# In Vitro Regulatory Effect of Epididymal Serpin CRES on Protease Activity of Proprotein Convertase PC4/PCSK4

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**Abstract:** PC4 or PCSK4 belongs to the 9-member superfamily of mammalian subtilases collectively called Proprotein Convertases or Proprotein Convertase Subtilisin/Kexins that convert inactive precursor proteins into their active mature forms by endoproteolytic cleavage. PC4-activity plays a crucial role in mammalian fertilization *via* activation of sperm surface proteins. PC4 knockout mice exhibit severely impaired male fertility due to premature sperm acrosome reaction. Regulation of sperm-PC4 activity during its storage and transport through epididymis is an important determinant for ultimate egg-binding and fertilizing capacities of sperms. Herein we show that epididymal serpin CRES (cystatin related epididymal spermatogenic) recombinant protein inhibits PC4 activity *in vitro* in a differential manner when measured against the fluorogenic substrate Boc-RVRR-MCA depending on its oligomeric state. Thus while CRES-dimer exhibits K<sub>i</sub> ~8  $\mu$ M, the corresponding monomer showed K<sub>i</sub> > 100  $\mu$ M. Both forms also blocked PC4-mediated processing of human proIGF-2 in human placenta tropoblast cell line with dimer being more efficient. Using specific inhibitors and substrates, we also demonstrated the presence of PC4-like activity and CRES protein in varying levels in the fluids of various epididymal compartments. Our observations suggest a potential function of CRES as a regulator of PC4 in sperm-egg interaction and fertilization.

**Keywords:** Proprotein convertase 4, proprotein convertase subtilisin kexin 4, serpin, epididymal fluid, cystatin related epididymal spermatogenic, inhibitor, protein aggregation, proprotein processing.

# INTRODUCTION

Proprotein Convertase 4 or PC4 also known as Proprotein Convertase Subtilisin/Kexin 4 (PCSK4) belongs to the class of mammalian serine proteases homologous to the yeast kexin and bacterial subtilisin [1]. PCs are Ca<sup>2+</sup>, and pH dependent enzymes with a specialized function of endoproteolytic processing of large inactive precursor proteins into their smaller bioactive forms. These include proneuropeptides, prohormones, pro-growth factors, pro-receptors, adhesion and other proteins [1, 2]. Currently there are 9 members of PC family, namely PC1/3 (PCSK1), PC2 (PCSK2), furin (PCSK3), PC4 (PCSK4), PC5/6 (PCSK5), PACE4 (PCSK6), PC7/8 (PCSK7), SKI-1/S1P (PCSK8), and more recently NARC1 (PCSK9). Each PC member shows some distinguishing structural features that include 1) a signal peptide, cleaved shortly after the start of biosynthesis in the rough endoplasmic reticulum, 2) a chaperone-like pro-domain regulating the catalytic activity of the cognate enzyme;

3) a catalytic domain responsible for protease activity; 4) a P (protease)-domain that properly folds the enzyme during maturation [2]. In addition some PCs contain a transmembrane and a cytosolic domain as in furin, PC5, PC7, SKI-1 [1, 2] and possibly PC4) [3]. The maturation of all PCs, including PC4, follows a two-step process. First, they undergo autocatalysis in neutral-pH ER, but the cleaved propeptide remains attached to the enzyme which still remains in inactive form. Then, a second internal cleavage occurring in *trans* Golgi network produces a fully activated PC [2].

Although many PC enzymes exhibit redundant and widespread tissue-localization, PC4 is uniquely expressed in the reproductive tissues [2-8] namely in testis, sperm surface, and epididymis in the male reproductive system, as well as in placenta, ovary, and oocytes in the female reproductive system. In majority it is found in sperm surface head and acrosome [reviewed in 3]. PC4 plays a role in the processing of sperm-surface precursor proteins such as ADAMs (A Disintegrin And Metalloproteinase), growth factors such as Insulin-like Growth Factor (IGF)-1 & 2 and hormonal peptide Adenylate Pituitary Cyclase-Activating Polypeptide (PACAP) that are key to sperm-egg fusion leading to fertilization. PC4's functional role in these events is now getting slowly unraveled. Knock-Out (KO)

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studies revealed that PC4 is critical for male fertility [9]. In female, impaired processing of PC4 substrate, pro-IGF-2, results in lack of mature IGF-2 leading to a high risk of developing fetal growth restriction and preeclamsia in prenatal mothers [8]. PC4 KO in male mice leads to infertility, accelerated sperm capacitation, premature sperm acrosome reaction, reduced sperm hyperactivation and inability to fertilize oocytes. In female mice, similar KO leads to subfertility, reduced folliculogenesis and reduced ovulation [9]. These observations are accompanied by lack of ovarian and testicular functions as well as reduction in spermatid PACAP regulated MAPKs [9].

Owing to these findings, study of PC4 activity and its regulation became crucial for better understanding of reproductive biology and fertilization. So far, pro-PACAP [7], pro-IGF-2 [8] and ADAM-2 [10] are the only three confirmed physiological substrates processed by PC4, although in vitro studies proposed more potential substrates, including ADAM-5, Cyritestin (ADAM-3) and fertilin  $\alpha$ , (ADAM-1). We and others showed that PC4 cleaves best after KXXR1 or KXKXXR1, where X is any amino acid [11-13]. A lot of interest has grown in recent vears to design PC4 inhibitors because of their potential applications in the prevention of sperm maturation, sperm-egg fusion, fertilization as well as in the study of biochemical property of the enzyme itself. A number of natural or physiological inhibitors of PCenzymes have been reported in the literature. These include 7B2 and its peptides for PC2 [14], proSAAS and its peptides against PC1 [15] and finally the serpin (serine proteinase inhibitor) dspn-4 for drosophila furin activity [16]. So far, no natural inhibitor of PC4 has been described. However recently a new serpin called CRES (cystatin related epididymal spermatogenic) has been discovered in epididymal fluid [17], but the corresponding enzyme partner has not yet been identified. However based on localization, tissue expression profiles and biochemical studies [17-20] it is likely that CRES may be an endogenous regulator of PC4. Serpins represent a family of protease inhibitors that have conserved structure [21]. Serpins regulate protease activity responsible for processing of precursor proteins into their biologically active forms which participate in many physiological I events like coagulation, aggregation, apoptosis and inflammation [22]. The inhibitory action of serpin is attributed to its reactive-site loop (RSL) domain, which interacts with the catalytic segment of the protease in question [23]. This induces a conformational change in the serpin leading to partial insertion of its "RSL" into the β-sheet of the protease. This is followed by a proteolytic cleavage forming a stable covalent adduct by reacting with a nearby active residue such as Cys. This results in loss of enzyme activity. Though not always the case, a serpin can also behave as a substrate of proteases where the insertion of "RSL" into β-sheet takes place slowly. In this case, the protease escapes inhibition by completing the cleavage of the serpin [24]. Most "RSL" loop is characterized by the presence of one or multiple S-S bonds that are crucial for the inhibitory action of serpin [23, 24]. The serpin CRES belongs to the family2 of cystatin superfamily of cysteine proteases and is closest to cystatin-8 [20]. It shows characteristic sequence homology and conserved motif such as the C-terminal "PW" motif, and four conserved Cys residues. Because it lacks the crucial catalytic "QVG" sequence, CRES consequently does not inhibit cysteine proteases [17]. So far CRES present in endocrine system has been shown to inhibit serineprotease PC2 (PCSK2), confirming its identity as a cross-class serine-protease inhibitor [25]. But the partner enzyme for CRES present in epididymis and acrosome (cap for mice and equatorial segment for human) [18, 19] is not known but it might be PC4. It is established that acrosome houses a number of hydrolytic enzymes, exemplified by serine- and cysteine-class proteases proacrosin and cathespin, which are released during acrosome reaction to digest zona pellucida before sperm nucleus can enter the egg [26]. Recently CRES knock out mouse has been generated and overall its sperm exhibited fertility defect, unable to undergo progesterone stimulated acrosome reaction, possesses decreased levels of protein tyrosine phosphorylation leading to a defect in sperm capacitation [27]. These observations suggest a potential role of CRES in sperm maturation and fertilization and may enforce the notion that CRES may be a natural regulator of PC4. In this study we present data showing the presence of PC4-like activity in mouse epididymal fluid and demonstrated by in vitro and cell culture experiments that PC4 is inhibited by recombinant CRES depending on its oligomeric state.

