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The role of carbonic anhydrase IX overexpression in kidney cancer

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Abstract

Carbonic anhydrase IX (CA IX) is a membrane isoenzyme, the overexpression of which is associated with clear cell carcinoma of the kidney. Its overexpression is restricted mainly to cancer, as it is absent in corresponding normal tissues making it a potential cancer biomarker. Several recent studies have shown that CA IX, apart from its classical enzyme activity of reversibly hydrating carbon dioxide extracellularly to facilitate the net extrusion of protons from inside to outside the cell, it can also be a key player in the modulation of cell adhesion processes and participate in the regulation of cell proliferation in response to hypoxic environment to ultimately contribute to tumour progression. Here, we have shown that the sole tyrosine moiety of CA IX present in its intracellular domain can be phosphorylated in an epidermal growth factor dependent manner, suggesting that it can feed into the growth factor receptor dependent signalling pathways. Our studies suggest that the tyrosine phosphorylated CA IX can interact with the regulatory subunit of PI-3-Kinase, contributing to Akt activation. These studies have revealed a positive feed back loop that can form the basis of a vicious cycle that could contribute to the progression of clear cell renal carcinoma and poor prognosis. These studies show that CA IX signalling may be a part of both the hypoxia driven and hypoxia independent pathways that occur in the cancer cell. Finally, our studies emphasize the need for a more refined strategy using signal transduction therapeutics to inhibit the cell surface carbonic anhydrases for the management of this malignancy.
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Keywords: Carbonic anhydrase IX; Carbonic anhydrase XII; Cell signaling; Epidermal growth factor receptor; Renal cell carcinoma; Clear cell

1. Introduction

Renal cell carcinoma (RCC) accounts for roughly 2% of all cancers, with the highest rate of occurrence within the United States and Northern Europe [1,2]. RCC is well known clinically for its lack of early warning signs, resulting in significant occurrence of metastatic disease progression at first diagnosis. Recent advances in our understanding of the molecular pathogenesis of RCC, greatly aided by the introduction of cDNA microarray technology, has provided a clearer picture of the underlying molecular and genetic alterations in RCC [3,4]. Currently, RCC is viewed not as a single entity, but as a mixture of several sub-types of diseases with different biological attributes and unique molecular signatures that happen to occur in a single organ, *i.e.*, the kidney [5,6]. Extensive biochemical analyses including the molecular profiling of these RCC subtypes revealed the association of a class of carbonic anhydrase isoenzymes (CA IX and CA XII) which are membrane bound zinc metalloenzymes with clear cell carcinoma of the

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kidney [7–9]. Initial studies focused on their role as a molecular marker, since their expression was mostly restricted to cancer cells, the only exception being their normal expression in polarized epithelial cells lining the gastrointestinal tract and pancreas [10–12]. For clear cell carcinoma of the kidney, CA IX protein appears to play a significant role in cancer adaptation to hypoxic environments and may be involved in tumour progression [13,14].

CA IX is a novel member of the phylogenetically well preserved carbonic anhydrase family [15,16]. It is a single span transmembrane glycoprotein that possesses an extracellular catalytic domain which is preceded by a novel proteoglycan domain. It catalyzes the reversible hydration of CO_2 ($CO_2 + H_2O \rightarrow HCO_3^- + H^+$). There are 15 isoenzymes of CA identified so far and these are found in diverse subcellular locations such as the cytoplasm and mitochondria [17]. Of these enzymes, CA IX is membrane bound and its overexpression on the cell surface is seen in a number of solid tumours, particularly in clear cell RCC and including cervical, ovarian, colorectal, head and neck, bladder and non-small cell lung carcinomas [18,19]. In all these malignancies, the expression of CA IX is invariably linked to the development of tumour hypoxia, which is mediated by the transcription factor HIF-1. Other HIF-1 target genes induced by intratumoural hypoxia include glucose transporter-1 (GLUT-1), several glycolytic enzymes and angiogenic growth factors such as vascular endothelial growth factor (VEGF) that are essential for survival and adaptation to hypoxic environments. Studies with clear cell RCC revealed that the CA IX expression is associated with mutation(s) in the von Hippel-Lindau tumour suppressor gene (*VHL*) or promoter suppression by methylation with subsequent loss of VHL protein function or expression [20,21]. CA IX protein can also be overexpressed in RCC clear cell tumours that arise in a sporadic manner [22]. In RCC cell lines that overexpress CA IX, the expression could be suppressed if the wild-type *VHL* gene was reintroduced by transfection [23]. Several landmark investigations have elucidated the role of VHL in the regulation of HIF-1α [24–26]. Thus, the regulation of CA IX expression; its function as a HIF-1α responsive gene; the recent discoveries on the molecular biology of the loss of VHL function; and its relatively high frequency of occurrence among subtypes of RCC has made clear cell RCC the most characterized subtype of all RCCs. This has had serious implications for our understanding of hypoxic tumour metabolism and the mechanisms by which the tumour cell manages to maintain intracellular pH homeostasis.

The transmembrane CA IX protein possesses cell surface enzyme activity which functions to convert CO_2 that has diffused to the extracellular space back into bicarbonate and protons. This would enable the chloride-anion exchanger on the cell membrane to transport these newly generated HCO_3^- anions back into the cytoplasm. This coupled transport process, with the net export of protons is absolutely essential for hypoxic cancer cells to buffer their intracellular pH to near neutral conditions necessary for their biosynthetic reactions. Thus, the function of membrane bound CA IX would contribute to extracellular acidosis, which in turn contributes to the activation of cell surface proteases such as cathepsin B and matrix metalloprotease 9 (MMP-9), release of growth factors bound and latent in the extracellular matrix and helps to suppress the immune function of effector T-cells [27–29]. These findings not only make CA IX protein an integral marker for hypoxia, but also make it an attractive target for therapy [30].

