibly also the function of CA IX protein. Provided that TACE/ADAM17 substrates are all biologically relevant molecules and their soluble ectodomains are generally not decoys (Arribas and Esselens, 2009) it is worth investigating whether there is any role for CA IX ectodomain in paracrine signaling and if so, what its cellular targets are.

4. Endocytosis of cell surface receptors

Receptor endocytosis is another important process regulating the abundance of cell surface receptors and/or signal transduction mediated by these molecules. This process includes receptor down-regulation induced either by ligands or by internalizing antibodies (Sorkin and von Zastrow, 2009).

Most of the molecules are internalized via clathrin-dependent endocytosis (Sorkin, 2004). They are first recruited to clathrin-coated pits by directly interacting with the clathrin coat adaptor complex AP2 or by binding to other adaptor proteins, which in turn interact with the clathrin heavy chain and/or AP2. Clathrin-coated pits invaginate inwards with the help of several accessory proteins and pinch off to form a clathrin-coated vesicle. Several clathrin-independent pathways of endocytosis also exist, although the precise mechanisms and structural components involved in these pathways are not well understood (Doherty and McMahon, 2009).

Endocytic vesicles derived from both clathrin-dependent and clathrin-independent endocytosis fuse with early endosomes, which mature into multivesicular bodies or late endosomes. Fusion of late endosomes and multivesicular bodies with lysosomes carrying proteolytic enzymes results in cargo degradation (Nickerson *et al.*, 2007).

Endosomes can undergo recycling that may proceed by two different routes: by a relatively fast pathway via endocytic

recycling compartment, or by a slow pathway via perinuclear recycling compartment (PNRC, pH 5.6). The PNRC is concentrated near the microtubule organizing center in most cell types. Disruption of microtubules by nocodazole can block transport to PNRC as shown by experiments with transferrin as a marker of clathrin-dependent endocytosis and recycling pathways (Baravalle *et al.*, 2005). After endocytosis into early endosomes, transferrin rapidly leaves this compartment ($t1/2 \approx 2.5$ min) and enters the PNRC from which it is recycled ($t1/2 \approx 7$ min). The recycling of transferrin is typically biphasic with distinct fast (from early endosomes) and slow components (via the PNRC). It has also been shown that the efficiency of recycling can be modulated by cell density. As described by Foerg *et al.* (2007), massive, compound-unspecific slow-down of endocytosis occurs in confluent MDCK cells. Interestingly, treatment of dense cells with IFN-γ/TNF-α results in restoration of the density-restricted endocytosis possibly by a cytokine-induced redistribution of lipid rafts.

It is becoming more and more apparent that endocytosis has many effects on signal transduction and that these cellular processes employ intertwining molecular networks (Jekely *et al.*, 2005). The endocytosis of many signaling receptors is triggered by ligand-induced activation and uses the same basic endocytic machinery as other cargo molecules (Sorkin and von Zastrow, 2009). Endocytosis may attenuate signaling from the cell surface but on the other hand, signaling can also proceed efficiently from the endosomes, where the regulatory proteins have closer spatial proximity to their interacting partners and/or substrates and alternatively, where proteolytic enzymes are enriched and activated by low pH (Joffre *et al.*, 2011). For example, endocytosis affects biological activity of Notch by its co-localization with γ-secretase in endosomes, which increases the cleavage of its intracellular domain and its downstream signaling (Vaccari *et al.*, 2008). It has also been proposed that Notch ectodomain shedding occurs in the lumen of the late endosomes. This provides a paradigm for spatial and temporal coordination of endocytosis and shedding as interconnected processes that regulate abundance and functioning of the nominally cell surface proteins (Le Borgne, 2006).