# MATERIALS AND METHODS

# Materials

Leishmania tarentolae gene expression kits were obtained from Jena BioscienceGmbH, Germany,(http:// www.jenabioscience.com/cms/en/1/browse/1119\_lexsy \_literature.html). DEAE (Di-Ethyl Amino Ethyl)sepharos and Arg-sepharose 4B resins were both purchased from Amersham Bioscience, Piscataway, NJ, USA. Polyclonal PC4 antibody was obtained from Alexis Biochemicals, catalogue number: ALX-210-218-R050 (San Diego, CA, USA) and it was raised against the PC4 fragment m (mouse) PC4<sup>23-393</sup> that contains its catalytic and middle domains. This sequence bears a 99.5% homology with the corresponding domain of h (human) PC4 [28]. Reagents for western blot and SDS-PAGE analyses were purchased from BIO-RAD Labs (Hercules, CA, USA). Chemiluminescence reagents (PerkinElmer LAS Inc, Shelton, CT, USA) were used for detection of immuno-reactive bands. Images were captured using Kodak X-OMAT Blue autoradiography film (PerkinElmer LAS Inc., USA). Matrix-Assisted Laser Desorption Ionization (MALDI) and Surface Enhanced Laser Desorption Ionization (SELDI) time-of-flight (tof) mass spectra (MS) were recorded using Voyageur reflector (Framingham, MA, USA) and Ciphergen (Fremont, CA, USA) instruments respectively. The corresponding mass spectra plates were purchased from the respective companies. 1-Cyano 4-hydroxy cinnamic acid (CHCA) and Sinapic

acid (SPA), used as energy absorbing matrices for mass spectrometry were purchased from Sigma Chemical Company, St Louis, MO, USA. The fluorogenic substrate Boc-RVRR-MCA was bought from Bachem Inc. (King of Prussia, PA, USA). Rec (recombinant)-mCRES plasmid was kindly provided as a gift by Dr Gail A. Cornwall. The plasmid construct was expressed in Escherichia coli (E. coli) as described in [29]. All reversed phase high performance layer chromatography (RP-HPLC) purification of protein samples were performed using Varian HPLC instrument (ProStar, Varian, Foster City, CA, USA). Each sample solution was pre-cleaned through filters 32 mm diameter and pore size 0.8/0.2 µm (Life sciences, Long Beach, Ca, USA) prior to injecting into HPLC column [28].

# Production and Purification of Rec-rPC4 Enzyme

Using the L. tarentolae expression system, rec (recombinant)-r (rat) PC4 was produced and purified in large quantity as described in [28] with some modification. Typically, culture medium of 1000 ml containing rec-rPC4 protein was concentrated by 40fold using Centricon filter (cut-off MW=10 kDa) to provide ~25 ml of crude concentrate medium. It was then purified by chromatography through DEAEagarose column alone without the subsequent argininesepharose column chromatography as done previously [28]. The elution profile of chromatography is shown in Supplementary Fig. (1) in a schematic form. All buffer-A (consisting of 25 mM TRIS + 25 mM MES + 2.5 mM CaCl<sub>2</sub>, pH 7.4) wash fractions 1-95 (~1 ml each) were Following combined (#1-95). this wash. chromatography was further continued with elution buffers consisting of a step-wise gradient of NaCI concentration, starting with 30 mM NaCI [pool # (1-80)], followed subsequently by 60 mM NaCl [#(1-60)], 70 mM NaCl [#(1-74)] and finally 200 mM NaCl [#(1-67)]. Thus 381 fractions with a total volume of ~381 ml were collected in 4 separate combined pools as indicated above.

# Production of Rec-m (Mouse) CRES

CRES cDNA encoding for mCRES (lacking the signal-peptide 1-21 amino acids) has been cloned into a champion pET directional pET101/TOPO vector (Invitrogen, USA) that contained a T7 promoter, a  $V_5$ epitope, and 6xHis tag at 3' end. The mCRES cDNA was a generous gift from Dr. Gail A. Cornwall, Texas Technological University, USA. The plasmid was used to transform Star BL21 One Shot Competent E. coli cells (Invitrogen, Carlsbad, CA, USA) by heat shock. The cells were grown first in 5 ml LB (Luria Bertani) medium then later scaled to 2 L. Cells reached optimal optical density (OD) after 4 h of growth, and IPTG (isopropyl β-D-1-thiogalactopyrano-side) was added to induce protein expression. Cells were collected by centrifugation after 1.5 h induction and were stored at -80°C. The next day, the cells were suspended in purification buffer (250 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8) and lysed by repeated sonication in the presence of lysozyme.

The lysate was centrifuged, and the clear supernatant containing rec-mCRES protein was collected for analysis by SDS-PAGE and mass spectroscopy.

