



HAL
open science

Reconstitution of carbonic anhydrase activity of the cell-surface-binding protein of vaccinia virus

Anna Ohradanova, Daniela Vullo, Juraj Kopacek, Claudia Temperini, Tatiana Betakova, Silvia Pastorekova, Jaromir Pastorek, Claudiu T Supuran

► **To cite this version:**

Anna Ohradanova, Daniela Vullo, Juraj Kopacek, Claudia Temperini, Tatiana Betakova, et al.. Reconstitution of carbonic anhydrase activity of the cell-surface-binding protein of vaccinia virus. *Biochemical Journal*, 2007, 407 (1), pp.61-67. <10.1042/BJ20070816>. <hal-00478832>

HAL Id: hal-00478832

<https://hal.science/hal-00478832v1>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Reconstitution of carbonic anhydrase activity of the cell surface binding protein of vaccinia virus

Anna Ohradanova*, Daniela Vullo[†], Juraj Kopacek*, Claudia Temperini[†], Tatiana Betakova*, Silvia Pastorekova*, Jaromir Pastorek*[‡] & Claudiu T. Supuran^{†‡}

*Centre of Molecular Medicine, Institute of Virology, Slovak Academy of Sciences, Dubravská cesta 9, 845 05 Bratislava, Slovak Republic

[†]Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino, Italy

[‡]Equal senior contributors

Corresponding author: claudiu.supuran@unifi.it

Tel.: +39-055-4573005

Fax: +39-055-4573385

Page heading title: Vaccinia virus carbonic anhydrase

Synopsis

The N-terminal region of a 32-kDa cell surface-binding protein, encoded by D8L gene of vaccinia virus, shows sequence homology to carbonic anhydrases (CAs, EC 4.2.1.1). The active CAs catalyze the reversible hydration of CO₂ to bicarbonate participating in many physiological processes. The CA-like domain of vaccinia protein (vaccCA) contains one of the three conserved histidines required for coordination to the catalytic zinc ion and for enzyme activity. Here we report the engineering of catalytically active vaccCA mutants by introduction of the missing histidines into the wild type protein. The wild type vaccCA was inactive as a catalyst and does not bind sulfonamide CA inhibitors. Its position on a phylogram with other human CAs shows a relationship with the acatalytic isoforms CA X and XI, suggesting that the corresponding viral gene was acquired from the human genome by horizontal gene transfer. The single mutants (vaccCA N92H/Y69H) showed low enzyme activity and low affinity for acetazolamide, a classical sulfonamide CA inhibitor. The activity of the double mutant, vaccCA N92H/Y69H, was much higher, of the same order of magnitude as that of some human isoforms, namely CA VA and CA XII. Moreover, its affinity to acetazolamide was high, comparable to that of the most efficient human isoenzyme, CA II (in the low nanomolar range). Multiplication of vaccinia virus in HeLa cells transfected with the vaccCA N92H/Y69H double mutant was almost twice more efficient than in wild type vaccCA transfectants, suggesting that the reconstitution of the enzyme activity improved the virus life cycle.

Keywords: carbonic anhydrase, vaccinia virus, *in vitro* mutagenesis

Abbreviations: hCA, human carbonic anhydrase; vaccCA, vaccinia virus CA-like protein, vaccinia virus cell surface-binding protein; GST, glutathione S-transferase, IPTG, isopropyl β-D-thiogalactoside, PBS, phosphate-buffered saline; E-I complex, enzyme-inhibitor complex; RT-PCR, reverse-transcription polymerase chain reaction; PFU, plaque forming unit.

Introduction

Poxviruses constitute a family of viruses characterized by a large, linear dsDNA genome, a cytoplasmic site of replication and complex virion morphology [1, 2]. The most notorious member of the family is the variola virus, the causative agent of smallpox, whereas the laboratory prototype virus used for the study of poxviruses is vaccinia, the virus used as a live, naturally attenuated vaccine for the eradication of smallpox [1-3]. The poxviruses have a long history of causing disease, and their biological effects in humans and other mammals have been extensively studied [4]. In the 80s and 90s, genetic engineering techniques were applied to vaccinia in order to create replicating recombinant vectors that could express inserted genes encoding other proteins, which were able to elicit a specific adaptive immune response to foreign antigens in animal models, creating the basis of gene delivery approaches for the treatment of diseases (mainly viral infections and tumors) [4, 5]. Although intensively studied, much is still to be learned about vaccinia virus, considering the fact that its approximately 200 kbp long genome encodes for more than 200 different proteins, some of which have not been much investigated up to now [1, 6].

In 1990, Maa et al [7] identified a 32-kDa protein of vaccinia, which was shown to be a part of the virus envelope binding machinery to cells. The gene encoding this 32-kDa viral protein was then mapped and sequenced, and was found to encode a 35,426-Da protein with a large N-terminal domain, showing sequence homology to α -carbonic anhydrases (CAs, EC 4.2.1.1), a family of zinc enzymes widespread all over the phylogenetic tree [8-10], and a C-terminal domain with sequence similarity to the attachment glycoprotein VP7 of rotavirus and some other transmembrane proteins [7]. The 32-kDa protein was basic, with a pI around 8.7, and was probably dimeric due to a disulfide bridge involving the unique cysteine residue at amino acid position 262 [7]. It was also shown to be a vaccinia virus antigen, with predicted antigenic sites located near amino acids 108-110 (carbonic anhydrase domain) and 298-299 (transmembrane domain). This protein was found to bind to cell surface chondroitin sulphate of cultured cells and participate in efficient virus replication in both cultured cells and in infected animals [7-9, 11]. As there are no other known viral CAs or CA-like proteins [10, 12-14], we decided to investigate this 32 kDa protein discovered by Maa et al. [7]. In this paper we report the cloning of vaccinia CA-like proteins (vaccCAs) in which we have mutated two amino acids belonging to the putative enzyme active site in such a way as to bind zinc ions, the metal cofactor essential for

catalytic activity in all α -CAs [10, 12, 13]. We were able to engineer a variant of vaccCA possessing enzyme activity for the physiologic reaction catalyzed by CAs, i.e., CO₂ hydration to bicarbonate, of the same order of magnitude as exhibited by some human CAs (hCAs). Furthermore, this active vaccCA was also inhibited by sulfonamide CA inhibitors (some of which are clinically used drugs [15, 16]) in the low nanomolar range, indicating that the engineered protein is indeed a viral CA.