Antibody-mediated receptor endocytosis using internalizing monoclonal antibodies (MAbs) that are specific for tumor-associated antigens represents an efficient and important strategy of anticancer immunotherapy. Existing therapeutic approaches include MAb-mediated inhibition of the ligand binding or receptor dimerization, blocking of downstream signal transduction and acceleration of natural receptor internalization (Bianco *et al.*, 2005). Antibody-induced receptor endocytosis can be exploited either for the attraction of the immune response by T cells or for the delivery of immunotoxin conjugates into tumor cells (Trnkova *et al.*, 2012; Jaracz *et al*, 2005; Sharkey and Goldenberg, 2006). Several immunoconjugates, particularly those that incorporate internalizing antibodies, have demonstrated impressive activity in preclinical models as well as in clinical settings (Reichert, 2011).

5. CA IX endocytosis via internalizing monoclonal antibody

Almost exclusive expression in tumors, cell surface localization and functional involvement in cancer make CA IX a suitable target for anticancer treatment, particularly for immunotherapy. There are several monoclonal antibodies against CA IX with a potential therapeutic application including at least two antibodies capable of internalizing by inducing CA IX endocytosis. One of them, G250 MAb has nevertheless been examined mainly on the basis of its capacity to act through binding CA IX at the cell surface and attracting antibody-dependent cell-mediated cytotoxicity (ADCC) response that leads to cytotoxic killing of CA IX-expressing tumor cells. Pre-clinical studies as well as early stages of the clinical trials with G250 MAb (commercially known as RENCAREX®) have suggested promising therapeutic efficacy of MAb and its excellent tolerability in patients with renal cancer (Siebels *et al.*, 2011; Bleumer *et al.*, 2004). Moreover, the recently accomplished third phase of the clinical trial performed in adjuvant treatment setting in patients with non-metastasized clear cell renal cell carcinoma (so called ARISER study), showed clinically and statistically significant improvement in disease-free survival in the patient population with a high CA IX level treated with RENCAREX® compared to both placebo and patients with a low CA IX score (see http://www.wilex.de/press-investors/announcements/press-releases/20130226-2/). This is in agreement with earlier investigations, which showed that the efficient targeting of tumor cells is affected by antigen heterogeneity and requires certain CA IX antigen density (Steffens *et al.,* 1999).

Our group has generated several monoclonal antibodies against human carbonic anhydrase IX (CA IX), which are useful for immunodetection of CA IX (including the well-known M75 antibody frequently used for immunohistochemical staining of CA IX in hypoxic tumors, Zatovicova *et al.*, 2003). Among them, the antibody VII/20 directed to the catalytic domain of CA IX can trigger the antibody-mediated endocytosis (Fig. 1), which leads to depletion of about 30% of CA IX molecules from the cell surface in the period of 3 hr (Zatovicova *et al.*, 2010). During this initial period as well as in the following 24 hr, the antibody signal is intracellular and overlaps with the CA IX protein signal suggesting that the antibody remains associated with the antigen. Following this, antibody-CA IX antigen complex recycles back to the cell membrane and is again exposed at the cell surface. This

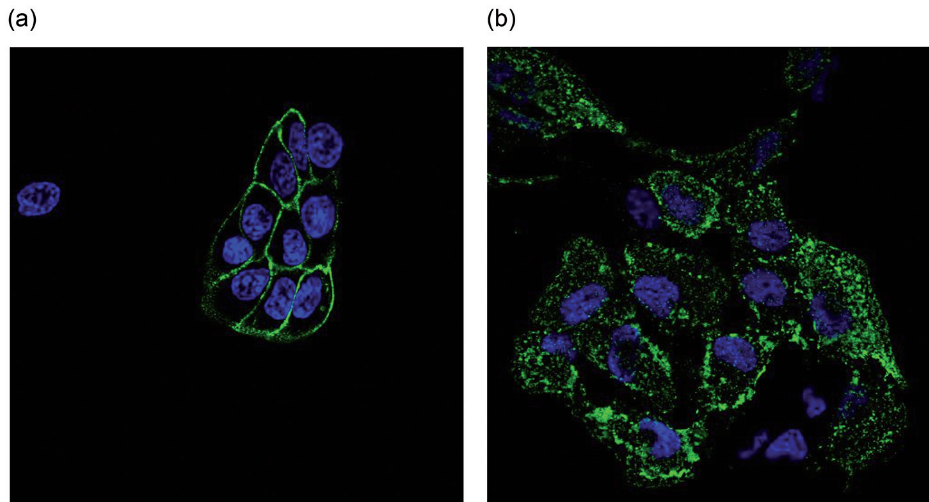

Fig. 1

Endocytosis of CA IX induced by the monoclonal antibody VII/20 analyzed by immunofluorescence staining and confocal microscopy