# Purification of Rec-mCRES by RP-HPLC

Purificat-ion of crude rec-mCRES protein obtained from bacterial culture was performed by RP-HPLC using a semi-preparative C<sub>4</sub>-column (Varian, 10x25 cm size). During HPLC purification, proteins were separated using a linear gradient of 1% solvent B/min from 10% to 90% solvent B in solvent A [solvent B = 0.1% TFA in ACN (acetontrile); solvent A = 0.1% TFA in water]. Fractions were collected and analyzed as the elution was monitored by using an on-line UV detection system with wavelength fixed at 225 nm. Two peaks corresponding to monomer (Rt ~ 22 min) and dimer (Rt ~ 34 min) of mCRES protein (for characterization see below) were collected.

# Characterization of Purified rec-mCRES

(a) SDS-PAGE analysis: Tris-glycine gels with 12% resolving and 4% stacking phases were used for SDS PAGE analysis of mCRES mono and domer peaks. Denaturation of protein samples were achieved by heating to 95°C for 10 min with sample loading buffer containing 1% SDS, 10% glycerol, 10 mM Tris-HCI and 5% of reducing agent dithiothreitol (DTT) at pH 6.8. The gel was immersed in 1X running buffer (500 mM Tris, 0.1% SDS), and was run at 150V for about 1 h. The See Blue Plus2 pre-stained standard (Invitrogen Company, San Jose, CA, USA) was used as marker. When the separation was finished, coomassie dye staining solution (Bio-Rad, Hercules, CA, USA) was added to the gel for visualization of protein bands.

(b) Mass Spectrometry analysis: SELDI-tof MS was performed on 2  $\mu$ I of purified rec-mCRES sample solution (mono and dimer) in 0.1% TFA/water and sinapic acid as a matrix using Ciphergen protein chips instrument as previously described [12].

(c) Proteomic analysis: Following identification by SELDI-tof MS, rec-mCRES was further characterized by digestion of purified monomeric mCRES with Endoproteinase Lys-C enzyme followed by mass spectrometric analysis using SELDI-tof. All digest peaks were analysed by using the proteomic website www.expasy.ch. The observed molecular weights of various digest fragments were compared with those expected from the known amino acid sequence of recmCRES protein.

Digestion of rec-mCRES protein by rec-rPC4 enzyme: Purified rec-mCRES dimer (5  $\mu$ g in 5  $\mu$ l water) was incubated with purified rec-rPC4 (20  $\mu$ l, specific activity=179  $\mu$ mol AMC released per hour per  $\mu$ g protein per  $\mu$ l) at 37°C in a shaker incubator. A small sample of aliquot (5  $\mu$ l) was withdrawn from the mixture at times 0 h, 1 h, 2 h and 4 h and treated with glacial acetic (2  $\mu$ l) to stop the reaction. The samples were then subjected to SELDI tof MS and the data were analyzed for cleavage products of mCRES protein by rec-rPC4 enzyme.



**Fig. (1).** (A) Full length of CRES PCR product without signal peptide with expected size of 376 base pairs (BP). It was amplified using bluescript vector DNA containing mouse germ cell full-length mCRES cDNA (Gene bank accession number: NM\_009978). (B) It displays the the map of construct expressing CRES-V<sub>5</sub>-His<sub>6</sub> fusion protein.

PC4 assay and  $K_m$  determination: PC4 activity assay was monitored *in vitro* by using the fluorogenic substrate Boc-RVRR-MCA (50  $\mu$ M final concentration) in a 96-well plate (Dynatech, Millipore, USA) using the Fluorescence Spectrophotometer instrument (Spectra Max Gemini XS, Molecular Devices, USA) as described [28]. For determination of K<sub>m</sub> (Michaelis-Menten constant), several concentrations of Boc-RVRR-MCA ranging from 5-150  $\mu$ M were used as described in [28].

Protein assay: Total protein content in a sample was measured by using Bradford's optical density method. Each sample was mixed with bicinchoninic acid (BCA) reagent (Pierce Chemicals, USA) and optical density (OD) value was measured using Multiskan® Spectrum (Thermo) plate following the protocol of the manufacturer.

# Inhibition of rPC4 by Rec-mCRES and Kinetic Analysis

Determination of  $K_i$  and  $IC_{50}$  values:  $K_i$  (inhibition constant) and IC<sub>50</sub> (concentration needed to achieve 50% inhibition of enzyme activity) determinations were carried out graphically with RVRR-MCA substrate as described in [12, 13, 28] using Grafit software program version 4.09 (Erithacus software limited, Staines, UK). The measured relative fluorescence unit (RFU) values were converted to pmol AMC released per hour using a standard curve. K<sub>i</sub> determination was achieved by plotting 1/V against [I] (Dixon plot) at three different substrate concentrations namely 12.5, 25 and 50 µM, where V= velocity of reaction and [I] = inhibitor concentration. The data were analysed by linear regression using Sigma Plot program version 11.0. IC<sub>50</sub> value was also determined for each substrate concentration using the sigmoidal graph (plot of V vs log [l]).

Collection of mouse epididymal fluid samples and their analyses for PC4-like activity: Four young adult C57BL/6J (Jacksons Lab, Bar Harbor, ME, USA) male mice of same age (~3 months), housed under identical conditions were sacrificed and their epididymis sections were separated. Under the microscope, fluid samples from various sections of the epididymis tube namely caput, corpus, cauda and the vas deferens for each mouse were collected as cleanly as possible. The fluids were squeezed directly into cold PBS buffer (500 µl) which were then vortexed at 4<sup>o</sup>C for 5 min. Sperms and suspended tissues were then separated from the supernatant by centrifugation and the clear supernatant liquids were used for further characterizations. Protein and enzyme assays were performed on each sample as described above. The specific activity of each epididymal sample was then calculated and compared with that of others. Out of the total measured activity, attempt was made to estimate the actual PC4-like activity by using our PC4-specific inhibitor namely the prodomain peptide PC475-84, as well as the inhibitor (leupeptin) of trypsin-like enzymes which does not inhibit PC-like activity to any significant extent even at 100 µM concentration used in this study [12, 13, 28].