Several investigations have recently focused on another aspect of the CA IX protein, namely, its role in the regulation of intercellular communication processes. Svastova and colleagues [31] have recently found that CA IX protein has the capacity to modulate E-cadherin mediated cell adhesion processes, *via* its interaction with β-catenin, which could play a significant role in hypoxia induced tumour progression. This capacity is conferred to CA IX protein by its proteoglycan domain (PG) which resides immediately upstream of the carbonic anhydrase (CA) domain. Its described role in decreasing the binding of E-cadherin to β-catenin has important consequences for the destabilization of adherence junctions, a property attributed to the acquisition of increased invasive behavior of the tumour cell [32,33]. Thus, these observations place CA IX in the class of crucial molecules that regulate cell–cell interaction pathways. The carbonic anhydrase domain of CA IX is shared, although in a catalytically inactive form, with the extracellular domain of certain receptor type protein tyrosine phosphatases (RPTP) [34–36]. The CA domain of RPTP-β is implicated as the ligand for the adhesion molecule contactin and plays a role in the regulation of cell–cell adhesion of aggrecans and similar molecules [36]. Thus, the extracellular domain modules of CA and PG in CA IX could play important roles in regulating cell–cell interactions apart from its established enzymic activity. But the cytoplasmic tail portion of this protein has not yet been shown to participate in signal transduction processes, even though it has been known to possess the necessary characteristic features and has been predicted to do so [31,37]. In this study, we have investigated the possibility that the intracellular domain of the CA IX protein could be involved in cell signalling pathways that go on to characterize the clear cell RCC. Our studies reveal that this CA IX protein is indeed an active participant in the growth factor receptor mediated signal transduction pathways in clear cell RCC and have identified a positive feed back loop which could form the basis of a vicious cycle that might contribute to the progression of renal cell carcinoma and to poor prognosis. Finally, our studies strongly suggest the need for a more

refined strategy to inhibit the cell surface carbonic anhydrases to achieve maximum therapeutic benefit.

2. Materials and methods

2.1. Cell culture

SKRC-01, SKRC-08 and SKRC-17 RCC cell lines were a kind gift from Neil Bander (Weill Medical College, Cornell University, NY). Of these cells, the 01 and the 08 lines overexpressed CA IX protein whereas the SKRC-17 cell line did not. The cell lines were regularly maintained at 37 °C in a 95% air and 5% CO_2 incubator in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 2 mM non-essential amino acids (NEAA), 50 IU/ml penicillin and 50 μg/ml streptomycin sulfate and 2.5 μg/ml fungizone. All reagent kits, recombinant proteins, antibodies and other reagents such as trypsin for replating the cells were used according to the manufacturer's recommendations.

2.2. EGF dependent phosphorylation of CA IX

SKRC-01 cells, grown to 50% confluency in 60 mm culture dishes, were serum starved by growing them in serum-free medium supplemented with 0.1% FBS overnight. The medium was then changed to serum-free medium for a further 2 h. Recombinant EGF (rEGF, Santa Cruz) was dissolved in 10 mM acetic acid containing 0.1% BSA at a stock concentration of 50 μg/ml and increasing amounts of rEGF at final concentrations of 0–50 ng/ml were used to stimulate the serum starved cells for 30 min. Radioimmunoprecipitation assay buffer (RIPA) used in these studies consisted of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS containing the protease inhibitor cocktail (Roche Diagnostics) supplemented with 2 mM phenylmethyl sulfonyl fluoride (PMSF) and 1 mM activated sodium orthovanadate. Total RIPA lysates were prepared and equivalent amounts of RIPA lysates were processed for immunoprecipitation. Briefly, the RIPA lysates that have to be immunoprecipitated were treated with 20 μl of washed 50% suspension of protein A-agarose (Santa Cruz) for 30 min at 4 °C to eliminate non-specific protein binding. The beads were removed by centrifuging at 1000*g* for 1 min and supernatants were retained. To the supernatants, a polyclonal antibody against CA IX (Santa Cruz) was added at 1:1000 dilution and subjected to gentle mixing in a rocker at 4 °C overnight. The immune complexes were collected by addition of 20 μl of 50% suspension of protein A-agarose. The samples were then rocked gently at 4 °C for 1 h. Immunoprecipitates were subjected to gel electrophoresis and blotted onto PVDF membranes. The blocked membranes were treated with a monoclonal antibody against phosphotyrosine (PY-20, Santa Cruz Biotechnology, CA) at 1:500 dilution. The membranes were washed and the blots were finally treated with goat anti-mouse immunoglobulin (IgG) conjugated to horse radish peroxidase (Santa Cruz at 1:3000 dilution). The signals were revealed with enhanced chemiluminescence. As a negative control, the initial immunoprecipitation was done by replacing the polyclonal antibody to CA IX with normal rabbit serum (Santa Cruz, 1:500 dilution) and following through the entire procedure. As a control for the amounts of protein loaded on each SDS–PAGE gel, the PVDF membranes with the transferred immune complexes from the polyclonal antibody (to CA IX) were probed with M75 monoclonal antibody to CA IX at a 1:1000 dilution (a kind gift from Bayer Corp, West Haven, CT) and the signals were visualized by enhanced chemiluminescence as described earlier. Parallel experiments were performed to determine the kinetics of the loss of tyrosine phosphorylation when the same SKRC-01 cells were serum starved and stimulated with 50 ng/ml EGF for 30 min as described earlier. The stimulus was then removed and the extent of phosphorylation was followed further for 90 min.

2.3. Preparation of lipid rafts from SKRC-01 cells

Lipid rafts were prepared from renal cancer cells according to the method of Goebel with few modifications [38]. Briefly, around 4×10^7 cells were lysed in MES lysis buffer containing 25 mM MES (morpholinoethane sulfonic acid), 150 mM NaCl, 0.5% Triton X-100 and 2 mM EDTA for 30 min on ice and sonicated very briefly (3 one second pulses). An equal amount of 85% sucrose made in MES buffered saline (MBS) containing protease inhibitor cocktail at 1× concentration (Roche Diagnostics, Indianapolis, IN). Ultracentrifuge tubes were underlayed with 6 ml of 5% and 6 ml of 35% sucrose in MBS and finally the lysed cell suspension was underlayed with the help of a syringe and needle below the 35% sucrose layer. The tubes were spun at 104000*g* at 4 °C for 20 h. The lipid rafts located at the interface of 5% and 35% sucrose layers were collected as 1 ml fractions. A 2 μl aliquot of the fractions was routinely spotted on to nitrocellulose membranes and processed with cholera toxin B-subunit conjugated with horse radish peroxidase (HRP) using the enhanced chemiluminescence method (ECL) to detect rafts.

2.4. Co-immunoprecipitation of PI-3Kinase with CA IX

Equal aliquots of the RIPA cellular extracts prepared from serum starved SKRC-01 cells and those prepared by stimulating the serum starved cells with 20 and 40 ng/ml EGF as described earlier were immunoprecipitated with M75 monoclonal antibody to CA IX (1:500

dilution) and the immune complexes were collected with Protein A/G Agarose (Santa Cruz). The denatured immune complexes were separated on 7.5% SDS–PAGE gels, transferred to PVDF membranes and blocked and probed with a polyclonal antibody to the p85 subunit of PI-3-Kinase (Lab Vision, Freemont, CA). As a negative control, equivalent aliquots of the RIPA extracts used for the above experiment was separated on another gel and probed for the presence of the p85 subunit of PI-3-Kinase using the same antibody as described above. In some cases, the lipid raft fraction isolated from the SKRC-01 cells that were serum starved and stimulated with 40 ng/ml EGF was also immunoprecipitated with M75 monoclonal antibody and probed for the co-immunoprecipitating PI-3-Kinase (p85 subunit).