Experimental methods

Cell culture, viral and bacterial strains

HeLa cells derived from human cervical carcinoma were cultivated in DMEM supplemented with 10% FCS (BioWhittaker, Verviers, Belgium), 100 IU/ml penicillin, 100 µg/ml streptomycin sulfate and 0.25 µg/ml Amphotericin B (BioWhittaker) in a humidified atmosphere with 5% CO₂ at 37 °C.

Vaccinia virus, strain CR19 (wild type), was a kind gift of Dr. Gustav Russ, Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovakia. *E. coli* strains JM109 and BL21-CodonPlus (DE3)-RIPL (Stratagene, La Jolla, U.S.A.) were used for cloning and protein expression, respectively.

Plasmids, primers and cloning

The gene for vaccinia virus carbonic anhydrase-like protein (vaccinia virus cell surface-binding protein – the National Center for Biotechnology Information (NCBI) accession number J05190) was amplified from a boiled culture medium containing viral particles by PCR using oligonucleotides VaccCAs and VaccCAa (sequences of all primers used are listed in Table 1; nucleotides introduced for cloning are lowercase; mutations are boldface) using Phusion polymerase (Finnzymes, Espoo, Finland). The PCR product was first subcloned into the EcoRV site of pBluescript KS- (Stratagene), creating the plasmid pBKS-vaccCAwt. The target fragment was retrieved by digestion with restriction enzymes EcoRI and XhoI and cloned into pGEX-4T-1 (Amersham Biosciences, Chalfont St Giles, United Kingdom), creating a plasmid designated pGEX-4T-1-vaccCAwt.

Mutants of vaccinia virus CA-like protein designated N92H and Y69H, according to the amino acids mutated, were generated by two-step PCR-directed mutagenesis using the pBKS-vaccCAwt plasmid as a template. One reaction of the first round was performed using sense mutagenic oligonucleotide N92Hs or Y69Hs, respectively, and antisense pBluescript specific external primer R. In the second reaction, antisense mutagenic primers N92Ha or Y69Ha were used with sense pBluescript specific external primer F. For the second round, PCRs were carried out using sense and antisense pBluescript specific external primers for both mutants and the PCR products from the first round were used as templates. Final PCR products of single mutants were gel purified, processed with EcoRI and XhoI and inserted into pGEX-4T-1 vector linearised with EcoRI and XhoI, creating plasmids pGEX-4T-1-N92H and pGEX-4T-1-Y69H.

Double mutant N92H/Y69H of vaccinia virus CA-like protein was similarly generated later using plasmid construct pGEX-4T-1-N92H as a template. First PCRs were carried out using sense and antisense Y69H-mutagenic primers and a corresponding pGEX-4T-1 specific external primers pGEXA and pGEXS. Obtained PCR product was inserted into pGEX-4T-1 vector using EcoRI and XhoI yielding a plasmid designated pGEX-4T-1-vaccCAadm.

All PCR reactions were performed using the following amplification program: an initial denaturation at 98 °C for 30 s, then denaturation at 98 °C for 10 s, annealing at 62-68 °C for 30 s and extension at 72 °C for 20-35 s for a total of 30 cycles, and finally 5 min at 72 °C. All pGEX-vaccinia constructs were confirmed by sequencing using ABIPrism BigDye terminator V3.1 sequencing kit for fluorescent detection and ABIPrism 3100 genetic analyser (Applied Biosystems, Foster City, U.S.A.).

For eukaryotic expression, cDNAs of the wild type and the double mutant of vaccinia virus CA-like protein were cloned from the plasmids pGEX-4T-1-vaccCAwt and pGEX-4T-1-vaccCAadm, respectively, into pCMV1 expression vector (Genlantis, San Diego, U.S.A.) using EcoRI and NotI enzymes.

Recombinant protein expression and purification

Verified plasmid constructs pGEX-4T-1-vaccCAwt, -N92H, -Y69H and -vaccCAadm were transformed into BL21-CodonPlus (DE3)-RIPL cells. An expression of GST-fusion proteins

was induced with 0.4 mM IPTG at 30 °C for 4 hours. Recombinant proteins were purified using Glutathione Sepharose 4B medium and cleaved from their GST-tag overnight at 4 °C with thrombin (Sigma-Aldrich) while still attached to Sepharose beads; as reported earlier by us for the purification of several mammalian CA isozymes [17-19]. After elution from the beads (which retained the GST part of the fusion protein), the concentration of recombinant proteins was determined by measuring its absorption A_{260} as well as by SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining. For the double mutant possessing good CA activity (see later in the text), the obtained bacterial lysate was applied to a prepacked Glutathione Sepharose 4B column (Amersham), the column was extensively washed with ice-cold PBS and then the GST-vaccCA_{adm} fusion protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris-HCl pH 8.0. Finally the GST part of the fusion protein was cleaved with thrombin and the vaccCA_{adm} was further purified by sulfonamide affinity chromatography [20], the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO₂ as substrate [21]. The CO₂ hydrase activity has been measured as described below.