Cell culture work: The placenta cell (HTR-8/SV neo cell from human trophoblast), a gift from Dr. Charles H. (Queen's University, Kingston, Graham Ontario, Canada), were grown in the RPMI-1640 medium (Rosewell Park Memorial Institute medium) (Sigma Aldrich Chemical Company, USA) containing 10% FBS (Fetal Bovine Serum) (Gansera Intentional Inc, ON, Canada) and 0.6 µl/ml of antibiotic gentamicine sulphate (Invitrogen Corporation, ON, Canada) in (GTM) presence of 5% CO<sub>2</sub> at 37<sup>0</sup>C. Cells were grown until they attained at least 80% confluency and then the passage of the cells was done into the 6-well plates to grow to near confluency again in the next day when the old medium was removed. Plates were then rinsed with 1XPBS buffer followed by the addition of RPMI-1640 serum free medium containing varying concentrations of rec-mCRES protein. The next day the culture media and the cell pellets were collected in separate tubes for each well within the 6-well plates. The media samples were centrifuged before storage at  $<4^{\circ}$ C to remove all the cell debris and suspended insoluble particles. The pellet was also stored separately in a similar manner. The media samples were subjected to analysis by western blot using hIGF-2 antibody, raised against the whole mature protein (R&D systems, USA) with 1:500 dilution. This will

reveal the changes in the relative amounts of mature and proIGF-2 (a PC4-specific substrate). It is expected that the endogenous level of unprocessed proIGF-2 in placenta cell medium will be enhanced in the presence of rec mCRES protein, if the latter inhibits the PC4mediated cleavage of human pro-IGF2. The above experiment was also repeated by externally adding recrPC4 enzyme into the medium keeping all other conditions unaltered. Equal volume of enzyme sample (10 µl, specific activity = 179 µmol AMC<sup>-1</sup>h<sup>-1</sup> µmol protein, equivalent to 500U, where one U or unit = 1 pmol released per hour per µl sample) was added to medium containing 1x10<sup>3</sup> cells/ml) was used.

#### RESULTS

# Production and Purification of Active Rec-rPC4 Enzyme

Enzymatically active rec-rPC4 was obtained by Leishmania expression system and purified through DEAE-agarose column using buffer containing sodium chloride of increasing concentrations as described in section above. The measured specific activity profiles based on enzyme activity and protein content of various column fractions were used to determine fractions that containg most PC4 activity. This is shown in Supplementary Fig. (2A). which revealed that higher salt concentration pools that containing most PC4 specific activity. This suggested the possibility of the presence of several conformationally active forms of PC4. Out of all the eluted fractions, 28-pools exhibited high PC4-protease activity per  $\mu$ g of total protein when assayed against the fluorogenic substrate Boc-RVRR-MCA.

However only pools #15, 21, 22, 23, 24 (see Supplementary Fig. 1) contain most pure active PC4 protein with pool #15 and #24 being the purest. This is further confirmed by SDS-gel electrophoresis with coomasie staining and western blot analysis (Supplementary Fig. **2B**, **C**) which clearly confirmed the presence of active PC4 protein. The two major coomassie stained bands at ~ 65 and 52 kDa was consistent with the molecular sizes of two mature active forms of PC4 as reported earlier [28]. The western blot data further confirmed the conclusion.

Supplementary Table **1** summarizes the fold purification of PC4 achieved in various combined pools following DEAE-agarose column chromatography. It showed that both pools #15 and #24 represent most purified PC4 enzyme with ~511 and ~677 fold of purification compared to the crude concentrate enzyme sample. In comparison, pools #21, 22 and 23 are the next pure samples with fold purifications being >450. **Pool #15**, with activity 500 units where 1 unit =1µmol AMC released per hour per µl and specific activity ~179 µmol AMC<sup>-1</sup>h<sup>-1</sup>µmol total protein was used for all our *in vitro* work presented in this study.

#### Production and Purification of Rec-mCRES

A full length mCRES PCR product without the signal peptide sequence but containing both  $V_5$  and His<sub>6</sub> tags with the expected size of 376 base pairs (BP) (Fig. **1A**) was amplified using bluescript vector DNA containing mouse germ cell CRES. The northern blot analysis showing the presence of mRNA of CRES-V<sub>5</sub>-His<sub>6</sub> fusion protein is displayed in Fig. (**1B**).



**Fig. (2).** MALDI tof mass spectrum of rec-mCRES crude protein following its production by E coli expression system. The peaks at m/z 7,050; 14,055; 28,135; 42,113 and 56,003 were due to M/2, M, 2M, 3M and 4M respectively. The expanded portion of each observed peak was displayed at the bottom with regions indicated in the spectrum. It shows the propensity of CRES to ologomerize like many other serpins. The small variations in mass values between the calculated and the observed ones as well as from one experiment to another were due to calibration issue but they are still within the accepted error limit.

The expressed rec-mCRES protein present within the cell membrane inclusion bodies was recovered by extracting the cell pellet (10 gm) repeatedly with 6M aqueous guanidine HCI (4 x 10 ml) followed by vortex and sonication. The clear filtered liquid contained recmCRES protein as revealed by the presence of a strong peak in the mass spectrum at ~14,055 Da, consistent with the calculated mass (14,288 Da) (Fig. **2**). This is further confirmed by the presence of additional peaks at ~7, 28, 42 and 56 kDa respectively for M/2, 2M (dimer), 3M (trimer) and 4M (tetramer) respectively.

The RP-HPLC of crude rec-mCRES protein exhibited two peaks at retention times (Rt) 22 min and 34 min (Fig. **3**) which were attributed by mass spectrometry (Fig. **4**) to monomeric and dimeric forms of CRES protein, respectively. The monomer exhibited strong peaks at m/z ~7,276 and 14,578 Da for half and full molecular weight of monomer CRES respectively. A weak peak at m/z ~29,000 was also observed for the formation of dimer during mass spectrometry. The dimer on the other hand showed a strong peak at m/z 29,114 for the molecular weight of the dimer and a weak peak at m/z 14,601 for half the molecular weight of the dimer. The purities of these two collected peaks were further tested by rechromatography under identical condition using an analytical C<sub>4</sub> column which showed largely a single peak for each form (Fig. 3B, C). Thus it is evident that recmCRES protein exists in mono and dimeric forms. The presence of additional higher oligomeric CRES-forms as indicated by mass spectrum of crude sample could not be detected by RP-HPLC. The conversion of CRES monoto dimeric form was found to be quite rapid as judged by RP-HPLC and mass spectrometry. Nearly 10 mg of each mono- and dimeric CRES proteins was obtained from 200 ml solution of crude CRES sample. This represented ~ 0.066 mg of CRES protein per ml of crude extract solution.

#### **Characterization of Rec-mCRES**

The identity of HPLC-purified rec-mCRES mono and dimers was confirmed by digestion experiment of



**Fig. (3).** (A) RP-HPLC chromatogram of crude rec-mCRES sample. Two major peaks eluting at retention time (Rt) 22.3 min and 33.6 min were collected and were identified by mass spectra as CRES monomer and dimmer respectively. (**B**) It shows the rechromatogram of purified CRES monomer (Rt = 22.3 min) whereas (**C**) shows the rechromatogram of purified CRES dimeric protein (Rt = 33.6 min). The rechromatograms confirmed the purity of each rec-mCRES form. Both purified mono and dimer showed the presence of trace amount of other form suggesting the tendency of CRES monomer to undergo self association even under normal condition, The peak depicted with \* is impurity present in the dimeric form of CRES.