2.5. Phosphorylation status of Akt

To determine the activation of PI-3-Kinase by interaction with the tyrosine phosphorylated carbonic anhydrase IX protein, SKRC-17 cells which do not express CA IX protein were transiently transfected with vector only (pSG5C) or with wild-type CA IX cloned into pSG5C using the Transfast transfection kit (Promega Corporation, Madison, WI) exactly as described by Zatovicova and coworkers [39]. The cells that underwent transfection were maintained in the CO_2 incubator for 64 h. At that time, the complete medium was replaced with a serum free medium supplemented with 0.1% FBS to mimic serum starvation conditions and the PI-3-Kinase inhibitors LY 294002 and wortmannin were added at the indicated concentrations and the incubation continued for 8 more hours. Before completion of this experiment (*i.e.*, at 72 h), the transfected cells in the presence or absence of the inhibitors were stimulated for 30 min in the presence of recombinant EGF (50 ng/ml). Whole cell extracts were made with the RIPA buffer and equivalent amounts of the extracts were analyzed on 7.5% denaturing polyacrylamide gels as described earlier. The transferred proteins on the PVDF membranes were probed with phosphospecific antibodies for Ser 473 or Thr 308 of Akt (1:1000 dilution, Akt sampler kit, Cell Signalling Technologies, Beverly, MA). Identical amounts of the extracts were run on another gel and probed with the antibody to unphosphorylated Akt (1:1000 dilution, Akt sampler kit, Cell Signalling Technologies, Beverly, MA) using the same blotting and probing conditions, as described above to verify that equivalent amounts of proteins in each sample had been analyzed.

2.6. Site directed mutagenesis of CA IX and stable transfection studies

The single tyrosine at position 449 of the wild-type CA IX protein was changed to phenylalanine using the Quick Change XL mutagenesis kit (Stratagene, La Jolla, CA) and the mutation (CA IX YF) was confirmed by subsequent sequencing. The "sense" (S) and the antisense (A) primers used for creating this mutation were synthesized from MWG-Biotech AG (Charlotte, NC). The S primer was 5′-CAA AGG GGG TGT GAG CTT CCG CCC AGC AGA GGT AG-3′ and the A primer was 5′-CTACCT CTG CTG GGC GGA AGC TCA CAC CCC CTT TG-3′. SKRC-17 cells constitutively expressing either wild-type CA IX or the C IX YF mutant were obtained by co-transfection of the recombinant plasmids with the mammalian expression vector pCDNA 3.1(neo) (Invitrogen, Carlsbad, CA) in a 10:1 ratio using the TransFast transfection kit (Promega Corp, Madison, WI) exactly according to the instructions by the manufacturer. The cells were selected for growth at a G418 concentration of 600 μg/ml and isolated with the use of cloning cylinders. The transfected clones were tested for CA IX expression and expanded further. Six individual cell populations were analyzed for CA IX expression to rule out the effect of clonal variation. As negative controls, the same SKRC-17 cells were transfected with empty vector pSG5C and pCDNA 3.1 and individual clones were selected for G418 resistance.

2.7. Analysis of HIF-1α in relation to CA IX expression and EGF stimulation

SKRC-01, 08 and 17 cells were serum starved as described earlier and stimulated with 50 ng/ml recombinant EGF. The same experiment was also performed with the SKRC-17 cells stably expressing the empty vector, wild-type CA IX plasmid and the CA IX YF mutant plasmid. RIPA lysates were prepared from all the cells at the end of each stimulation experiment. For SKRC-01, 08 and 17 lysates, equivalent proteins were separated on denaturing gels, immunoblots were prepared and probed for the presence of CA IX, Akt, phosho Akt (ser 473), HIF-1α and HIF-1β. The polyclonal antibodies for HIF-lα and HIF-1β were purchased from Novus Biologicals, Littleton, CO. The expression levels of total Akt and HIF-1β in these blots also served as a control amount of total protein separated on each gel. Immunoblots generated from the EGF stimulated and stably transfected lysates of SKRC-17 cells harboring the negative control, wild-type CA IX and the mutant CA IX were probed for the relative expression of Akt and the phosphorylated Akt (ser 473) using the phospho-Akt pathway sampler kit as described above.

3. Results

3.1. Intracellular domain of CA IX can be phosphorylated in an EGF dependent manner

Since epidermal growth factor receptor (EGFR) signalling is critically modulated by its localization in cholesterol rich membranes, and since its overexpression

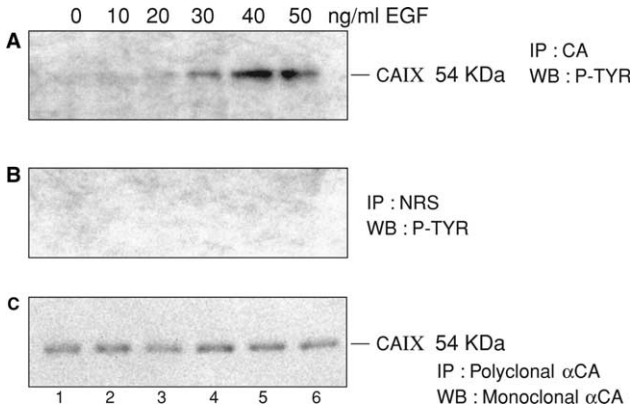

Fig. 1A–C. Study showing EGF dependent phosphorylation of membrane associated carbonic anhydrase IX. SKRC-01 cells were serum starved and stimulated with increasing concentrations of EGF for 30 min. Whole cell lysates were immunoprecipitated with polyclonal antibody to CA IX and the blots were probed with a monoclonal antibody to phosphotyrosine (panel A). As a negative control, the same experiment was repeated with normal rabbit serum instead of the polyclonal antibody to CA IX shown in the panel B. The panel C shows that equivalent amounts of protein were loaded when the same amounts of protein loaded for panel A were run on another gel and probed with the monoclonal antibody to CA IX (M75).

