

SUPPLEMENTARY MATERIALS AND METHODS

Construction of recombinant CA9 sequences – For cloning, BglII and HindIII restriction enzyme sites were added to the 5' and 3' ends, respectively. The PCR reactions were carried out using Phusion polymerase (Finnzymes, Espoo, Finland) according to the manufacturer's instructions. PCR was performed in a PTC 2000 thermal cycler (MJ Research, Waltham, MA), and the program consisted of a single 98°C denaturation step for 2 min, followed by 33 cycles of denaturation at 98°C for 10 s, annealing at 65°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 8 min. In the first PCR reaction, the template was the full-length human CA9 cDNA in the pOTB7 vector (IMAGE Clone 4865275, Geneservice Ltd, Cambridge, UK). Supplementary Table I provides the PCR reactions, annealing temperatures and primers that were used to construct the sequences that encode both the PG+CA and CA recombinant proteins. In some reactions (indicated on Supplementary Table I), 10% DMSO was used as an additive. For the PG+CA form, the PCR reactions (P2-P5) were as mentioned above. For the CA form (PCR reactions P6-P9), the initial denaturation step lasted for 2 min, the extension step for 30 s and the final extension for 7 min. The PCR products were run on 1.2% agarose gel and then purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire, UK).

Construction of recombinant baculoviruses – The final PCR products were digested with BglII and HindIII enzymes, while the pFastBac1 vector was digested with BamHI and HindIII (BglII and BamHI produce the same overhangs). The PCR product was ligated into the vector using T4 ligase (Invitrogen), and then the recombinant plasmids were transformed into TOP10 cells (Invitrogen). The transformed bacteria were incubated on LB plates containing 50 µg/ml ampicillin at 37°C overnight, and colonies containing the insert were identified by colony PCR. A positive clone for each construct was grown overnight in LB medium containing ampicillin, and then the plasmids were isolated using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To confirm the coding sequence for the recombinant CA IX proteins, sequencing was performed using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reactions Kit, version 3.1 (Applied Biosystems, Foster City, CA). Sequencing reactions were amplified by cycle sequencing on a thermal cycler, and the analysis was performed with an ABI PRISM Genetic Analyser 9100 (Applied Biosystems). The expression cassettes were recombined from the donor vectors (pFastBac1) to baculovirus genomes through site-directed transposition by transforming the DH10Bac cells (which contain the bacmid baculovirus shuttle vector and a helper plasmid that produces the proteins needed for transposition) with the donor vector. For transformation, a streak of DH10Bac cells was resuspended in 100 µl of cold 100 mM CaCl₂ and incubated on ice for 15 min. Then, 100 ng of donor plasmid was added to the suspension, and the cells were incubated on ice for 30 min. Heat shock was performed at 37°C for 2 min, and then 450 µl of SOC medium (Invitrogen) was added. The cells were grown at 37°C for 4 h in an orbital shaker. The cells were centrifuged at 6000 g (20°C, 1 min), resuspended in 100 µl of SOC medium and spread on LB plates that were prepared according to the Bac-to-Bac kit instructions. Recombinant bacmid selection and transfection of the insect cells were performed according to manufacturer's instructions.

SUPPLEMENTARY TABLE I. Details of the SES-PCR reactions that were used to construct the recombinant CA9 sequences.

PCR Reaction	Template	Primers	Annealing T / °C	DMSO
P1	Vector	F1+R1 5'-CTG CTG CTT CTG ATG CCT GTC C-3' (F1) 5'-CCT CTG GCT GGC TTC TCA CAT TCT-3' (R1)	65	-
P2	P1	F2+R2 5'-CAC CAC CAT CAC CAC CAT CAC CAC CTG GTG CCC CGT GGT TCC CAG AGG TTG CCC CGG ATG CAG-3' (F2) 5'-TTC GCC <u>AAG CTT</u> TTA GTC ACC AGC AGC CAG GCA GGA ATT CAG CTG GAC T-3' (R2, HindIII site underlined)	50	-
P3	P2	F3+R2 5'-CTG TCC CTG CTG CTG CTG ATG CCC GTG CAC CCC CAG CGT CTG CAC CAC CAT CAC CAC CAT CAC-3' (F3)	55	-
P4	P3	F4+R2 5'- CCC CTG CTG ATC CCC GCT CCC GCT CCC GGT CTG ACC GTG CAG CTG CTG CTG TCC CTG CTG CTG CTG ATG-3' (F4)	45	+
P5	P4	F5+R2 5'- GGC <u>CAG ATC</u> TAT GGC TCC CCT GTG CCC CTC CCC CTG GCT GCC CCT GCT GAT CCC CGC TCC C-3' (F5; BglII site underlined)	50	+
P6	P1	F6+R3 5'-CAC CCC CAG AGT CAT TGG CGC TAT GGA GGC-3' (F6) 5'-ATG GTG GTG GGA ACC ACG GGG CAC CAG AGG GAA GGA GGC CTC AAT CAC TCG CCC ATT-3' (R3)	50	-
P7	P6	F7+R3 5'-CAG CTG CTG TCC CTG CTG CTG CTG ATG CCC GTG CAC CCC CAG AGT CAT TGG CGC TAT-3' (F7)	50	-
P8	P7	F4+R3	50	+
P9	P8	F5+R4 5'-TTC GCC <u>AAG CTT</u> TTA GTG GTG ATG GTG ATG GTG GTG GGA ACC ACG GGG CAC CAG-3' (R4; HindIII site underlined)	45	+

SUPPLEMENTARY FIGURE LEGENDS

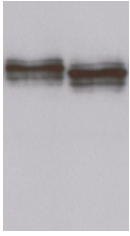
SUPPLEMENTARY FIG. 1. **Western blot analysis of the PG+CA form with (+) and without (-) PNGase F treatment.** Both lanes contained 10 ng of protein and were detected with the M75 antibody. PNGase F treatment caused a shift in both bands of the doublet indicating that the N-linked glycosylation is not responsible for the double band.

SUPPLEMENTARY FIG. 2. **Calibration curves of the size exclusion chromatography used for the PG+CA form.** A and B show the calibration curves of the molecular weight (MW) and the hydrodynamic radius (Rh), respectively. The curves were constructed using four standard proteins, and the results for P1 and P2 of the PG+CA form were calculated from the curves. The hydrodynamic radii of the standard proteins were determined by dynamic laser light scattering. $K_{av} = (V_e - V_o) / (V_t - V_o)$, where V_e = protein elution volume, V_t = column volume and V_o = void column volume.

SUPPLEMENTARY FIGURE 1

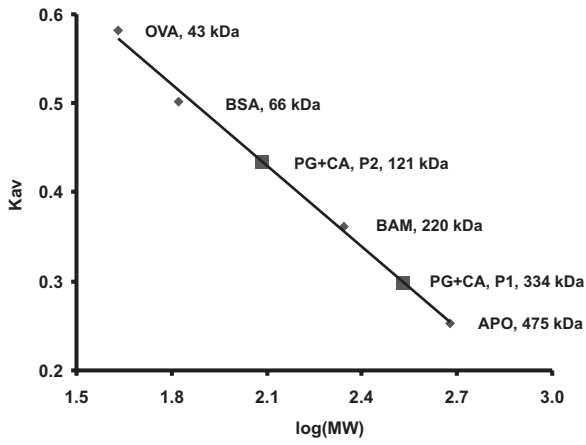
PNGase F

- +



SUPPLEMENTARY FIGURE 2

A



B