**Fig. (4).** SELDI-tof mass spectra of crude rec-mCRES (A) as well as the purified samples of CRES monomer, Rt~22 min (B) and CRES dimer Rt~34 min (C). The crude sample exhibited peaks at m/z~14,476 Da and 28,922 Da for the presence of mono and dimeric CRES protein. Interestingly the purified samples of CRES mono and dimer also exhibited a mixture of similar peaks in their mass spectra. For monomer sample this is likely due to the conversion of mono to dimer during laser bombardment of mass spectrum. For dimer, the monomeric peak was actually the M/2 peak of dimer. In the monomer mass spectrum one can notice a strong peak at m/z~7276 owing to M/2 peak of the monomer.

each protein with Endoproteinase Lys-C enzyme followed by analyses of mass spectra at various times ranging from 0 to 4 h. Several fragment peaks were observed as shown in the expanded portions of mass spectrum (Fig. 5A, left panels) for 1 h crude digest sample. The peaks were observed within the the following molecular weight ranges: (i) 5,600-5,800 Da, (ii) 5,200-5,500 Da, (iii) 4,900-5,000 Da and (iv) 4,500-4,700 Da. The analysis of the data suggested cleavages of mCRES protein at various post Lys residue sites as indicated in Fig. (5B). These results confirmed the identity and amino acid sequence of recmCRES protein. These cleavage sites of rec-mCRES for Lys-C enzyme are shown by letters A, B, C, D, E, F and G. It is important to point out that the peaks found in the mass spectrum of rec-mCRES digest by Lys-C are absent as expected in the control mass spectrum These include peaks at m/z 5,716, 5,700, 5,338, 5,360 data suggested which are consistent with those expected with the known sequence of mCRES protein.

# PC4-Protein and its Activity in Mouse Epididymal Fluid

Western blot analyses of fluids collected from various regions of mouse epidydimis showed the presence of varying levels of PC4 protein in them. The results are presented in Fig. (6) for 2 representative mice tested. It showed that PC4 content is highest in caput followed successively by corpus, cauda and vas deferens. This calculation was based on equal amount of loaded protein (5 µg). Moreover, attempts have been made to measure PC4-like activity in these fluids by using the fluorogenic substrate Boc-RVRR-MCA. Protease activity was measured in the absence and presence of PC4 inhibitor hPC4<sup>75-84</sup>, trypsin inhibitor leupeptin, or a mixture of the two inhibitors. Roughly PC4-like activity was calculated as equal to RFU in presence of leupeptin + PC4<sup>75-84</sup> minus RFU in presence of leupeptin alone. Using this estimation, the PC4-like activity of each of the four epididymal regions was calculated and the averaged results for 4 mice are



**(B**)



**Fig. (5).** (A) Expanded portion of SELDI-tof mass spectra of 1h crude digest of purified rec-mCRES monomer by Endoproteinase Lys-C enzyme. The region of spectra depicted was between the mass range 4,000 to 6,000Da (the 4 left panels i, ii, iii and iv). The corresponding control mass spectra of rec-mCRES monomer and Lys-C alone under identical condition are shown on the right panels. Important digest peaks namely at m/z 5700, 5377, 4952 and 4591 (major), not observed in the mass spectra of the control samples confirmed the identity of rec-mCRES as thoroughly explained in (**B**).

presented in Fig. (7) and Table 1. It was noted that PC4 like activity is relatively high in caput (~288  $\mu$ mol/h/ $\mu$ g x 10<sup>-6</sup>) and corpus, (~354  $\mu$ mol/h/ $\mu$ g x 10<sup>-6</sup>) compared to cauda (~164  $\mu$ mol/h/ $\mu$ g x 10<sup>-6</sup>) and vas deferens (~50  $\mu$ mol/h/ $\mu$ g x 10<sup>-6</sup>) based on data with 4 mice tested. Earlier presence of CRES in caput was demonstrated both mRNA and protein levels [30, 31]. So far little is known about the relative levels of CRES protein in corpus, cauda and vas deferens in comparison to that in caput. However based on mRNA, CRES expression appears to be in comparable level in these regions [27, 32].



**Fig. (6).** Western blot results of various mouse epididymal fluid samples using mPC4 specific antibody. The results were shown as representative examples for 2 out of 4 mice tested in the study. For each lane a total of  $5\mu g$  protein was loaded. The data indicated a gradual decline in PC4 level from caput to vas deferens *via* corpus and cauda.

#### Inhibitory Effect of Rec-mCRES on PC4 Activity

Purified mono and dimeric rec-mCRES were separately tested for their effects on PC4 activity *in vitro* using fluorogenic substrate.

Effect of CRES dimer. Fig. (8) showed that CRES dimer efficiently inhibited PC4 activity *in vitro* when measured against Boc-RVRR-MCA substrate. The measured IC<sub>50</sub> values were found to be dependent slightly on the concentration of the substrate used and as indicated in the figure these are 31.3±3; 25.2±4 and 24.5±4  $\mu$ M respectively for 12.5, 25 and 50 mM substrate concentration respectively. These measured IC<sub>50</sub> values were then used to calculate K<sub>i</sub> (inhibition constant) using Cheng-Prusoff equation [33] K<sub>i</sub> = IC<sub>50</sub>/(1+ ([S]/Km) valid for competitive inhibition where

[S] = concentration of the substrate and K<sub>m</sub>=Michaelis– Menten constant which was found to be  $12.9\pm2 \ \mu M$  as measured [28] *(not shown)*. Our three independent calculated K<sub>i</sub> values based on IC<sub>50</sub> were 15.8, 8.56 and 5.03  $\mu$ M (average ~9.84  $\mu$ M). This average value was found to be fairly in agreement with that measured from Dixon plot (5.8, 10.72 and 26.6  $\mu$ M, and average ~14.4  $\mu$ M) (Fig. **9A**) This finding as well as the linear nature of double reciprocal Lineweaver-Burke plots at three different substrate concentrations with a common intersection point on y-co-ordinate (Fig. **9B**) confirmed the competitive nature of PC4 inhibition by CRES dimer.



Fig. (7). Average specific activity measured in four epididymal regions of mice. The data shown here as bar graphs, was derived from samples collected from 4 mice of identical age and genetic background. The samples were optimized based on measured total protein content by Bradford's assay and protease activity by the fluorogenic peptide Boc-RVRR-MCA (50  $\mu$ M). Samples containing ~10  $\mu$ g total protein were used for each assay. Tabular representation of the data is shown in Table 1.