is well documented in the poor prognosis of renal cell carcinoma, we wished to see the effect of EGFR dependent signalling on the phosphorylation status of CA IX [40,41]. The results of these studies are shown in Fig. 1A–C . The CA IX expressing SKRC-01 cells were serum starved and stimulated with increasing amounts of recombinant EGF and the RIPA extracts were made from these stimulated cells. These extracts were used in immunoprecipitation experiments with a polyclonal antibody to CA IX and the immune complexes collected were run on a denaturing polyacrylamide gel. The proteins transferred to PVDF membranes were probed for the presence of phosphotyrosine using a commercially available monoclonal antibody. This resulted in the visualization of the tyrosine phosphorylated version of CA IX as shown in Fig. 1, panel A. As a negative control, we immunoprecipitated the same extracts with a commercially available non-immune rabbit serum, processed the immune complexes collected and the resulting blots were probed with the same anti-phosphotyrosine antibody as described earlier which is shown in Fig. 1, panel B. The presence of equivalent amounts of CA IX used for all the lanes as a loading control are shown in Fig. 1, panel C after probing the blots with the M75 monoclonal antibody. These results indicate that CA IX is capable of receiving stimulatory signals from the epidermal growth factor receptor and participate in the ensuing signalling pathways. Since CA IX is also a very stable protein, we wanted to see how this new CA IX function is regulated. The kinetics of loss of CA IX tyrosine phosphorylation is shown in Fig. 1D revealed a complete loss of signal after 75 min post stimulation.

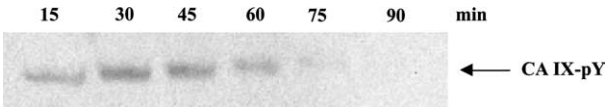

Fig. 1D. Kinetics of the loss of phosphorylation of CA IX in EGF stimulated SKRC-01 cells using conditions as in Fig. 1A–C. The initial EGF stimulus was removed after 30 min and the loss of CA IX-pY signal was followed up to 90 min.

3.2. Co-immunoprecipitation of tyrosine phosphorylated CA IX and p85 of PI-3-Kinase

Some of the data from this study have indicated a functional cross-talk between CA IX and EGFR signalling pathways and suggests that the tyrosine phosphorylated version of CA IX could participate in the phosphatidyl inositol-3 Kinase (PI-3-Kinase) signalling. To investigate this possibility, we immunoprecipitated the serum starved and EGF stimulated extracts of SKRC-01 cells with the M75 monoclonal anti-CA IX antibody and probed the resulting blots for the possible association with PI-3-Kinase. For this, we used a polyclonal antibody to the p85 subunit of the PI-3-Kinase which is shown in Fig. 2, upper panel. This figure shows that in the absence of any stimulatory signal, under completely serum starved conditions, there is no association of PI-3-Kinase with the CA IX protein. This would be expected since the C-terminal Y is not phosphorylated under these conditions (lane 1). This figure also shows that there is an EGF concentration dependent increase in the amount of associated PI-3-Kinase (lanes 2 and 3). In some experiments, the association of the tyrosine phosphorylated CA IX with PI-3-Kinase was also verified in the membrane raft preparations made from the EGF stimulated SKRC-01 cells (upper

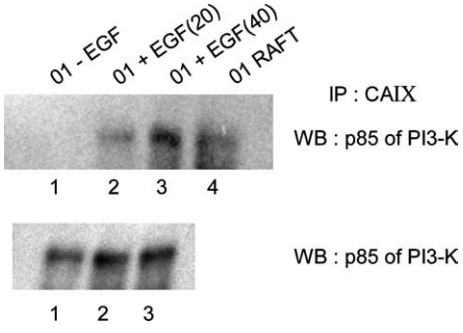

Fig. 2. Co-immunoprecipitation of p85 subunit of PI-3-Kinase with the tyrosine phosphorylated CA IX. SKRC-01 cells stimulated in the presence or absence of EGF were solubilized and immunoprecipitated with M75 MAb to CA IX. The blots were probed with a polyclonal antibody to the p85 regulatory subunit of PI-3-Kinase (upper panel lanes 1, 2 and 3). As a loading control, identical blots were probed for the presence of the total p85 subunit of PI-3-Kinase (lower panel). In some cases, the lipid raft membrane fractions from EGF stimulated SKRC-01 cells were solubilized and processed for immunoprecipitation with CA IX and immunoblotting with p85 (upper panel, lane 4).

panel, lane 4). This shows that CA IX is recruited to the lipid rafts where it could participate in signal transduction processes. To verify that equivalent amounts of proteins were loaded in the co-immunoprecipitation experiments, equivalent amounts of protein extracts were run on an independent gel and the resulting blot was probed for the presence of the p85 subunit of PI-3-Kinase (lower panel). Based on these observations, we infer that CA IX could be an active participant in the PI-3-Kinase signalling pathways.

3.3. Activation of Akt by CAIX-pY and PI-3-Kinase interaction

We then wished to see whether the PI-3-Kinase activation by association with CA IX could be reproduced in a CA IX negative RCC cell line such as SKRC-17 upon transfection with a wild-type CA IX plasmid and to investigate whether this associated PI-3-Kinase could be pharmacologically blocked. The results of these studies are shown in Fig. 3. SKRC-17 cells were transiently transfected with either the wild-type CA IX containing plasmid or the vector alone and were treated with inhibitors in the presence or absence of recombinant EGF. Initial transfection experiments incorporating negative controls which included empty vector plasmid (pSG5C)

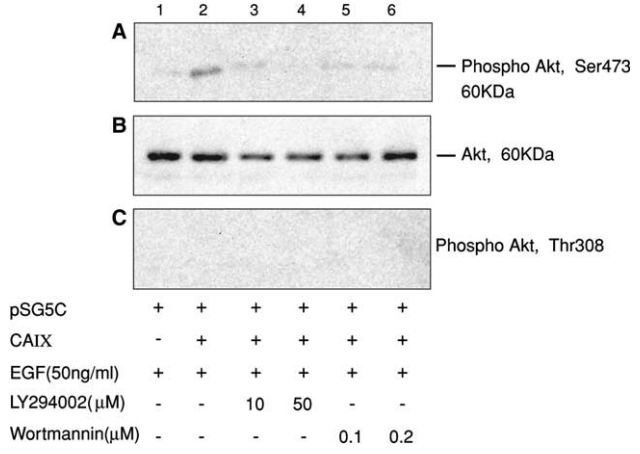

Fig. 3. Effect of pharmacological PI-3-Kinase inhibition on the phosphorylation status of Akt. SKRC-17 cells (CAIX negative) were transiently transfected with the plasmid pSG5C-wtCAIX and serum starved before the start of the study. The cells were pretreated for 8 h with wortmannin and LY294002 at the indicated concentrations. At the end of this pretreatment period, the cells were stimulated with EGF and whole cell extracts were made with radioimmunoprecipitation (RIPA) buffer. Immunoblot assays were performed using antibodies against phosphorylated Akt (for serine 473 and threonine 308). As a measure of the loading controls, blots were probed for total unphosphorylated Akt protein, shown in panel B. Preliminary work with either vehicle only controls (DMSO) or EGF unstimulated controls showed no phosphorylation of either Ser 473 or Thr 308 in serum starved conditions (data not shown). Under the EGF stimulated conditions, the phosphorylation of Akt at Thr 308 was not observed (panel C).

