Src induces expression of carbonic anhydrase IX via hypoxia-inducible factor 1

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Abstract. Carbonic anhydrase IX (CA IX) belongs to the physiologically important enzymes which contribute to tumor physiology. Tumor-associated expression of CA IX is induced mainly due to its strong transcriptional activation via hypoxia-inducible factor 1 (HIF-1). Therefore, CA IX can serve as a surrogate marker of hypoxia and a prognostic indicator. HIF-1 is a master transcription factor that mediates essential homeostatic responses to cellular and systemic hypoxia by activating transcription of multiple genes including those encoding glycolytic enzymes and vascular endothelial growth factor. In addition to hypoxia, HIF-1α expression can be up-regulated by growth factors and oncogenic signals (e.g. Src oncogene). Consequently, induction of the HIF-1α transcription factor up-regulates target gene expression. The results from the present study suggest that Src oncogene induces CA IX expression under normoxic, as well as hypoxic conditions. Moreover, we demonstrate that Src-mediated induction of CA IX expression is critically dependent on HIF-1α activity. Transcriptional activity of the CA9 promoter was significantly increased by expression of v-Src or c-Src. The effect was more prominent in normoxia, most likely because of already high level of HIF-1α expression in hypoxia. By co-transfection with dominant-negative HIF-1α we confirmed that Src-induced stimulation of CA9 transcription is mediated via HIF-1α. Consistent with this, Src-expressing HeLa cells displayed higher levels of HIF-1α protein. Finally, these results indicate a novel regulatory pathway responsible for increased CA IX expression in tumor cells and define CA IX as a new downstream target for Src oncogene.

Introduction

Carbonic anhydrases (CAs) are zinc metalloenzymes that catalyze the reversible conversion of CO₂ to bicarbonate and proton. There are 15 human CA isoforms that regulate diverse physiological processes based on ion transport and pH balance (1). Carbonic anhydrase IX (CA IX) is a cancer-related enzyme involved in regulation of pH homeostasis, cell proliferation and adhesion. The expression pattern of CA IX is characterized by limited expression in normal tissues contrasting with its broad distribution in many different tumors including carcinomas of uterine cervix, kidney, oesophagus, lung, breast, colon, brain and other types of neoplasia that are generally derived from tissues which contain no or low level of CA IX (2). On the other hand, physiological expression of CA IX is restricted to the epithelia of gastrointestinal tract, particularly to the glandular gastric mucosa (3).

The usefulness of CA IX as a tumor biomarker, as a prognostic indicator and as a potential tumor therapeutic target have been reviewed (1,2). As mentioned above, CA IX expression is induced in different solid tumors in response to hypoxia or inactivating mutation of the von Hippel-Lindau (VHL) tumor suppressor gene. CA9 belongs to the group of genes with the strongest hypoxia response mainly due to the unique localization of its hypoxia response element (HRE) just -3/-10 nucleotides from the transcription start site (4). In addition to hypoxia, expression of CA IX is also enhanced by high cell density and modulated by the PI3K (phosphatidylinositol 3-kinase) and MAPK (mitogen-activated protein kinase) pathways (5-7).

Hypoxia is a reduction in the normal level of oxygen tension and occurs during acute and chronic vascular disease, pulmonary disease and cancer. Hypoxia-inducible factor 1 (HIF-1) is important for the transcriptional activation of genes under low concentration of oxygen. It consists of the oxygen-regulated HIF-1α subunit and constitutively expressed partner factor HIF-1β (also known as ARNT) (6). Under normoxic conditions, HIF-1α is targeted for ubiquitin-mediated degradation by the proteasome. This process is primarily regulated...
by prolyl hydroxylases (PHDs) which hydroxylate proline residues (Pro402 and Pro564 of HIF-1α) localized within an oxygen-dependent degradation domain (ODDD) and permit binding of the VHL protein (pVHL), a component of an E3 ubiquitin ligase complex (9,10). Additionally, HIF-1α is hydroxylated at asparagine residue (Asn805) within the C-terminal transactivation domain (C-TAD) by Factor inhibiting HIF-1 (FIH-1) which prevents binding of the p300/CBP co-activator (11,12). Under hypoxic conditions, hydroxylation and acetylation are inhibited and therefore, pVHL cannot target HIF-1α for degradation. After accumulation of HIF-1α in the nucleus and dimerization with HIF-1β, HIF-1 mediates essential homeostatic responses to cellular and systemic hypoxia by activating transcription of multiple genes (e.g. VEGF, Glut1) whose promoters contain HREs (5'-RCGTG-3') (13). In addition to changes in oxygen tension, HIF-1α expression is also regulated by growth factors and oncogenic signals e.g. Ras or Src (14). Previous studies support the hypothesis that Src onco gene acts as an inducer of HIF-1α (15-17). The effect of Src seems to be mediated at least in part by a pVHL-independent mechanism (15), involving the up-regulation of HIF-1α protein synthesis (17).