The antibody was added to MDCK cells constitutively expressing CA IX on the cell surface. The cells were incubated at 4°C to allow for antibody-antigen binding on the cell surface and prevent endocytosis. The cells were then washed and either fixed with ice-cold methanol (left panel) or transferred to 37°C to induce endocytosis for 3 hr and then fixed (right panel). Both samples were rehydrated and incubated with the ALEXA 488-conjugated secondary anti-mouse IgG. Distribution of the VII/20 primary antibody was visualized by confocal microcopy and was found at the cell surface in a condition that prevented endocytosis (a), whereas it was clearly intracellular following induction of endocytosis (b).

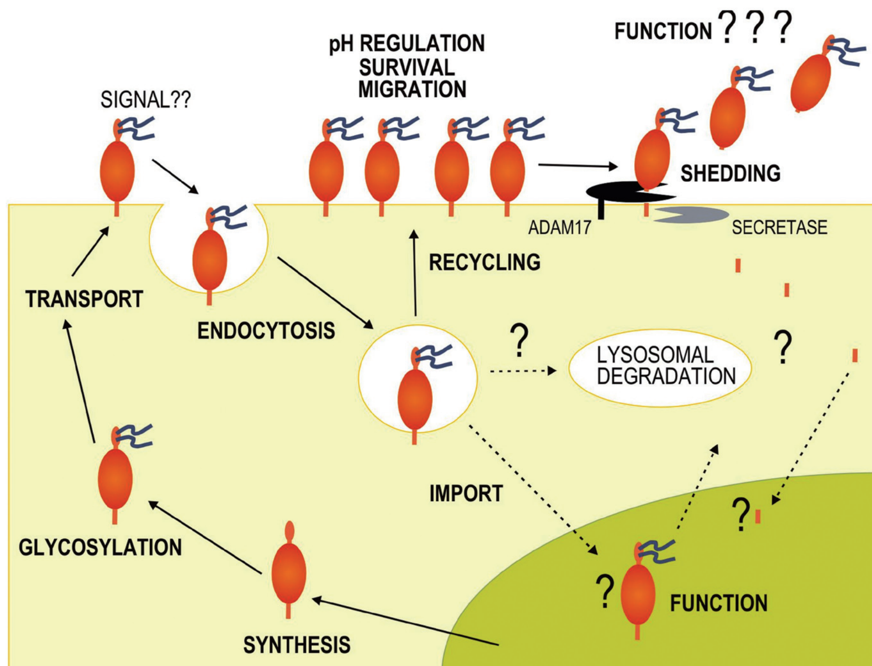

Fig. 2

Schematic illustration of the transport routes of the CA IX protein

Starting from the bottom left, CA IX is synthesized in a hypoxia-dependent manner, glycosylated and transported to the plasma membrane. There, CA IX can fulfil its biological role connected with pH regulation and related protection from hypoxia/acidosis as well as facilitated cell migration/invasion. Cell surface CA IX can undergo endocytosis in response to various signals, such as the disruption of cell adhesion, hypoxia, internalizing antibody and/or so far unknown natural ligand (top left). Endocytosis of CA IX is followed either by recycling back to the plasma membrane, or by import (via interaction with the transport proteins) to the nucleus (bottom right). Alternatively, or simultaneously, CA IX ectodomain can be shed by the TACE/ADAM17 protease from the cell surface to the extracellular space in response to severe hypoxia and other stress stimuli. Ectodomain shedding can be followed by the cleavage with γ-secretase that releases the intracellular portion of CA IX that remains in the intracellular space, although its exact destination is not yet known. It also remains to be clarified whether, how and under which conditions CA IX functions in various intracellular compartments and in the extracellular space.

offers opportunity for the repeated ADCC but also for the reconstitution of the CA IX function, although it is not yet fully clear how antibody binding affects CA IX function.