Effect of CRES monomer: In contrast to dimer, recmCRES monomer inhibited PC4 activity only weakly. In fact no significant inhibition was observed even at 100  $\mu$ M concentration (Fig. **10**, upper panel) using the same substrate Boc-RVRR-MCA at three different concentrations. In all cases there was a slight inhibition of PC4 activity in a dose dependent manner at low concentration levels (between ~0.5-5  $\mu$ M), beyond which we observed a slow regeneration of activity followed again by weak inhibition at higher

 
 Table 1. PC4-Like Activity Measured in Fluids Collected from Caput, Corpus, Cauda and Vas Deferens Regions of Mouse Epididymal Fluid

	PC4-Like Activity in Various Epididymal Regions pmol AMC h <sup>-1</sup> mg <sup>-1</sup>				
Mouse No.	Caput	Corpus	Cauda	Vas Deferens	
Mouse #1	240	601	112	45	
Mouse #2	279	230	139	30	
Mouse #3	346	232	242	26	
Mouse #4	288.3	354.3	164.3	50.5	

Four different mice of same age and background were used and protease activity was measured with Boc-RVRR-MCA (50 mM) substrate in the absence and presence of PC4 specific inhibitor namely PC4<sup>75-84</sup> peptide derived from its prodomain. PC4 like activity was calculated by subtracting the lower activity (in presence of inhibitor) from the higher activity (no inhibitor present)



**Fig. (8).** IC<sub>50</sub> value determination for inhibition of rec-rPC4 by rec-mCRES dimer using three different substrate (Boc-RVRR-MCA) concentrations (50, 25 and 12.5 μM) as indicated in the graphs. The sigmoidal nature of each graph where velocity of reaction was plotted against the logarithmic of inhibitor (rec-mCRES dimer) concentration is quite evident.



Fig. (9). (A) Dixon plot showing PC4 inhibition by rec-mCRES dimer protein at three different substrate (Boc-RVRR-MCA) concentrations (S1, S2, S3) as indicated in the graph. (B) Double reciprocal Lineweaver-Burk plot showing competitive inhibition of PC4 by rec-mCRES dimer. The three concentrations of rec-mCRES dimer as used are shown in the graph. AMC=7-amino 4 methyl coumarin.

concentration ranges (between ~20-100  $\mu$ M) The explanation for this unique behavior is not clearly understood. Interestingly in the presence CRES dimer also, PC4 activity is first slightly activated within the low concentration range ~0.5 - 5  $\mu$ M and then inhibited strongly in a dose dependent manner within the higher concentration range 5 - 100  $\mu$ M (Fig. **10**, lower panel). This observation was found to be consistent with all three substrate concentrations used.

#### Cleavage of Rec-mCRES by Rec-rPC4 Enzyme

Since CRES belongs to serpin class of proteins of cystatin type, it is likely to contain a cleavage site within its reactive site loop for the enzyme (PC4) it inhibits. To

test this possibility, purified rec-mCRES monomer is digested with active rec-rPC4 enzyme for various lengths of time ranging from 0 - 4 h and the crude digests were analysed by SELDI-tof mass spectrometry. The results shown in Fig. (**11A**, **B**) indicated the presence of several additional peaks not found in the mass spectra of rec-mCRES or PC4 alone. The peaks observed are m/z 28,766 Da (CRES dimer), 14,383 Da (CRES monomer), 7,273 Da (M/2 peak of CRES monomer), 9,656 Da (M/3 peak of CRES dimer), 9,226 Da (the N-terminal fragment following cleavage at **NCKK↓PLN**. The peak at m/z 4,613 Da is the M/2 of the above cleaved N-terminal fragment (m/z 9,226). This suggested that PC4 cleaves rec-mCRES at the C-



**Fig. (10).** Effects of rec-mCRES mono and dimer at various concentrations ranging from 0-100 μM on PC4 activity *in vitro* as measured against three different concentration levels ([S]) of Boc-RVRR-MCA were shown in comparative bar graphs. The graph confirms that in contrast to CRES monomer, the corresponding dimer inhibited PC4 activity quite efficiently. The error bars are drawn from three independent experimental values.

terminus of double lysine residues namely at  $KK^{92} \downarrow P$  (Fig. **11C**), although we failed to detect the corresponding C-terminal fragment peak in the mass spectrum. This represents a unique and rare but not unusual cleavage site for any PC-enzyme.

# Effect of Rec-mCRES on PC4 Mediated Cleavage of Pro-IGF2.

Following in vitro results showing inhibition of PC4 activity by rec-mCRES dimer, we became interested to examine effect of rec-mCRES on PC4 mediated cleavage of its known physiological substrate proIGF-2 [8]. To test this we selected human placenta tropoblast cell line which expresses both PC4 and proIGF-2, making it an ideal model for the experiment. The western blot results using IGF-2 antibody shown in Fig. (12) suggested that rec-mCRES inhibited proIGF-2 cleavage in a dose dependent manner as confirmed by the gradual appearance of the bands for precursor proIGF-2 and/or its intermediate form with the addition of increasing amount of rec-mCRES protein. Interestingly the effect was observed with both mono and dimeric CRES. However while CRES dimer was found to be effective in blocking the direct conversion of ~24 kDa proIGF-2 into ~8 kDa mature IGF-2, the monomer seems to block both this as well the conversion of ~16 kDa IGF-2 intermediate form (known as big IGF-2) into its ~8 kDa mature form. The results

were compared with the control sample done in parallel in the absence of any added rec-mCRES protein. It was noted that the commercial IGF-2 antibody used in this case could only detect proIGF-2, its intermediate form and the synthetic mature IGF-2 but not any mature IGF-2 produced in the culture medium. As expected in the control lane, no proIGF-2 band could be detected suggesting its complete cleavage by the endogenous PC4 into mature IGF-2 protein. Similar results were also obtained when the same experiment was repeated in the presence of added rec-rPC4 along with the CRES protein (compare Fig. (12A-D) for CRES monomer and dimer). Our data suggested that rec-mCRES blocks both PC4 mediated cleavages of proIGF-2 as well as intermediate IGF-2 into mature IGF-2, depending on its oligomeric state.

#### Aggregation of Rec-mCRES

Fig. (13A, B) showed the SELDI-tof mass spectra of rec-mCRES protein following 3 days of incubation in water of pH 7.4 at room temperature. In addition to the peak at ~14 kDa for monomeric CRES, there were also peaks at m/z ~ 28, 43, 58, 72, 85 and 99 kDa corresponding to the formation of di, tri, tetra, penta, hexa and heptamers. This is also evident from the SDS-PAGE results of the same sample (Fig. 13C) where the formation of CRES di, tri, penta and heptamer bands could be easily detected upon



**Fig. (11).** (A) <u>Top four panels</u>: Mass spectrum analysis of rec-mCRES upon incubation with a fixed amount of active recrPC4 at various time points 0 - 4 h. (B) <u>Bottom two panels</u>: It shows the mass spectra of two controlsone consisting of rec-mCRES and the other rec-rPC4 alone. (C) It shows the site of cleavage of re-mCRES sequence by PC4 enzyme as confirmed by the presence of N-terminal fragment peak (shown underlined) at m/z 9,226 in the mass spectrum shown in (B).

coomassie staining. Interestingly the data seems to support the notion that rec-mCRES rapidly forms the dimer as the major relatively stable oligomeric form which then undergoes further oligomerization likely at a slower pace. Thus the dimeric form appears to be the most stable form. The nature and the mechanism of this oligomerization or self-association property of CRES have not yet been fully understood.