Src family kinases (SFKs) are membrane-associated non-receptor tyrosine protein kinases that share a common domain structure. The SFKs comprise nine members which are either expressed in most tissues (Sck, Fyn, Yes) or more selectively expressed in particular tissues (Blk, Lck, Hck, Lyn) (reviewed in ref. 18). Because SFKs affect both cell cycle progression and cytoskeletal organization, deregulation may lead to constitutive activation and cellular transformation (19). The founding member of the family, Src, is a transforming product of Rous sarcoma virus (the first identified oncogenic retrovirus) that induces transformation of avian cells and sarcoma formation in chickens (20,21).

Src protein tyrosin kinases are composed of six distinct functional domains: SH4 (Src homology 4), a unique domain, SH3, SH2, SH1 and a C-terminal regulatory region (19). The kinase domain (SH1), found in all proteins of the Src family, is responsible for the tyrosine kinase activity and has a central role in binding of the substrates (18). Phosphorylation of tyrosine (Tyr416) within the kinase domain regulates the catalytic activity of Src. v-Src differs from c-Src on the basis of structural distinctions in the C-terminal region responsible for regulation of kinase activity. The loss of amino acids, that normally bind to the SH domains and stabilize a ‘closed’ or inactive conformation of the c-Src molecule, resulted in an opened, active conformation of v-Src (19,22). The kinase activity of Src is tightly regulated and Tyr245 is a critical regulatory tyrosine residue in the C-terminal tail. When Tyr245 is phosphorylated, kinase domain is maintained in closed conformation and c-Src is inactive. Dephosphorylation or mutation of Tyr245 activates c-Src and induces cellular transformation (19). The kinase that phosphorylates Tyr245 is Csk, C-terminal Src kinase (23).

c-Src, a cellular counterpart of v-Src, is overexpressed and/or activated in a wide variety of human cancers, including colon, breast, lung, head and neck and pancreas (22). Numerous studies have defined the normal function of c-Src in regulation of cell adhesion and migration as well as in growth factor signaling. Src activation promotes tumor progression, metastasis and angiogenesis, while blockade of the Src kinase activity results in decreased tumor cell proliferation, migration and invasion (22).

Because hypoxia and oncogenic mutations are both commonly present in tumors, we decided to examine the effect of Src oncogene on CA IX expression which is strictly dependent on HIF-1α activity.

Materials and methods

Cell culture. HeLa cells derived from human cervical carcinoma were cultivated in DMEM supplemented with 10% FCS (fetal calf serum; BioWhittaker, Verviers, Belgium) and 40 μg/ml gentamicin (Lek, Ljubljana, Slovenia) in a humidified atmosphere with 5% CO2 at 37°C. Hypoxic treatments were performed in an anaerobic workstation (Ruskinn Technologies, Bridgend, UK) in 2% O2, 5% CO2, 10% H2 and 83% N2 at 37°C.

Antibodies and plasmids. Mouse monoclonal antibody M75, specific for the human MN/CA IX protein, was characterized earlier (24). Mouse monoclonal antibody 327 that recognizes Src was purchased from Oncogene (Cambridge, MA, USA), mouse monoclonal anti-HIF-1α antibody was from BD Transduction Laboratories (San Jose, CA, USA) and goat polyclonal anti-actin antibody was from Santa Cruz Biotechnology (Prague, Czech Republic) and anti-goat or anti-mouse peroxidase-conjugated antibodies were from Dako (Glostrup, Denmark).

Human promoter constructs were generated by an insertion of PCR-amplified -50/+37 and -174/+37 CA9 genomic fragments upstream of the firefly luciferase gene in pGL3-Basic luciferase reporter vector (Promega, Madison, WI, USA) (7). pRL-TK Renilla vector (Promega) served as a transfection efficiency control. Expression plasmid mutHIF-1α (pcDNA1-mutHIF-1α) coding for deletion mutant of HIF-1α (aa 402-564) was created by PCR from the original expression plasmid pcDNA1-HIF-1α and described previously (25).