in the absence of stimulating EGF (*i.e.*, CA IX- and EGF-) showed no phosphorylation of either ser 473 or Thr 308 of the Akt enzyme, the target of PI-3-Kinase. It is well known that the activation of PI-3-Kinase is triggered by the binding of its SH2 domain containing p85 regulatory subunit to phosphorylated tyrosine residues of activated growth factor receptors or their substrates [42,43]. Thus, in Fig. 3A in lane 2 from left, there is a significant increase in the ser 473 phosphorylation of Akt under EGF stimulated conditions as studied by using a phospho-specific antibody for this species, upon transient transfection of CA IX, when compared to a relatively decreased phosphorylation level of the same protein in the absence of transfected CA IX but in the presence of EGF (Fig. 3A lane 1). This implies that the activation of PI-3-Kinase and subsequent phosphorylation of ser 473 on Akt by EGF stimulation of CA IX expressing cells could be additive and could act as a synergistic mechanism in the activation of PI-3-Kinase. These inferences could have significant implications with respect to therapeutic interference. Akt phosphorylation could be pharmacologically reduced by PI-3-Kinase inhibitors, namely LY294002 and wortmannin. (Fig. 3A, lanes 3–6 from left). At the indicated concentrations, LY294002 is shown to be a better inhibitor of both the base level and the CA IX stimulated PI-3-Kinase activity when the phosphorylation status of Akt ser 473 is studied. As other negative controls, the use of equivalent amounts of the vehicle dimethylsulfoxide (DMSO) that was used to dissolve these inhibitors did not have any effect on the PI-3-Kinase activity (data not shown). As loading controls, equivalent amounts of protein extracts were run on another gel and probed for the presence of unphosphorylated Akt as a measure of total Akt, which is shown in Fig. 3B. We could not see however, any phosphorylation of threonine 308 of Akt in these transfection studies (Fig. 3C). Nevertheless, these studies lead us to conclude that the introduction of the membrane bound CA IX in these CA IX negative RCC cells led to an additive activation of PI-3-Kinase and subsequent activation of Akt under conditions of EGF stimulation.

3.4. CA IX when stably transfected, shows elevated Akt phosphorylation under EGF stimulated conditions

Since the above studies were done under conditions of transient transfection, that may or may not reflect physiological conditions, we next wished to see whether the phenomenon of Akt phosphorylation could be seen in SKRC-17 cells (which are CA IX negative) when they are transfected to express the human CA IX protein in a constitutive manner. For this purpose, the pSG5C-CA IX plasmid was co-transfected with pCDNA3.1-neo plasmid at the same ratio as described by Svastova and G418 resistant cells were selected [39,44]. In parallel,

SKRC-17 cells stably expressing the CA IX YF mutant protein was also selected under identical conditions. As negative controls for these stable transfection experiments, SKRC-17 cells expressing empty vectors pSG5C and pCDNA3.1-neo were also selected. The cells were serum starved and stimulated with 50 ng/ml recombinant EGF as described earlier. RIPA lysates prepared from these cells were subjected to denaturing gel electrophoresis and immunoblots were probed for the expression of total and phosphorylated Akt (ser 473) proteins. The results of a typical experiment are shown in Fig. 4A. While the total Akt amounts that was followed in each experimental condition was equivalent, the differences in the level of Akt phosphorylation was more significant in wild-type CA IX expressing SKRC-17 cells with higher Akt phosphorylation (Fig. 4A, lane 2) compared to the same cells without CA IX expression, both in the presence of EGF (Fig. 4A, lane 1). Whereas, when the same cells expressed the YF mutant of CA IX, significantly less phosphorylated Akt (Fig. 4A, lane 4) was detected. The corresponding negative controls for these CA IX proteins in the absence of EGF (serum starvation) showed basal levels of Akt phosphorylation (Fig. 4A, lanes 3 and 5). These results suggest that among other factors such as EGF/EGF-R induced phosphorylation of Akt, CA IX phosphorylation may be another important factor contributing to the phosphorylation/activation status of Akt and that the mutation of this single tyrosine to phenylalanine in the intracellular domain of CA IX abrogates this Akt

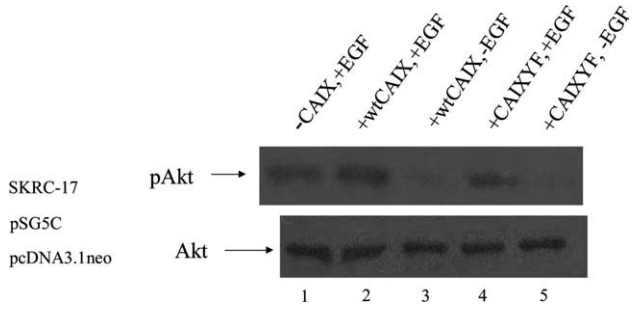

Fig. 4A. Effect of serum starvation and EGF stimulation in stably transfected SKRC-17 cells expressing CA IX as seen by the differences in Akt phosphorylation. Upper panel: immunoblot using the phosphospecific Akt (ser 473) antibody as probe; Lower panel, an identical blot using antibody for the unphosphorylated Akt protein as probe, which also serves as a loading control. Lane 1: SKRC-17 cells transfected with empty vector pSG5C and pCDNA3.1 in the presence of EGF (50 ng/ml); lane 2: cells expressing wt CA IX in the presence of EGF; lane 3: G418 resistant cells expressing the wild-type CA IX in the absence of EGF (*i.e.* serum starvation); lane 4: CA IX YF mutant expressing cells in the presence of EGF and lane 5: CA IX YF mutant expressing cells in the absence of EGF.