# DISCUSSION

# **Rec-rPC4** Purification

Our study showed that active recombinant soluble rPC4 can be produced as before [28] by *Leishmania toreantola* expression system but unlike previously it was now purified in a much efficient manner by a single DEAE ion exchange column chromatography using slow stepwise salt gradient method. The purified enzyme was eluted in two separate batches one with 70 mM NaCl containing buffer and the other with 200 mM NaCl containing buffer. Both were found to be

similar by activity, gel electrophoresis and western blot measurements suggesting that these may represent different conformational forms of PC4 with similar specific activity. Previously rec-rPC4 purified by two step DEAE-sepharose and Arginine-sepharose 6B columns exhibited  $K_m = 91.7 \pm 12.0 \mu$ M,  $V_{max} = 658.9 \pm 34.9 p$ Mh<sup>-1</sup> and  $V_{max}/K_m = 7.18 \times 10^{-9} h^{-1}$ . In the present study, our rec-rPC4 purified by single step DEAEcolumn and eluted with 70 mM NaCl/buffer exhibited  $K_m$  = 13  $\mu M,$   $V_{max}$  = 176.8±8.04  $pMh^{\text{-1}},$   $V_{max}/K_m$  = 13.6 x 10^{\text{-9}} h^{\text{-1}}. This is nearly 1.9-fold higher than the previous value, although the individual  $K_{\text{m}}$  and  $V_{\text{max}}$  values for the present pool are respectively 7-and 3.7 folds lower than the previous values. This indicated ~2-fold higher activity for the present PC4 batch compared. This may be linked to the use of arginine-sepharose column in previous study where the final eluted enzyme contained a significant amount of free arginine in the eluted enzyme sample. The presence of free arginine in the eluting buffer may likely have inhibitory effect on rec-PC4 enzyme. Such notion may require confirmation



**Fig. (12).** Effect of rec-mCES monomer and dimer protein on PC4 mediated cleavage of pro-hIGF2 in human placenta tropoblast cell line in the presence of a fixed amount of added rec-rPC4 enzyme. The cells were cultured in DMEM medium in the presence of a fixed amount of added rec-rPC4 enzyme. Varying quantities of rec-mCRES mono or dimer protein was added. A control experiment without any added CRES protein was performed in parallel. Immunoblots using IGF-2 specific antibody was performed on concentrated media from each sample and the results are in (**A**) and (**B**) for mono and di-meric rec-mCRES protein respectively and their quantification was done based on three independent experiments as shown in the (**C**) and (**D**). Both CRES mono and dimer blocked the cleavage of 24 kDa proIGF-2 into its 8 kDa mature form. It is interesting to note that while in the presence of increasing amounts of recCRES monomer one can notice the appearance of 16 kDa IGF-2 intermediate form as well as 24 kDa proIGF-2, this is not the case with CRES dimer (E) modified from [8] and shows the details about the known PC4-cleavage sites in pro-IGF-2.  $\bullet$  = Glycosylation site.

in future study where removing free arginine and measuring the activity can be conducted. Both PC4 samples exhibited multiple bands at ~65, 52 and 38 kDa in western blot corresponding to the presence of pro, full length and truncated mature PC4 forms respectively. Similar results (*data not shown*) were obtained with the 200 mM NaCl pool of PC4, suggesting similarity in the two purified PC4 samples.

#### Production of Rec-mCRES

Our study showed that *E-coli* expression system combined with RP-HPLC produced rec-mCRES protein in pure form. Both mono and dimeric forms were obtained under the conditions used. Proteomic analysis using Endoproteinase Lys C digestion followed by mass spectrometry confirmed the identity of rec-mCRES protein. mCRES is 121 amino acid long secreted protein [without the signal peptide of 21 amino acids] with a calculated molecular weight of 14,040 Da. However our CRES-construct contained a C-terminal V<sub>5</sub> as well as His<sub>6</sub> tags with a few additional amino acids as indicated in Table **2**. The expected molecular

weight of rec-mCRES based on this construct is ~17,682 Da which is ~3,372 Da higher than the observed value. In fact our purified rec-mCRES protein exhibited molecular ion peak in the mass spectrum at m/z ~14,476 and ~28,922 Da for the mono and dimer respectively. We explain this size difference by proposing that our rec-mCRES may have lost both V<sub>5</sub> and His<sub>6</sub> tags during its expression. Complete removal of these two tags and an additional C-terminal peptide segment "ELNSKLE" may account for the observed mass difference of ~3,372 Da. In that case, the recmCRES should have a theoretical molecular weight 17,682 - 3,372 = 14,310 Da that agrees guite well with the observed value of 14,476 Da (1.1% deviation). This fact and the mass spectral data of Lys-C digest of recmCRES where we only detected fragments from the Nand mid-terminal regions (Fig. 5), seem to suggest a possible shedding or cleavage in rec-mCRES at any of the sites indicated by a vertical arrow  $KD \downarrow V^{140} \downarrow E \downarrow LN$ . This cleavage may likely be mediated by an endogenous protease and therefore our purified rec-



Fig. (13). (A) SELDI-tof mass spectrum of rec-mCRES following incubation for lengths of time at room temperature showing the formation of more and more higher oligomers as a result of self aggregation. (B) Expanded portion of the mass spectrum in the region between m/z 55,000 to 90,000 Da. (C) Coomasie stained 15% SDS polyacrylamide gel electrophoresis of purified re-mCRES protein mono and dimer at room temperature.

During RP-HPLC purification of crude rec-mCRES protein it was observed that even though CRES dimer (~28 kDa) and monomer (~14 kDa) were eluted at retention times almost 12 mins apart from one another, a second HPLC chromatograms of each (Fig. 3B, C) and MS data (Fig. 4) revealed that each oligomeric form contains a minute amount of the other form possibly due to their interconversion. This likely supports the notion that CRES monomer has a strong tendency to self-associate to form the dimer. This observation was previously reported by Cornwall et al. [34, 35]. Interestingly the purified dimer showed in the mass spectrum and RP-HPLC the presence of a weak peak for the monomer. Self incubation experiment confirmed that on prolonged storage CRES monomer undergoes significant self-assembly to yield dimer (major) along with higher oligomers (minor) consisting of tri, tetra, penta, hexa and even heptamers (Fig. 13). Such self-aggregating potential has been reported in the literature for several other serpins such as cystatin c, antitrypsin and antithrombin [36, 37]. It was also established that such aggregation is triggered and propagated by the initial formation of dimers which is a crutial step for further oligomerisation.