CA activity/inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA/vaccCA catalysed CO₂ hydration activity [21]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM HEPES (pH 7.5) as buffer, 0.1 M Na₂SO₄ (for maintaining constant the ionic strength), following the CA-catalyzed CO₂ hydration reaction for a period of 10-100 s. The CO₂ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5-10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10-20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear

least-squares methods using PRISM 3, from Lineweaver-Burk plots, as reported earlier, and represent the mean from at least three different determinations [21].

Generation of HeLa cells expressing vaccCA proteins

HeLa cells plated in 35 mm Petri dishes were transfected with 2 μ g of the phCMV1-vaccCAwt, phCMV1-vaccCAadm plasmids and an empty phCMV1 vector using GenePorter transfection reagent (Genlantis, San Diego, U.S.A.). Two days after transfection, the cells were split (onto seven 60 mm Petri dishes) and subjected to a two-week selection in a medium containing Geneticin G418 (Invitrogen, Carlsbad, U.S.A.) in the concentration of 600 μ g/ml. The resistant colonies were pooled and tested for the expression of ectopic genes by RT-PCR using vaccCAs and Y69Ha primers and primers for β -actin that served as internal standard (see the list in Table 1).

Plaque assay

The effect of the wild-type and the double mutant of vaccinia CA-like protein on vaccinia virus replication was compared after the infection of stably transfected HeLa cells where ectopically expressed vaccinia CA-like protein variants could be packed into virus particles. Confluent HeLa cell monolayers grown in 96-well tissue culture dishes were infected with 5-fold serial dilutions of vaccinia virus starting at a multiplicity of infection (MOI) of 1 PFU per cell. After 1 h of adsorption, the monolayers were washed and covered with DMEM containing 0.5 % methylcellulose. After two days, the cells were stained with crystal violet and PFU/ml for the vaccinia virus grown in different transfectants was determined.

Computer analysis of CA sequences

Amino acid sequence alignments were carried out using the program CLUSTAL W, version 1.83 [22]. Protein phylogeny tree reconstructions were performed using the MEGA3.1 software [23]. Trees were obtained using the distance/neighbor joining method and bootstrap statistical analyses were performed with 1000 replicates. 3-D protein models were obtained with the program PHYRE, a successor of the program 3D-PSSM [24].

Results and discussion

By catalyzing the interconversion between carbon dioxide and bicarbonate, with generation of a proton, the CAs operate on three very simple molecules/ions involved in a variety of critical life processes [10, 12-15]. Among them, the most important ones are pH regulation, respiration, secretion of electrolytes, biosynthesis of some important biomolecules such as urea, glucose, lipids and pyrimidines, excretion of acid and salts, carcinogenesis, signalling, etc. [10, 12-15]. Different isozymes among the 16 presently known isoforms in mammals participate to such processes. These isoforms display very diverse cellular localization, catalytic activity and susceptibility for inhibition/activation by various endogenous or exogenous regulators of activity. In humans only 15 of the 16 mammalian isoforms are present, due to the fact that CA XV is encoded by a pseudogene in all primates investigated so far [25]. Among these remaining 15 isoforms, 12 possess catalytic activity for the CO₂ hydration reaction, whereas CA VIII, X and XI (also denominated CARPs, for CA-related proteins) are devoid of this activity due to a lack of one or more histidine residues coordinating the catalytically critical Zn(II) ion within the enzyme active site [26]. Indeed, CA I-III, VII and XIII are soluble cytosolic isozymes, CA IV and XV are extracellular membrane-anchored enzymes by means of glycosylphosphatidylinositol (GPI) tails, CA VA and VB are mitochondrial enzymes, CA VI is the only secreted CA isoform, initially identified in sheep saliva and parotid glands, whereas CA IX, XII and XIV are transmembrane proteins with the active site situated outside the cell [10, 15, 25, 26].

Whereas the physiological function of most catalytically active CAs is rather well understood, the three CARPs (i.e., isozymes CA VIII, X and XI) still represent a biochemical and physiological enigma [26]. The CARPs lack one or more histidine residues required to bind the zinc ion, which is essential for the CO₂ hydration activity of all CAs. The Zn(II) ion within the enzyme active site is tetrahedrally coordinated to the imidazole moieties of three His residues (His94, 96 and 119, CA II numbering [10]), whereas the fourth ligand is a water molecule/hydroxide ion which acts as a strong nucleophile on the substrate (CO₂) bound within a hydrophobic pocket in its neighborhood [10, 12-18]. The CARPs were shown to be consistently expressed in the brain, and CARP VIII was also found to be expressed in some peripheral tissues [26]. Similarly to the

catalytically active transmembrane isoforms CAs IX and XII, CARP VIII has also been reported to be overexpressed in certain types of carcinoma [26].

An inactive CA-like domain has also been identified in the N-terminal regions of two receptor protein-tyrosine phosphatases, RPTP β and RPTP γ [27, 28]. RPTP β is expressed on the surface of glial cells and utilizes the CA domain to bind the cell recognition molecule contactin on neuronal cells, thus supporting its role in neuronal adhesion and neurite outgrowth [29]. RPTP γ is expressed in a variety of tissues. It appears developmentally regulated and is implicated in hematopoietic differentiation [30]. Both these phosphatases contain only one of the three His residues that bind zinc and are crucial for CA activity. On this basis it has been proposed that their CA domains function rather as a protein-binding pocket.