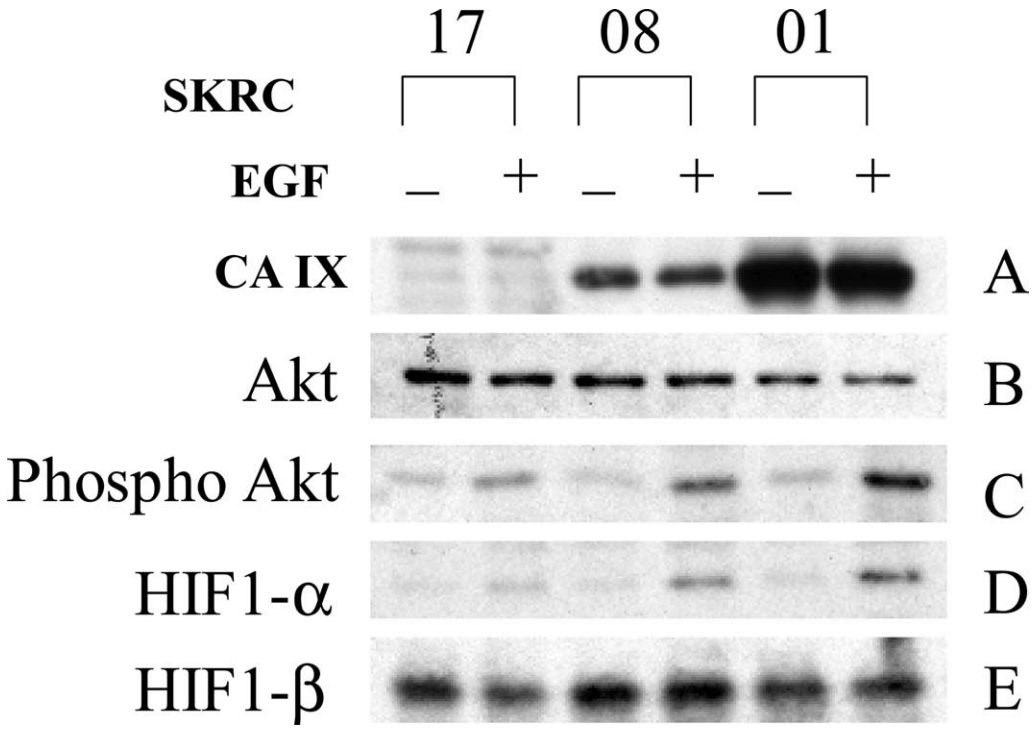

Fig. 4B. Correlation of Akt phosphorylation with the level of expression of CA IX and HIF-1 protein subunits under normoxic conditions in SKRC cells. SKRC-17 (CA IX negative), -08 (low expression of CA IX) and -01 cells (high expression of CA IX) were serum starved and stimulated with 50 ng/ml EGF as described earlier. Thirty minutes post stimulus, cell lysates were prepared, subjected to denaturing gel electrophoresis and immunoblots were probed for the expression of CA IX (panel A), total unphosphorylated Akt (panel B), phosphoAkt (ser 473, panel C), HIF-1α subunit (panel D) and HIF-1β subunit (panel E) using specific antibodies. The signals were developed using the corresponding secondary antibodies conjugated with horse radish peroxidase (HRP) and enhanced chemiluminescence (ECL). Panels B and E also served as loading controls.

activating function of CA IX. Finally, we wished to see whether the extent of Akt phosphorylation can be correlated with the level of CA IX expression in cells naturally overexpressing CA IX and to see whether the relative increase in the extent of Akt phosphorylation can be translated to an increase in the expression of HIF-1α levels in these SKRC cells that inherently differ in their levels of CA IX expression. For these experiments, the same SKRC-01 and 08 cells which are CA IX positive and SKRC-17 cells which are CA IX negative were chosen and the results are shown in Fig. 4B. These cells were serum starved and stimulated with 50 ng/ml recombinant EGF as described earlier and the relative levels of expression of CA IX (panel A), total Akt (panel B), phosphorylated Akt (panel C), HIF-1α (panel D) and HIF-β (ARNT protein, panel E) were followed by immunoblotting with specific antibodies. The results presented in Fig. 4B essentially reinforce the concept that in CA IX overexpressing cells, growth factor stimulation results in a relative increase in Akt phosphorylation and an increase in the expression level of HIF-1α, whereas the expression level of HIF-1β is unchanged. Since all these experiments were done under normoxic conditions, these results will have important implications for hypoxia dependent and independent modes HIF-1α expression in the hypoxic core and tumour periphery where elevated CA IX expression could be seen [37].

4. Discussion

Even though CA IX expression is widely accepted as a marker of hypoxic regions in tumours, there are increasing number of studies which suggest that CA IX expression is regulated at multiple levels. Parallel studies that have focused on the expression of CA IX and pimonidazole staining for hypoxic regions revealed a non-overlapping pattern of expression of CA IX with hypoxic regions with the CA IX positive areas extending beyond regions of hypoxia [45,46]. Varying amounts of HIF-1α can be detected at mildly hypoxic and even under normoxic conditions in normal tissues and in cell lines [47]. CA IX expression was also found to be regulated by cell density [48]. Its expression is very low in sparse and rapidly proliferating HeLa cell cultures whereas its synthesis is induced in dense cultures, very likely triggered by intermediate oxygen tensions or transient hypoxia. This process has recently been shown to involve the activation of the PI-3-Kinase pathway [48]. Apart from this, CA IX was also expressed in necrotic regions which are known to be hypoxic [49]. But in these necrotic and perinecrotic regions, other mechanisms such as the production of TNF-α, the reactive oxygen species (ROS) and NF-kB plays a role in the production of HIF-1α which in turn induces the expression of its target gene, namely CA IX [50]. This is more so in non-clear cell carcinomas of the kidney such as the papillary type 1 tumours, whereas in clear cell carcinomas with *VHL* gene inactivation either in an inherited manner or in a sporadic manner, there is a near uniform expression of CA IX throughout the tumour [49,51]. Thus, the multiple levels of regulation of expression of CA IX can be visualized as follows: (1) factors such as frank hypoxia in the core of the tumour or *VHL* gene mutations in clear cell RCC tumours that force HIF-1α stabilization; (2) pericellular hypoxic or mildly hypoxic regions which are not hypoxic enough to induce HIF-1α stabilization but induce CA IX at intermediate HIF-1α levels through the participation of the PI-3-Kinase pathway; (3) regions where necrotic foci are observed where the expression of HIF-1α can be supplemented by the expression of factors unique to necrotic foci such as TNF-α, ROS and NF-kB; and (4) regions of the tumour which are well supplied by oxygen where the expression of HIF-lα can be induced under normoxic conditions through mechanisms such as the overexpression of several growth factor receptors. Several clinical studies show a clear relationship between high levels of CA IX expression in tumours and poor prognosis [52–54].