VII/20 antibody-induced CA IX endocytosis can be reduced or prevented by high cell density. However, the density block can be relieved by hypoxia, which is known to destabilize intercellular contacts either via decreased expression/functional inactivation of E cadherin or by activation of proteases which disrupt other molecules involved in cell-cell adhesion (Svastova *et al.*, 2003; Beavon, 2000; Esteban *et al.*, 2006). In fact, hypoxia can induce CA IX endocytosis in MDCK polarized epithelial cells even in the absence of the internalizing antibody. In these cells, CA IX endocytosis proceeds simultaneously with the hypoxia-induced endocytosis of E cadherin and is linked with the perturbation of intercellular contacts (Svastova *et al.*, 2003). Moreover, cell disconnection induced by depletion of calcium ions also results in endocytosis of E cadherin as well as in CA IX supporting the above-described connection between the density, hypoxia and endocytosis. This data also indicates that CA IX endocytosis can occur in natural conditions upon stimulation by physiological factors. It is quite possible that CA IX responds to binding of a natural ligand, however no such molecule has been identified so far.

In accordance with the observed recycling of the antibody-CA IX complex, our unpublished data suggests that CA IX undergoes endocytosis via clathrin-coated vesicles (CCV), but does not subsequently enter lysosomes and degradation, but rather goes to perinuclear recycling vesicles. However, recycling of CA IX is much slower compared to transferrin or EGFR, suggesting that it has a different regulatory impact.

Interestingly enough, a recent proteomic study searching for the interacting partners of CA IX revealed a group of intracellular proteins that belong to nuclear transport machinery, including XPO1 exportin and TNPO1 importin (Buanne *et al.*, 2013). Although the interaction occurs via the C-terminal region of CA IX, it involves entire (mature) CA IX molecule that can be detected in the nuclear and perinuclear regions of hypoxic cells through the catalytic domain-specific antibody. This suggests the existence of the CA IX protein subpopulation with a potential intracellular function, distinct from the crucial CA IX role at the cell surface. It is quite conceivable that this subpopulation represents an endocytosed fraction of the CA IX molecules, although the experimental evidence for this assumption is not available so far.

6. Conclusions and future directions

Based on the data described above, we can draw the main contours of the routes leading CA IX to and from the cell surface and regulating its abundance in various subcellular compartments as well as in the extracellular space (Fig. 2). We now know that CA IX, newly translated from hypoxia-induced transcripts, is transported to the cell surface, where it can either reside for a relatively long time in the plasma membrane, or can be stimulated to endocytosis (and consequent recycling), or can be cleaved by proteases to soluble ectodomain released to extracellular space and intracellular fragment remaining inside the cell. However, we are still missing answers to many questions related to these phenomena: Is there a natural ligand binding to CA IX, affecting its function, inducing signaling and mediating its endocytosis? What is the molecular identity of the ligand? Does CA IX signal from endosomes inside the cell? Why does CA IX recycle so slowly and why is its intracellular persistence so long? Is the intracellular fraction of CA IX that cooperates with nucleo-cytoplasmic machinery functionally involved in regulation of transcription/translation? What is the biological role of the shed CA IX ectodomain? Does the ectodomain mediate autocrine or paracrine signaling? Which cells/tissues are targets of CA IX signaling? What is the final destination of the cleaved intracellular fragment of CA IX? Does it play a biological role? Are there additional posttranslational mechanisms regulating the cell surface density of CA IX? How do all of these phenomena crosstalk in different tissues and under different physiological conditions? Extensive experimentation is clearly needed to elucidate the complex network of CA IX transport routes and functional aspects. This is very important because understanding of these phenomena is a necessary prerequisite for the rational clinical use of CA IX.

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