HeLa cells were transfected with vectors containing c-Src (pLNCX mouse Src), v-Src (pLNCX chick v-Src), constitutively active (CA) mutant (pLNCX chick Src Y527F) that lacks the negative regulatory Tyr527 and dominant-negative (DN) mutant (pCMV mouse Src Y295R Y527F) with inactivated ATP-binding site by mutation of Lys527 to Arg. Additionally, substitution of Phe for Tyr527 prevents the intramolecular interaction between phosphorylated Tyr527 and the SH2 domain, rendering it accessible to cellular binding proteins that interact with activated variants of Src under normal conditions. Plasmids containing cDNA for Src and mutant constructs were purchased from Addgene (plasmid no. 13658, 14578, 13660, 13657, Cambridge, MA, USA).

Transfection and luciferase assay. Cells were plated into 35-mm Petri dishes to reach ~70% density on the following day. Transient transfection was performed with 1 μg of promoter-containing luciferase construct and 50 ng of pRL-TK plasmid DNA using GenePorterII reagent (Genlantis, San Diego, CA, USA) according to the manufacturer’s recom-
mendations. To analyze the effect of mutHIF-1α or different Src vectors (v-Src, c-Src, CA Src, DN Src) on CA9 promoter activity, the equal amount of plasmids containing corresponding cDNAs were co-transfected. Cells transfected with control empty vectors (pCMV or pcDNA3.1) served as negative controls. The day after transfection, cells were trypsinized and plated in triplicates into 24-well plates. Transfected cells were allowed to attach for 24 h and then they were either transferred to hypoxia or maintained in normoxia for additional 24 h. Reporter gene expression was assessed 72 h after transfection using Dual-Luciferase Reporter Assay System (Promega) and luciferase activity was normalized against Renilla activity.

**Western blotting.** HeLa cells were plated in 35-mm petri dishes to reach ~70% density on the following day. Transient transfection was performed with 2 μg of Src-containing vector (v-Src or c-Src). Twenty-four hours after transfection, cells were trypsinized and plated in two 60-mm Petri dishes either for normoxia or hypoxia. Parallel control dishes were transfected with control empty vector and maintained in normoxia or hypoxia for the same time period. Twenty-four hours later, transfected cells were lysed in ice-cold lysis buffer (1% Triton X-100; 150 mM NaCl; 50 mM Tris, pH 7.5; 0.5% Nonidet P-40; 50 mM NaF) containing inhibitors of proteases (Roche Applied Science, Mannheim, Germany). Cell lysates were scraped, sonicated for 10 sec and centrifuged at 10,000 x g for 10 min at 4°C. Protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL, USA).

Total protein extracts (50 μg/lane) were separated in 10% SDS-PAGE and transferred onto PVDF membrane (Immobilon™-P, Millipore, Billerica, MA, USA). For CA IX detection, the membrane was incubated with M75 primary antibody diluted 1:2 in blocking buffer for 1 h. Secondary anti-mouse peroxidase-conjugated antibody (Sevapharma) was diluted 1:2 in blocking buffer for 1 h. For loading control, the membrane was incubated with M75 primary antibody diluted 1:500 (Immobilon™-P, Millipore, Billerica, MA, USA, http://rsb.info.nih.gov/ij/, 1997-2007). ImageJ can create density histograms and calculate area and pixel value statistics of user-defined selections. Amount of proteins was expressed as the ratio of the intensity of each band to the intensity of the related actin internal standard.

**Reverse transcription PCR.** Total RNA was isolated from cells using InstaPure reagent (Eurogentec, Seraing, Belgium) according to the manufacturer’s instructions. Reverse transcription was performed with M-MuLV reverse transcriptase (Finnzymes, Espoo, Finland) using random heptameric primers. The mixture of 3 μg of total RNA and random primers (400 ng/μl) was heated for 10 min at 70°C, cooled quickly on ice and supplemented with 0.5 mM dNTPs (Finnzymes), M-MuLV reverse transcriptase buffer and 200 U of reverse transcriptase M-MuLV, incubated for 1 h at 42°C, heated for 15 min at 70°C and stored at -80°C until further use.

**Real-time PCR.** Real-time PCR was performed with POWER SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) with gene-specific primers (CA9) and primers for β-actin that served as internal standards. The primers were as follows: CA9 S: 5'-CCGAGCGACGCAGCTTTGA-3' and CA9 A: 5'-GGCTCCAGTCTCGGCTACCT-3' (252 bp product); β-actin S: 5'-CAACCGCGAGAATGACCC-3' and β-actin A: 5'-GATCTTCATGAGGTAGTCG-3' (236 bp product).

The amplification of the samples was carried out in a final volume of 20 μl on StepOne™ Real-Time PCR System (Applied Biosystems) using the following program: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 1 min. All PCR reactions were performed in triplicates and repeated three times.

**Statistical analysis.** Results were analysed by two-tailed unpaired t-test (Student’s test) and P<0.05 was considered significant.