Clear cell RCCs as well as papillary RCCs exhibit a complex and heterogeneous expression of several growth factors and their receptors, of which the role played by the epidermal growth factor receptor appears to be very significant [55,56]. They are almost invariably characterized by an overexpression of EGF-R and the cognate ligand TGF-α. Several studies indicated the functional intactness of the stimulatory autocrine loop for this receptor which contributes to cancer development and progression, including cell proliferation, suppression of apoptosis, angiogenesis and the metastatic spread [57]. Several recent studies have shown that this EGF-R can mediate several signalling pathways on the basis of its residence in the cholesterol rich microdomains of the cancer cell [38,58]. Modulation of cholesterol levels in these microdomains has been shown to alter the EGF-Receptor function and trafficking and even contribute to its ligand-independent activation [59]. These observations suggest that EGF-R signalling from its location in the lipid rafts may have significant clinical implications and prompted us to test the possibility that CA IX could be phosphorylated by this receptor in a ligand dependent manner. We have found that this was indeed so *in vitro*. Upon ligand binding, the cytoplasmic tail of the EGF-R gets autophosphorylated and this process helps in the activation of the tyrosine kinase activity of the receptor. In addition, the P-Tyr residues in the activated receptor also act as docking sites to cytoplasmic signal transducing adapter molecules that contain the SH2 or the phosphotyrosine binding (PTB) motifs [60,61]. For the P-Tyr of CA IX, which

is not endowed with any tyrosine kinase (TK) activity it may simply serve as a docking site for the same or a different set of signal transducing adapter molecules. Hence, its localization at the lipid raft regions may offer CA IX with a unique opportunity to recruit and direct a signalling pathway which is similar or different to that orchestrated by the EGF-R. Thus, CA IX may play a role in amplifying or diversifying the oncogenic signalling processes elicited by the EGF-Receptor alone in renal cell carcinoma. In this context, knowledge of the complete spectrum of the signal transducing adapter molecules with which the tyrosine phosphorylated CA IX can interact becomes absolutely essential. This would offer unique opportunities to interfere with these signalling processes which may have significant therapeutic potential. Inhibition of multiple pathways such as CA-IX phosphorylation, HIF-1α targeted therapies, VEGF-Receptor targeted therapies and EGF-R targeted therapies (as opposed to monotherapy using the EGF-R antagonists only) would theoretically create an environment in the RCC cell that closely approximates to a restored pVHL function in clear cell carcinoma, even though in reality, there is a biallelic loss of this tumour suppressor gene or function. Thus, signal transduction therapeutics that involves several of these pathways will offer new avenues for therapeutic approach for RCC and may possibly synergize with existing therapies such as those with IL-2 and interferon-α.

Our results also implicate the involvement of transmembrane carbonic anhydrase IX in PI-3 -Kinase pathway and suggest that CA IX, PI-3-Kinase and EGF-R signalling may function in an integrated manner to provide a molecular basis for the up-regulation of HIF-1α under non-hypoxic conditions in this cancer. Observations by Kaluz and coworkers [48] previously indicated a requirement for PI-3-K activity for the cell density dependent CA IX expression which might provide a link between the cancer-restricted expression of CA IX with the well established role of the PI-3-Kinase pathway in tumourigenesis. The results reported in this study imply that the expression of CA IX and its signalling through the EGF-R pathway would activate the PI-3-Kinase pathway. This in effect would form the basis for a self-promoting signalling loop which might be a poor prognostic factor for clear cell RCC. This would also help in explaining why several tumours that have deregulated PI-3-Kinase activity also have elevated expression of CA IX [48].

Several novel features of Akt activation process need to be highlighted here. The motif in the intracellular portion of CA IX protein (...GVXYXPA...) does not conform to the canonical YXXM motif preferred by the SH2 domain of class IA PI-3-Kinase adapter p85 subunit. The reason for this is still not clear and it certainly warrants further studies. There could be several explanations for this observation which might be an exception to the rule. First, since occupation of both SH2 domains of the p85 subunit, preferably by two adjacent phosphotyrosine motifs of the binding protein is necessary for full activation of PI-3-Kinase, the binding of the GVXYXPA motif to PI-3-Kinase p85 subunit as seen in this study very likely brings up a relatively weaker activation of the PI-3K enzyme as it may bind to the p85 subunit with a lower affinity [62]. Second, it may also be possible that the GVXYXPA motif in CA IX protein interacts with another signal transducing adapter which in turn interacts with the p85 subunit of PI-3-Kinase. Third, a non-canonical interaction of the p85 subunit with other proteins such as HGF/SCF (hepatocyte growth factor/scatter factor) receptor and ErbB3-p85 subunit was reported earlier, which may influence endocytic sorting and internalization [63–65]. Finally, since CA IX is a very stable protein, unlike many other growth factor receptor proteins or signal transducing adapter proteins that undergo tyrosine phosphorylation, our observation that CA IX protein undergoes tyrosine phosphorylation in the first place is unique and we feel that the physiological significance of this observation may extend well beyond its role in PI-3Kinase activation. In this respect, the full spectrum of all the binding partners of phosphorylated CA IX needs to be characterized.

One of the most important functions of the activated Akt protein is to activate the mammalian target of rapamycin (mTOR) as shown by numerous studies [66–69]. The mTOR protein has been shown to be central homeostatic sensor receiving signals from a plethora of agents such as growth factors, amino acids, nutrients, intracellular ATP levels, oxygen levels, second messengers to integrate and coordinate the levels of ribosome biogenesis, cell cycle progression and translation initiation. Numerous pharmacological and genetic studies place the PI-3-Kinase activation process upstream of the mTOR pathway [70,71]. Among the many important functions of the activated mTOR protein, the most relevant for these studies is its ability to control the cap-dependent translation of certain mRNAs that have unique 5′-untranslated region secondary structure such as in cyclin D1 and c-myc mRNAs which help in the unrestricted progression from Gl to S phase of the cell cycle. Most notably, the HIF-1α protein is also synthesized in this manner [72]. In most cancers where the PI-3-Kinase pathway is deregulated, the up-regulated mTOR can contribute to hypoxia independent translation of HIF-1α [73,74]. But, in the case of renal cell carcinoma, with the loss of function of the *VHL* gene commonly seen in the clear cell type, there is net accumulation of this hypoxia driven transcription factor due to protein stabilization [6,70]. This leads to the increased expression of (apart from CA IX) its growth factor target genes such as TGF-α, VEGF and PDGF. These growth factors in turn contribute in activating