Similarly to CARPs and RPTP β/γ , the N-terminal CA-like domain of the cell surface binding protein of vaccinia virus identified by Maa et al [7] shows a high sequence homology with mammalian CAs, but lacks two of the zinc-coordinating histidines present in the active site of these enzymes. More precisely, a Tyr is present at position 69, and an Asn is found at position 92, instead of His at the corresponding positions 96 and 119 in CA II. Figure 1 shows the sequence alignment of the CA domains of all human isozymes (including CARPs), RPTP β/γ and vaccCA. The sequence homology between the CA domains of these proteins ranges from 22% to 67%. A phylogenetic analysis of these proteins (Figure 2) revealed that vaccCA is evolutionarily similar to CARPs XI and X, whereas it is more distantly related to human CARP VIII, CA XIII, CA VII, CA VA and CA VB. On the other hand, all other CAs are clustered on different branches of the phylogenetic tree, suggesting that they are less evolutionarily related to vaccCA.

In order to investigate the possibility of engineering a CA-like active site in this viral protein, we decided to mutate the vaccCA amino acids Tyr69 and Asn92 - corresponding to positions 96 and 119 in CA I and II - to histidines. Thus, we have generated three mutant vaccCAs, i.e., N92H vaccCA, Y69H vaccCA, and the double mutant, N92H/Y69H vaccCA (see Experimental section for details). GST-fusion proteins were obtained for the wild type and the three mutant vaccCAs that were purified by a procedure previously optimized for the production of high amounts of various CA isozymes for medicinal chemistry purposes. Two-column chromatographic passages were utilized, the first

involving a Glutathione Sepharose column which retained the GST-vaccCA fusion protein. After thrombin cleavage of the chimeric protein, a second column based on aromatic sulfonamide Sepharose allowed the purification of the proteins with CA-like activity (this second column could not be used for the wild type vaccCA which was not retained on sulfonamide affinity columns). The average yield after two purification procedures ranged between 1-3 mg per liter culture. The CO₂ hydrase activity of the obtained proteins has been measured by a stopped flow assay. Their inhibition by the prototypic sulfonamide CA inhibitor acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide, AAZ) was also assessed [10].

Data included in Table 2 show that the wild type vaccCA protein is devoid of CO₂ hydrase activity, as expected for a protein unable to bind zinc, being thus similar to CARP VIII; X and XI [26]. On the other hand, the two single mutants N92H and Y69H, and much more the double mutant, vaccCA N92H/Y69H show enzymatic activity typical of a CA-like protein. Nevertheless, the single mutants showed a low activity, with k_{cat} and k_{cat}/K_M values similar to those of CA III (the least effective catalyst among the mammalian CAs for the physiologic reaction [10]). This may be explained by the presence of only two His residues within the active site leading to a rather low affinity for Zn(II), and as a consequence, probably the active site of these enzymes is only partially occupied by zinc. This hypothesis is also confirmed by the finding that the sulfonamide CA inhibitor acetazolamide binds to these two proteins with a rather low affinity (inhibition constants in the range of 76-89 nM). However, the fact that the sulfonamide does bind represents a clear sign that the active site of the mutated viral proteins can be also formed when only two His residues are present, but the catalytic activity is low. In the case of the double mutant, vaccCA N92H/Y69H, the CO₂ hydrase activity is much higher as compared to the single mutants, with k_{cat} and k_{cat}/K_M values similar to those of some catalytically efficient human isoforms, such as CA VA and CA XII. The other enzymes considered in Table 2 are much better catalysts for this reaction, especially CA II, which is in fact one of the best catalysts known in nature (Table 2) [10]. Furthermore, acetazolamide binds with very high affinity to the double mutant, showing an inhibition constant of the same order of magnitude as that against CA II. This is clear proof that we were able to engineer the active site of a viral CA isozyme, which similarly to other α -CAs shows activity for the CO₂ hydration reaction and binds the typical CA inhibitors, sulfonamides such as acetazolamide, with nanomolar affinity.

How can we explain these findings? Due to the high sequence homology between vaccCA and other α -CAs, most of the amino acid residues needed for the construction of the active site architecture useful for CO₂ hydration are already present in the wild type vaccinia protein. By inserting the amino acid residues (two histidines) in positions equivalent to His96 and His119 in the CA II sequence, these features are further accentuated, leading to a viral CA with a moderate CA activity for the physiologic reaction (in the same range as the activity of the human isozymes CA VA and CA XII), which also binds the sulfonamide CA inhibitors efficiently. In fact, other residues in the neighborhood of the Zn(II) ion, more precisely Thr198 (CA II numbering), which forms a hydrogen bond to the water coordinated to Zn(II) as the fourth ligand in all other α -CAs [10, 12-17] and Thr199, which is involved in the binding of inhibitors, together with Thr199 [10, 12-17], are also present in vaccCA, and this probably helps in stabilizing the tetrahedral geometry of Zn(II) during the catalytic cycle. In the close proximity of another important residue for the catalytic cycle, His64 (in CA II), vaccCA (and CA III) contains Lys (see Figure 1). This residue is involved in the proton shuttling between the active site and the environment, with generation of the zinc hydroxide, catalytically effective species of the enzyme [10, 12-17]. Obviously, the ϵ -amino group present in Lys is a less efficient proton acceptor as compared to the imidazole moiety of a His residue, and this may explain the lower catalytic activity of vaccCA (double mutant), which is of the same order of magnitude as that of CA III. However, our double mutant is in fact approximately 83 times more efficient as a catalyst for CO₂ hydration as compared to CA III (Table 2). This is probably due to the fact that CA III has Phe197, a very bulky amino acid which highly restricts its active site, whereas the equivalent amino acid residues in vaccCA all possess smaller side chains, and thus the steric impairment is not present in its active site, allowing for a better catalytic efficiency.