**Results**

**Src induces CA IX expression.** Our initial aim was to determine whether Src influences expression of CA IX. For this purpose, HeLa cells were transiently transfected with different Src-expressing vectors and incubated for 24 h under both normoxic and hypoxic conditions. HeLa cells transfected with empty vector served as a control. Expression of CA9 was analyzed using real-time PCR (Fig. 1A). The introduction of Src oncogene (v-Src or c-Src) resulted in an increased CA9 transcription in normoxia, probably due to the stabilization of HIF-1α protein. In addition to the induction observed in normoxia, Src-stimulated expression of CA9 increased slightly also in hypoxia.

Subsequently, results obtained by real-time PCR were confirmed by Western blotting of corresponding protein level (Fig. 1B). To examine the CA IX protein level, we used a monoclonal antibody M75 which was characterized earlier (24). As expected, transfection of v-Src, as well as c-Src resulted in an increase in CA IX protein level in hypoxia, whereas the expression of CA IX was not detected in normoxia. Additionally, we also wanted to determine the protein level of HIF-1α in Src-expressing cells. As shown in Fig. 1B, transfection of HeLa cells with both v-Src and c-Src resulted in an elevation of HIF-1α protein expression in hypoxia. Specific monoclonal antibody did not, however, recognize HIF-1α protein under normoxic conditions. Besides CA IX and HIF-1α, the expression of Src proteins was also determined as a control for expression of Src plasmids.
experiments suggested that Src-mediated induction of CA9 expression is accomplished at the transcriptional level. Previous studies have determined the region -174 nt upstream from the transcription start site as a basal CA9 promoter (26).

In order to examine how Src oncogene affects the CA9 promoter activity, we performed luciferase assays. HeLa cells were co-transfected with equal amounts of the promoter-containing luciferase construct and different Src-expressing vectors. Control cells were transfected with an empty vector.

In accordance with our data, co-transfection of v-Src resulted in an almost 3-fold increase in the CA9 promoter activity under hypoxic conditions. As shown in Fig. 2, stimulated promoter activity was also detected in c-Src and constitutively active (CA) Src-expressing cells. In contrast, the expression of the dominant-negative (DN) Src-expressing vector had no effect on the CA9 promoter. Interestingly, higher promoter stimulation was observed in normoxia than in hypoxia. One possible explanation for the relatively weaker Src-mediated induction of the CA9 promoter is in the already high transactivation of CA IX in response to hypoxia (mainly due to increased HIF-1α activity). Thus, >12-fold stimulation of the CA9 promoter activity was measured after co-transfection with v-Src in normoxia (Fig. 2). Similarly, co-transfection with c-Src and CA Src led to a comparable induction (>7-fold and 4.5-fold). Therefore, the effect of Src-mediated induction of HIF-1α activity is more prominent in normoxia.

Discussion
Stabilization of HIF-1 protein is essential for its role as a regulator of gene expression under low oxygen conditions.
Besides hypoxia, the expression of several oncogenes has previously been found to increase activation of the HIF-1α pathway. Transformation with H-Ras has been reported to increase the level of HIF-1α protein and to induce expression of HIF-1α target genes (e.g., Glut1) via PI3K, in both normoxia and hypoxia (27). Earlier studies have already pointed to a correlation between HIF-1α expression and Src activity. Study of Jiang et al. (16) revealed increased levels of HIF-1α protein and its activity in v-Src-transformed cells. On the other hand, Seko et al. (28) demonstrated that both hypoxia and hypoxia/reoxygenation caused rapid activation of SFKs, c-Src and c-Fyn, which was followed by the activation of Ras.

The formation of new microvasculature is crucial for tumor development. Vascular endothelial growth factor (VEGF) is a powerful and directly acting angiogenic protein. The fact that VEGF belongs to one of the most prominent hypoxia-responsive genes that caused an interaction between the Src oncogene and HIF-1α transcription factor is mostly investigated through VEGF expression. The role of oncogenes Ras and Src in an increased VEGF expression and induced tumorigenicity was examined in an immortalized rat intestinal epithelial cell line (29). Conditioned media from v-Src-transfected cells could stimulate endothelial cell proliferation. Additionally, this increase was blocked by addition of antibodies to VEGF. Mukhopadhyay et al. (30) showed that genistein, an inhibitor of protein tyrosine kinase, blocks VEGF induction in U87 glioma cells and 293 kidney cells. Moreover, expression of either a dominant-negative mutant form of c-Src or Raf-1 markedly reduces VEGF induction. In keeping with previous study, Ellis et al. (31) revealed that an inhibition of c-Src expression in HT29 cells by an antisense expression vector resulted in a reduction of VEGF expression. Additionally, subcutaneous tumors from antisense transfecteds showed a significant reduction in vascularity. Thus, data from several studies confirmed that Src activity regulates the expression of VEGF in tumor cells. In contrast, Gleadle and Ratcliffe (32) demonstrated that hypoxic induction of target genes (VEGF, EPO and Glut1) did not appear, at least in Hep3B hepatoma cells, to be Src-mediated.