the mTOR pathway. Thus, in the clear cell RCC, mTOR can be up-regulated both by hypoxia driven as well as hypoxia independent pathways and our results place CA IX in the activation process of Akt in such a way that it may actually integrate both these HIF-1α dependent and independent pathways as shown in Fig. 5. Our results also provide a molecular basis of the positive feed back loops that are inherent in such integrated pathways and help in the visualization of a vicious cycle mediated by CA IX mediated signalling. In particular, the scheme put together in Fig. 5 helps in placing the many functions of VHL protein and its relationship to the CA IX mediated signalling in proper perspective. It also represents a working hypothesis for the significance of overexpression of CA IX in clear cell carcinoma of the kidney. For example: (1) the VHL protein has been shown to down-regulate the expression and transport activity of certain anion exchangers (AE) which are in complex with CA II or the membrane associated CA IV that facilitates bicarbonate transport [75]. This suggests that the transmembrane CA IX could also function in a complex in a similar fashion as other carbonic anhydrases; (2) VHL tumour suppressor protein is the main regulator for the expression of HIF-1α causing a down-regulation of CA IX expression [23]; (3) as a component of the hypoxic and non-hypoxic acidification machinery, CA IX might participate in pH dependent mechanism of nucleolar sequestration of VHL protein [76]. Thus, enhanced acidification of the extracellular environment may produce a feed back loop of a down-regulated VHL environment which might lead to HIF-1α stabilization; (4) pVHL protein has also been shown to be required for efficient blockade of the epidermal growth factor receptor and the autocrine loops that are established in RCC [77]; (5) moreover, expression of wild-type VHL in cells expressing a mutated endogenous VHL leads to decreased expression of TGF-α. TGF-α is a direct target for the VHL tumour suppressor which acts by decreasing the stability of TGF-α m RNA [78]. Thus, by facilitating both the EGF-Receptor blockade and targeting the TGF-α mediated autocrine loop, the wild-type VHL protein can down regulate the vicious cycle of CA IX mediated cell signalling as put forward in this study; and (6) in addition, wild-type pVHL binds to and inactivates certain atypical protein kinase C family members such as PKC zeta and delta [79]. In this

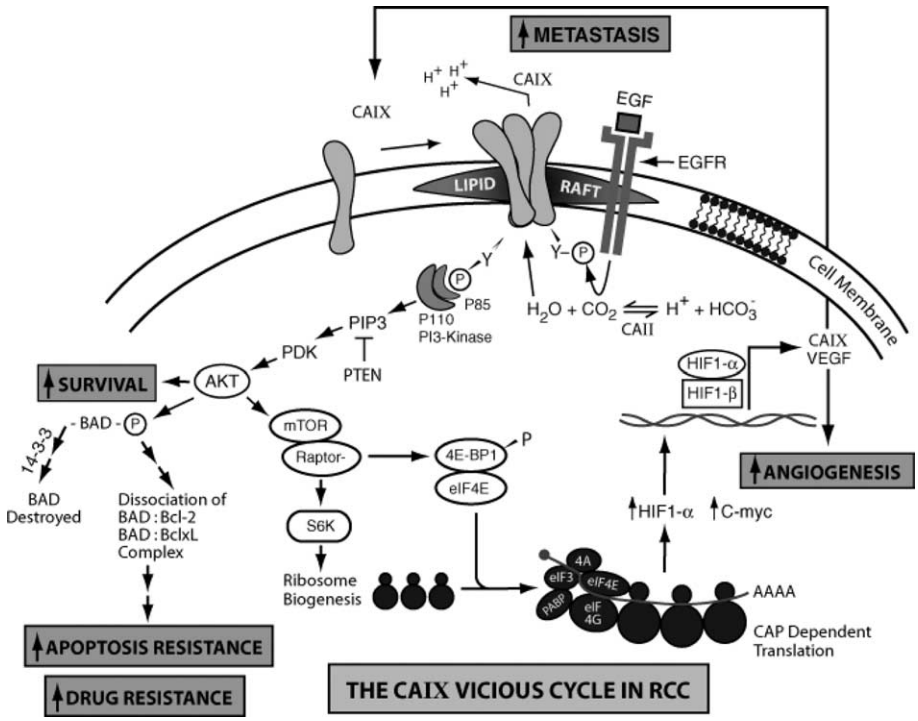

Fig. 5. A diagrammatic sketch of the major signalling pathways in clear cell carcinoma cell placing CA IX in the lipid rafts where it can get phosphorylated in a growth factor dependent manner and participate in the signalling processes regulated by PI-3-Kinase and mammalian target of rapamycin (mTOR). The CA IX protein recruited to the lipid rafts is depicted in its dimerized form. This figure also depicts the established role of PI-3-Kinase as a mediator of several survival, proliferation and apoptosis resistance pathways that lead to resistance to chemotherapeutic drugs. The major role of mTOR as an integrator of several signalling inputs is also presented with particular reference to cap-dependent translation of target proteins that include cyclin D1, c-myc and most importantly, HIF-1α. The placement of CA IX tyrosine phosphorylation in the midst of these cell signalling systems forms the basis of a vicious cycle, whereby CA IX mediated activation of Akt promotes the expression of HIF-lα which in turn promotes the expression of CA IX leading to poor prognosis in advanced cases of clear cell RCC. Increased acidification of the extracellular compartment contributes to increased invasive potential. The HIF-α target gene *VEGF* contributes to enhanced angiogenesis which is one of the hallmarks of clear cell RCC.

regard, it is very interesting to note that some recent studies have implicated PKC β II as the PDK II kinase that can activate Akt at serine-473 [80]. Thus, it would be logical to expect that pVHL would try to impede the Akt activation process which would in turn activate mTOR pathway as a consequence. Thus, all the phenomena described here go on to characterize the molecular signatures for the progression of clear cell carcinoma of the kidney and obviously, VHL inactivation serves the best interests of the cancer cell. Placement of CA IX as an active participant in the middle of these signalling pathways as shown by our studies may further help in the understanding of the role of VHL and its relationship to the overexpression of CA IX in these processes and justify the therapeutic interference of these pathways. Finally, it is entirely possible that when the enzymic activity is down-regulated by the use of specific CA IX inhibitors, the CA IX protein could still function in its signal transduction capacity. This warrants more investigations that focus on inhibiting CA IX in both its capacities to arrive at maximum therapeutic benefit. The patient's VHL and PTEN status will also determine the ultimate efficacy of such CA IX targeted therapies.

Conflict of interest statement

None declared.

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