To learn whether introduction of the CA catalytic activity into vaccinia virus cell surface binding protein could have any biological effect on infectious properties of the virus, we attempted to produce virions containing ectopically expressed vaccCA protein variants. Three stable transfectants of HeLa cells had been created that expressed either full length vaccinia virus CA-like protein or its catalytically active counterpart designated here as vaccCA_{adm}. As a control, HeLa cells had been transfected with an empty vector carrying a neomycin resistance gene. Prior to infection with CR19 strain of vaccinia virus, expression of vaccCA_{wt} and vaccCA_{adm} was confirmed by RT-PCR (data not shown). By plaque

assay, the titer of recombinant vaccinia virus containing vaccCA_{adm} in its envelope was determined as 4.3×10^7 PFU/ml, whereas the titer of the virus grown on HeLa cells expressing the wild type vaccCA was close to the titer of the control (2.4×10^7 PFU/ml comparing to 2.7×10^7 PFU/ml). The size of plaques was similar in all samples (see Figure 3).

Based on the recent knowledge, it is difficult to anticipate a possible function of the CA domain of vaccCA. This viral cell surface binding protein has been shown to contribute to virus entry, but the CA domain was considered dispensable for its binding capacity [7]. On the other hand, others have demonstrated that the N-terminal region (amino acids 1-264) of 32-kDa vaccinia virus CA-like protein is important in vaccinia virus adsorption to cells [11]. As a binding partner, a cell surface glycosaminoglycan chondroitin sulphate was presented [11], but the precise region of interaction between the two molecules has not been mapped so far.

To ascertain how the gained CO₂ hydratase function affected overall structure of the protein which may prove important for adsorption properties of the virus, we also derived 3-D models of the CA domain of vaccCA proteins in comparison with that of RPTP β and CA II, which is well characterized from the structural point of view. In a set of supplementary figures A-E we present the X-ray crystal structure of CA II and the corresponding 3-D model, which was obtained with the program PHYRE. This model well matches the crystal structure (compare Figures A and B). The model of RPTP β CA domain was also included for comparison (Figure C), since the pocket structure of the catalytically inactive CA domain of this protein was shown to serve as a receptor site that binds contactin and modulates adhesion and differentiation of neurons [27]. The vaccCA 3-D models were obtained from the amino acid sequences of the CA domains of the wild type protein (Figure D) and the catalytically active double mutant (Figure E). It may be observed that the globular shapes of CA II, RPTP β and vaccCA are quite similar. The active site of CA II forms a wide and deep pocket, but also the acatalytic RPTP β and vaccCA display a pocket-like structure. Interestingly, introduction of two mutations leading to the acquisition of zinc ion-binding capacity did not show any remarkable effect on the tertiary structure of the double mutant, suggesting that the formation of the deep pocket-like structure does not depend on the binding of the zinc ion, but seems to be affected by the amino acid composition of the entire CA domain. This, however, supports our experimental results

where no dramatic changes in properties of vaccCAwt and vaccCAadm virions were observed.

Genome analyses of poxviruses revealed the existence of numerous genes homologous to eukaryotic genes present in their host organisms. Therefore, it has been suggested that the evolution of poxviruses involved multiple events of gene acquisition [32]. Due to this horizontal gene transfer poxviruses express many proteins that are functionally and structurally similar to host proteins, such as DNA polymerase, thymidine kinase, ribonucleotide reductase, DNA topoisomerase, etc. It is well conceivable that vaccCA has also been acquired by means of a horizontal gene transfer. Its close similarity to CARP X and XI preferably points to an ancestral gene that led to CARPs (in mammals) and in parallel to vaccCA (Figure 2). However, taking into account the results of our mutagenesis study, it cannot also be excluded that the virus originally acquired an evolutionary even older gene, i.e., a common ancestor of the catalytic and acatalytic CA isoforms, which lost its catalytic activity over evolution in the virus, since it was not critical for the vaccinia virus life cycle.

In conclusion, we engineered the active viral CA active site by mutating two amino acid residues in the vaccCA protein. The double mutant vaccCA N92H/Y69H showed a catalytic activity of the same order of magnitude as that of some physiologically relevant human CA isoforms. The double mutant (and to a lesser degree also the two single mutants reported here) also bound sulfonamide CA inhibitors, such as acetazolamide, with a good efficacy. Moreover, when expressed in HeLa cells, the double mutant was able to slightly increase the titer of the virus. However, the biological significance of this finding cannot be judged merely on *in vitro* data from cell culture, as the carbonic anhydrase activity is particularly relevant at the physiological level in the living organisms.

Acknowledgement

This work was supported by the grants from Slovak Government (BITCET SP 337/2003) and from EU (EUROXY project LSHC-CT-2003-502932).