In the present study, we showed that Src activity induces the expression of CA IX in cells derived from cervical carcinoma. Transfection of HeLa cells with either v-Src or c-Src led to an increased CA9 promoter activity, which resulted in a substantial increase in CA9 mRNA levels under normoxic conditions. Moreover, Src-mediated stimulation of the promoter activity increased both CA IX mRNA and protein levels in concert with hypoxia. Using a luciferase reporter construct containing basal -174/+37 CA9 promoter, we showed that this effect was mediated at the transcriptional level. CA9 promoter activity was much higher in v-Src-expressing cells than in mock-transfected cells. The same induction of the CA9 promoter was observed using either c-Src or CA Src plasmids. On the other hand, the expression of the dominant-negative Src construct had no effect on the CA9 promoter activity.

In addition to the basal promoter construct, we also co-transfected the minimal -50/+37 CA9 promoter containing the SP1-binding site and HRE, which was found to be crucial for the response to cell density and hypoxia (26,33). As expected, the minimal CA9 promoter showed a significantly higher activity under normoxia in Src-expressing HeLa cells than in control cells (data not shown), suggesting that Src might mediate its effect through HIF-1α.

To confirm the role of HIF-1α in Src-mediated stimulation of the CA9 promoter activity, we performed a co-transfection of HeLa cells with a mutated form of HIF-1α which was previously described to act as a dominant-negative mutant. In three different rat cell lines incubated under hypoxia, co-transfection of mutHIF-1α resulted in a reduced or even abolished CA9 promoter activity (25). Consistent with this, the effect of Src expression on the CA9 promoter activity was completely abolished after co-transfection of HeLa cells with the mutated form of HIF-1α.

Having found that HIF-1α was important for Src-mediated induction of the CA9 promoter, we performed Western blotting with the specific monoclonal antibody against HIF-1α and revealed that Src-expressing cells contained higher levels of HIF-1α protein than control (mock-transfected) cells. This occurrence was probably due to an increase in HIF-1α protein level as a result of an enhanced rate of cap-dependent protein translation mediated by the PI3K-mTOR pathway and depending on the cell line also by the ERK-MAPK pathway (17). The former results were demonstrated in cancer cell lines HT29 and Saos-2 that possess high Src activity and an elevated expression of hypoxia-regulated targets including VEGF and PGK-1 in normoxia. It is noteworthy that both previously mentioned cell lines express considerably higher levels of CA IX under normoxic conditions. Another research group has reported an Src-mediated increase in HIF-1α protein levels as a result of elevated mRNA levels (16). Additionally, oncogene-induced stabilization of HIF-1α was also demonstrated to be affected by inhibition of prolyl hydroxylation. Chan et al. (15) revealed that introduction of v-Src or RasV12 oncogenes resulted in the stabilization of normoxic HIF-1α and the loss of hydroxylated Pro564. Conversely, a constitutive active Akt oncogene stabilized HIF-1α under normoxia independently of prolyl hydroxylation, suggesting an alternative mechanism for HIF-1α stabilization.

In addition to CA IX and HIF-1α proteins, the level of Src expression was determined by Western blotting. Even though, the amount of transfected plasmids was equal, the level of v-Src protein was much lower than the level of c-Src. The
fact that the active forms of Src (v-Src, CA Src) are less stable than either c-Src or kinase-inactive Src mutants has already been reported. Harris et al (34) revealed that Src proteins are regulated by ubiquitination and that steady-state levels of Src proteins are inversely proportional to their activity.

In most cases, up-regulation of HIF-1-target gene expression including CA9 can be explained by hypoxia. The essential role of the HIF-1 transcription factor in oxygen homeostasis suggests that genetic changes in tumor cells leading to an increased HIF-1α activity may be selected throughout the process of adaptation to hypoxia. Thus, oncogenic activation of genes involved in the signal transduction pathways (e.g. tyrosine kinase activity of Src) proposes an explanation for an increased expression of CA IX under normoxic conditions. In both situations, however, the elevation of CA IX expression promotes tumor adaptation through its role in cell adhesion and in balance of pH disturbances caused by tumor metabolism.

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