References

- 1 Condit, R.C., Moussatche, N. and Traktman, P. (2006) In a nutshell: structure and assembly of the vaccinia virion. *Adv. Virus Res.* **66**, 31-124
- 2 Moss, B. (2006) Poxvirus entry and membrane fusion. *Virology* **344**, 48-54
- 3 Tatsis, N., Sinnathamby, G. and Eisenlohr, L.C. (2004) Vaccinia virus as a tool for immunologic studies. *Methods Mol. Biol.* **269**, 267-288
- 4 Essajee, S. and Kaufman, H.L. (2004) Poxvirus vaccines for cancer and HIV therapy. *Expert Opin. Biol. Ther.* **4**, 575-588.
- 5 Moroziewicz, D. and Kaufman, H.L. (2005) Gene therapy with poxvirus vectors. *Curr. Opin. Mol. Ther.* **7**, 317-325
- 6 Resch, W., Hixson, K.K., Moore, R.J., Lipton, M.S. and Moss, B. (2007) Protein composition of the vaccinia virus mature virion. *Virology* **358**, 233-47.
- 7 Maa, J.S., Rodriguez, J.F. and Esteban, M. (1990) Structural and functional characterization of a cell surface binding protein of vaccinia virus. *J. Biol. Chem.* **265**, 1569-1577
- 8 Lai, C., Gong, S. and Esteban, M. (1991) The 32-kilodalton envelope protein of vaccinia virus synthesized in *Escherichia coli* binds with specificity to cell surface. *J. Virol.* **65**, 499-504
- 9 Rodriguez, J.R., Rodriguez, D. and Esteban, M. (1992) Insertional inactivation of the vaccinia virus 32-kilodalton gene is associated with attenuation in mice and reduction of viral gene expression in polarized epithelial cells. *J. Virol.* **66**, 183-189
- 10 Supuran, C.T., Scozzafava, A. and Conway, J. (2004) Carbonic Anhydrase – Its Inhibitors and Activators. CRC Press: Boca Raton, New York, London, pp. 1-363
- 11 Hsiao, J.C., Chung, C.S. and Chang, W. (1999) Vaccinia virus envelope D8L protein binds to cell surface chondroitin sulfate and mediates the adsorption of intracellular mature virions to cells. *J. Virol.* **73**, 8750–8761
- 12 Pastorekova, S., Parkkila, S., Pastorek, J. and Supuran C.T. (2004) Carbonic anhydrases: current state of the art, therapeutic applications and future prospects. *J. Enzyme Inhib. Med. Chem.* **19**, 199-229
- 13 Sly, W.S. and Hu, P.Y. (1995) Human carbonic anhydrases and carbonic anhydrase deficiencies. *Annu. Rev. Biochem.* **64**, 375-401

- 14 Tashian, R.E., Venta, P.J., Nicewander, P.H. and Hewett-Emmett, D. (1990) Evolution, structure, and expression of the carbonic anhydrase multigene family. *Progr. Clin. Biol. Res.* **344**, 159-175
- 15 Supuran, C.T., Scozzafava, A. and Casini, A. (2003) Carbonic anhydrase inhibitors. *Med. Res. Rev.* **23**, 146-189
- 16 Scozzafava, A.; Mastrolorenzo, A. and Supuran, C. T. (2004) Modulation of carbonic anhydrase activity and its applications in therapy. *Expert Opin. Ther. Pat.* **14**, 667-702
- 17 Pastorekova, S., Casini, A., Scozzafava, A., Vullo, D., Pastorek, J. and Supuran, C.T. (2004) Carbonic anhydrase inhibitors: The first selective, membrane-impermeant inhibitors targeting the tumor-associated isozyme IX. *Bioorg. Med. Chem. Lett.* **14**, 869-873
- 18 Svastova, E., Hulikova, A., Rafajova, M., Zatovicova, M., Gibadulinova, A., Casini, A., Cecchi, A., Scozzafava, A., Supuran, C.T., Pastorek, J. and Pastorekova, S. (2004) Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH. *FEBS Lett.* **577**, 439-445
- 19 Nishimori, I., Vullo, D., Innocenti, A., Scozzafava, A., Mastrolorenzom A. and Supuran, C.T. (2005) Carbonic anhydrase inhibitors. The mitochondrial isozyme VB as a new target for sulfonamide and sulfamate inhibitors. *J. Med. Chem.* **48**, 7860-7866
- 20 Khalifah, R.G., Strader, D.J., Bryant, S.H. and Gibson, S.M. (1977) Carbon-13 nuclear magnetic resonance probe of active-site ionizations in human carbonic anhydrase B. *Biochemistry* **16**, 2241-2247
- 21 Khalifah, R.G. (1971) The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. *J. Biol. Chem.* **246**, 2561-2573
- 22 Higgins, D., Thompson, J., Gibson, T., Thompson, J.D., Higgins, D.G., Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680
- 23 Kumar, S., Tamura, K., Nei, M. (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* **5**, 150-163

- 24 Kelley, L.A., MacCallum, R.M., Sternberg, M.J. (2000) Enhanced genome annotation using structural profiles in the program 3D-PSSM. *J. Mol. Biol.*, **299**, 499-520
- 25 Hilvo, M., Tolvanen, M., Clark, A., Shen, B., Shah, G.N., Waheed, A., Halmi, P., Hanninen, M., Hamalainen, J.M., Vihinen, M., Sly, W.S. and Parkkila, S. (2005) Characterization of CA XV, a new GPI-anchored form of carbonic anhydrase. *Biochem. J.* **392**, 83-92
- 26 Nishimori, I. (2004) Acatalytic CAs: Carbonic anhydrase-related proteins. In *Carbonic anhydrase - Its inhibitors and activators*, Supuran, C.T.; Scozzafava, A.; Conway J. Eds., CRC Press, Boca Raton (FL), USA, pp. 25-43
- 27 Peles, E., Schlessinger, J. and Grumet, M. (1998) Multi-ligand interactions with receptor-like protein tyrosine phosphatase β : implications for intercellular signalling. *Trends Biochem. Sci.* **23**, 121-124
- 28 Barnea, G., Silvennoinen, O., Shaanan, B., Honneger, A.M., Canoll, P.D., D'Eustachio, P., Morse, B., Levy, J.B., Laforgia, S., Huebner, K., Musacchio, J.M., Sap, J. and Schlessinger, J. (1993) Identification of a carbonic anhydrase-like domain in the extracellular region of RPTP gamma defines a new subfamily of receptor tyrosine phosphatases. *Mol. Cell Biol.* **13**, 1497-1506
- 29 Peles, E., Nativ, M., Campbell, P.L., Sakurai, .T, Martinez, R., Lev, S., Clary, D.O., Schilling, J., Barnea, G., Plowman, G.D., Grumet, M. and Schlessinger, J. (1995) The carbonic anhydrase domain of receptor tyrosine phosphatase β is a functional ligand for the axonal cell recognition molecule contactin. *Cell* **82**, 251-260
- 30 Sorio, C., Melotti, P., D'Arcangelo, D., Mendrola, J., Calabretta, B., Croce, C.M. and Huebner, K. (1997) Receptor protein tyrosine phosphatase gamma, PTP γ , regulates hematopoietic differentiation. *Blood* **90**, 49-57
- 31 Parkkila, A.K., Parkkila, S., Juvonen, T. and Rajaniemi, H. (1993) Carbonic anhydrase isoenzymes II and I are present in the zona glomerulosa cells of the human adrenal gland. *Histochemistry* **99**, 37-41.
- 32 Hughes, A.L. and Friedman, R. (2005) Poxvirus genome evolution by gene gain and loss. *Mol. Phyl. Evol.* **35**, 186-195

Table.1

List of primers used for cloning and RT-PCR analysis of vaccinia virus carbonic anhydrase-like protein and its mutant variants

Source DNA (GenBank Accession No.)	Name of primer	Sequence 5'→3'
Vaccinia virus cell surface-binding protein gene (J05190)	VaccCAs	gaattcATGCCGCAACAACATCTCCTATT
	VaccCAa	ctcgaGGAGGCTCAACAAGGTATCGAAT
	N92Hs	ACTCTGGAGAGATT C ATCTTGTTTCATTGG
	N92Ha	CCAATGAACAAGAT G AATCTCTCCAGAGT
	Y69Hs	CATCACTACATATA C ATTGGGGAAAGGAAG
	Y69Ha	CTTCCTTTCCCAAT G TATATGTAGTGATG
pBluescript KS- (X52329)	R	GGAAACAGCTATGACCATGATTACGCC
	F	GTGAGCGCGCGTAATACGACTCACTA
pGEX-4T-1 (U13853)	pGEXS	TACTTGAAATCCAGCAAGTATATA
	pGEXA	AGAGGTTTTTCACCGTCATCA
β-actin (NM_001101)	BactinS	CCAACCGCGAGAAGATGACC
	BactinA	AGGATCTTCATGAGGTAGTCAGTC

Table 2

Kinetic parameters for the CO₂ hydration reaction catalysed by the cytosolic α -hCA isozymes I-III, the mitochondrial isozymes VA and VB, the secreted isoform hCA VI (full length) and the transmembrane isozymes hCA IX (catalytic domain), and hCA XII (catalytic domain), at 20 °C and pH 7.5, vaccCA (wt) and the three mutants prepared during this study, and their inhibition data with acetazolamide **AAZ** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), a clinically used drug.

Isozyme	Activity level	k_{cat}^* (s ⁻¹)	k_{cat}/K_m^* (M ⁻¹ .s ⁻¹)	K_i (AAZ)* (nM)
hCA I	moderate	2.0x10 ⁵	5.0x10 ⁷	250 ± 13
hCA II	very high	1.4x10 ⁶	1.5x10 ⁸	12 ± 0.6
hCA III	very low	1.0x10 ⁴	3.0x10 ⁵	300,000 ± 5000
hCA VA	low	2.9x10 ⁵	2.9x10 ⁷	63 ± 4
hCA VB	high	9.5x10 ⁵	9.8x10 ⁷	54 ± 5
hCA VI	moderate	3.4x10 ⁵	4.9x10 ⁷	11 ± 1
hCA IX	high	3.8x10 ⁵	5.5x10 ⁷	25 ± 3
hCA XII	moderate	4.2x10 ⁵	3.5x10 ⁷	5.7 ± 0.4
vaccCAwt	devoid	-	-	-
vaccCA N92H	low	7.8x10 ⁴	(3.7±0.2)x10 ⁶	76 ± 6
vaccCA Y69H	low	8.3x10 ⁴	(2.3±0.1)x10 ⁶	89 ± 7
vaccCA N92H/Y69H	moderate	1.2x10 ⁵	(2.5±0.2)x10 ⁷	12 ± 1

* Mean ± standard error (from three different determinations). Kinetic data for hCA I – hCA XII are from the literature [10,12-17], whereas vaccCA data are reported here for the first time

Figure legends

Figure 1

Alignment of amino acid residues 64-128 (CA II numbering, relevant for the catalytic mechanism) of human CA isozymes I-XIV, human RPTP β/γ and vaccinia CA-like protein using the CLUSTAL W program. The zinc ligands of catalytically active isoforms are evidenced in black, whereas the corresponding residues in the non-catalytic CAs are in grey. The two missing, potential Zn(II) ligands of vaccCA (mutated to His in the experiments described in the paper) are indicated with arrows. For the alignment, the NCBI reference sequences were used with the following accession numbers: hCAI, NP_001729; hCAII, NP_000058; hCAIII, NP_005172; hCAIV, NP_000708; hCAVA, NP_001730; hCAVB, NP_009151, hCAVI, NP_001206; hCAVII, NP_005173; hCAVIII, NP_004047; hCAIX, NP_001207; hCAX, NP_064563; hCAXI, NP_001208; hCAXII, NP_001209; hCAXIII, NP_940986; hCAXIV, NP_036245, RPTP β , NP_002842; RPTP γ , NP_002832.

Figure 2

Unrooted phylogenetic tree of human CA isozymes I-XIV, human RPTP β/γ and vaccinia CA-like protein based on the alignment of carbonic anhydrase domains corresponding to amino acid residues 1-260 of hCA II. The tree was constructed using the distance (neighbor joining) method using MEGA3.1 software. A bootstrap of 1000 replicates was performed and the number on each node indicates the percentage with which each branch topology was supported. The bar shown represents the genetic distance.

Figure 3

A plaque assay of vaccinia virus, strain CR19, on HeLa cells expressing vaccCA protein variants. Representative wells of HeLa cells stably transfected with cDNA of vaccCAwt and vaccCA Δ m and infected with 5-fold serial dilutions vaccinia virus, strain CR19. After 1 h of adsorption, the monolayers were washed and covered with DMEM containing 0.5 %

methylcellulose. Two days later, the cells were stained with crystal violet and PFU/ml for the vaccinia virus grown in the tranfectants was determined.

Figure 1

hCAI	HSFHVNFEDNDNRSVLKGGPFSYRLFQFHFWG--STNEHGSEHTVDGVKYSAELEHVAHWNSA-KYS	130
hCAXIII	HSFNVDFFDDTENKSVLRGGPLSYRLRQVHLEHWG--SADDHGSEHTVDGVSYYAAELEHVHWNNSD-KYP	130
hCAII	HAFNVEFDDSQDKAVLKGGLTYRLIQFHFWG--SLDGQSEHTVDKKKYAAELEHLVHWN-T-KYG	128
hCAIII	KTCRVVFDPTYDRSMLRGGPLPYRLRQFHLEHWG--SSDDHGSEHTVDGVKYYAAELEHLVHWN-P-KYN	128
hCAVII	HSVQVDFNDSDDRTVVTTGGPLPYRLKQFHFWG--KKHDVGSEHTVDGKSFPSLEHLVHWNNAK-KYS	131
hCAVA	YLFQVEFDDATEASGISGGPLHYRLKQFHFWG--AVNEGGSEHTVDGHAYPAELEHLVHWNNSV-KYQ	127
hCAVB	YSFLVEFEDSTDKSVIKGGPLNYRLKQFHFWG--AIDAWGSEHTVDSKCFPAELEHLVHWNNAV-RFE	132
hCAVIII	HTIQVILK---SKSVLSGGPLEFELYEVRFHWG--RENQRGSEHTVNFKAFPMELEHLIHNST-LFG	125
hCAX	GRHVSRLRLDKEHLVNISSGPMShRLLEIRLHFG--SEDSQGSEHLLNGQAFSGEVOLIHYNHE-LYT	142
hCAXI	GRHVSFLPAPRPVNVVSGGPLSHRLSELRLHFG--ARDGAGSEHQINHQGFSAEVOLIHFNQE-LYG	142
hCAXII	HSVKLNLPDSMHIQ-GLQS--RYSATQLHLEHWG--NPNDPHGSEHTVSGQHFAAELEHIVHYNST-LYP	130
hCAXIV	HTVQLSLPSTLYLG-GLPR--KYVAAQLHLEHWG--QKGSPPGSEHQINSEATFAAELEHIVHYDSD-SYD	127
hCAIX	HSVQLTLPPGLEMALGPGR--EYRALQLHLEHWG--AAGRP-GSEHTVEGHRFPAELEHVHLST--AFA	124
hCAVI	HTVQISLPSTMRMTVADGT--VYIAQQMHLEHWG--GASSEISGSEHTVDGIRHVIETIIVHYNST--KYK	130
hCAIV	HSVMMLLEN---KASISGGGLPYQAKQLHLEHWS--DLPYKGSEHSLDGEHFAMEMHIVHEKEKGTSR	133
RPTPβ	KTVEINLTN--DYRVSGGVSVFKASKITLHFWGKCNMSSDGSEHSLGQKFPLEMOIYCFDAD-RFS	120
RPTPγ	KTVAILLKD---DYFVSGAGLRFKAEKVLEHFWGHSNGS-AGSEHSLGRRFPVEMOIFFYINPD-DFD	119
vaccCAwt	GKLVRINFKGG---YISGGFLEYVLSLHLEHWG--KEDDYGSNHLIDVYKYSGEINLVHWNKK-KYS	102

↑

↑

Figure 2

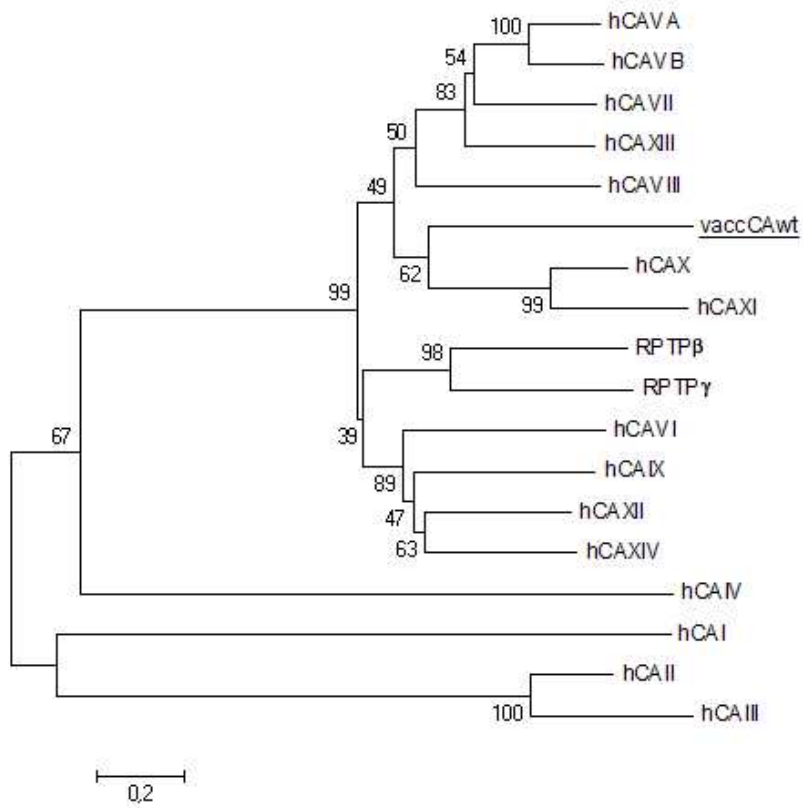


Figure 3