#### PC4-Like Activity in Mouse Epididymal Fluids

Our studies confirmed the presence of PC4 protein and its protease activity in the fluids of all four regions of mouse epididymis. Even though these fluids are not individually clean and to some extent are contaminated by the fluids from the neighbouring regions, the data revealed a much higher PC4-like activity in the caput region compared to those in cauda, corpus and vas deferens. Overall, PC4 protein was detected in all four regions with progressively lower amounts from caput to corpus, cauda and vas deferens. Moreover PC4-like activity per ug of total protein was found to be present in varving levels depending on the location. It is important to note that it is the protease activity of PC4 and not its actual amount that is more relevant to the ultimate fertilizing capacity of sperm since such activity is responsible for the proteolytic activation of sperm precursor proteins. So far available literature indicates that CRES is present in high amount in the proximal and distal caput region of epididymis based on mRNA and western blot data [29, 30]. mRNA results indicated that CRES is present in comparable level in caput, corpus and cauda regions of mouse epidydimis. However comparison of their protein contents has not yet been studied. Hence our findings in this respect are quite significant and novel which may provide some

Table 2.Possible Explanation of the Peaks Obtained Following Digestion of rec-mCRES Protein by Proteinase Lys-C<br/>as Shown in Mass Spectrometry in in Fig. (9A, B). The Various Cleavage Sites are Indicated by Vertical<br/>Arrows and by Letters "A. B, C, D, E, F,G and I" as Indicated Below. Two Letters Shown as D to H, E to H, C<br/>to G and A to B Indicated the Fragment within those Two Cleavage Sites



MVGVDQSKNEVK AQNYFGSINI SNANVKQCVW FAMKEYNK

Calc MW 4584, Obs 4591, 4623 (+2O)

insight into CRES and PC4 interaction and its net consequence on fertilizing potential of sperm. Presence of higher level of PC4 activity (our present data) and CRES [30, 31] in caput region of mouse epididymis may seem contradictory at the first sight. However one can rationalize this observation when other aspects are factored in. These include the fact that PC4 exists in both membrane bound and soluble secreted shed forms that are likely to exist in varying levels in various epididymal compartments depending on tissue and cell type. It is likely that the observed higher level of PC4 activity in caput may be associated with the presence of increased amount of soluble PC4 enzyme and/or low CRES aggregation. Thus it may be proposed that in caput, majority of sperm PC4 enzyme exists in the fluid in soluble form and the level of this activity is so high that despite the presence of high amount of CRES protein, the ultimate activity may still remain high. Alternatively it is also possible that in caput region most of the CRES may be present in monomeric form which is a weak inhibitor of PC4. Our data suggests that PC4like activity in mouse epididymal fluid is diminished gradually as the sperm travels to corpus, cauda and finally vas deferens before interacting with female egg. This observation may be explained by speculating that

CRES increasingly undergoes dimerization as it travels from caput towards vas deferens causing enhanced PC4 inhibition.

Overall sperm PC4 activity in various epididymal compartments is regulated by three factors. (i) How much PC4 enzyme remains in membrane bound and secreted forms? (ii) How much CRES protein is present the epididymal fluid? (iii) How much CRES protein is present in dimeric state? In addition to above, the age of mice is also an important factor as it may affect the level of CRES expression [31]. Additional studies are currently in progress to analyze more accurately PC4-like activity using PC4 specific fluorogenic substrates derived from IGF-1/2 and PACAP [12].

### PC4-Inhibitory Effect of Rec-mCRES

Our results suggested that rec-mCRES inhibits PC4 activity against both the fluorogenic substrate Boc-RVRR-MCA and proIGF-2 processing. The potency of this inhibition depends on the oligomeric state of CRES. Thus while CRES dimer exhibited more potent PC4-inhibitory activity with K<sub>i</sub> ~ 11.6  $\mu$ M and IC<sub>50</sub> ~ 20-35  $\mu$ M depending on substrate concentration, the monomer is only a weak PC4-inhibitor with measured  $IC_{50} > 200 \mu M$ . Our data using the human tropoblast placenta cell line indicated that both mono and di-meric CRES blocked with nearly equal efficiency the processing of proIGF-2 into its mature form. The inability of commercial IGF-2 antibody to detect physiologically produced mature IGF-2 (8 kDa) in cell experiment (despite the fact that it was able to detect commercial synthetic mature IGF-2) proved a major challenge for us to make a final conclusion. However it did suggest that CRES blocked PC4 mediated proIGF-2 maturation. However a close examination of Fig. (12A, B) revealed that while monomeric CRES was able to block effectively the processing of big-IGF-2 into mature IGF-2 in a concentration dependent manner, the dimeric CRES can block both the processing of intermediate IGF-2 as well as proIGF-2 into its mature form.

# Mechanism of PC4 Inhibition by CRES

Our data revealed that, consistent to serpins, recmCRES not only behaved as an inhibitor of PC4, but also acted as a substrate for PC4 as it is cleaved at the site **KK<sup>97</sup> PL** (Fig. **11C**). This type of cleavage at post di-lysine residues is quite uncommon but not unusual. In fact there is at least one report of cleavage at the carboxy terminal of double Lys residues by a PC enzyme, Thus it was shown that 267-mer human (h) Pro-opiomelanocortin (POMC) is cleaved by PC2 at KNAYKK<sup>265</sup>↓GE]. (Accession no AAH65832). The cleavage of recCRES by PC4 takes place at SRSNCKK<sup>97</sup> PL which is characterized by the presence of a P6 basic residue (R) which likely assists in recognition by the enzyme leading to cleavage. Interestingly the above mentioned POMC cleavage site by PC2 is also characterized by the presence of a P6 basic residue (Lys).

The reason why CRES dimer unlike its monomer inhibited PC4 so potently nor the mechanism of this inhibition is well understood. However our study revealed that the above inhibition is likely competitive in nature as previously reported for inhibition of PC2 by CRES. But no studies have so far been conducted on cleavage of CRES by PC2 and the effect of CRES dimerisation on PC2 inhibition. Our data confirmed that CRES is indeed cleaved by PC4 while inhibiting its activity - a feature commonly shared for protease inhibition by serpins [22, 23]. However unlike other serpins, we did not observe formation of any covalent adduct between rec-mCRES and rec-rPC4. Although most serpin-protease binding is of irreversible nature, there are several of examples in the literature where reversible binding is also observed. One such example is the serpin ovalbumin which inhibits cathepsin G and elastase in a reversible manner [38]. Further studies are needed to better clarify the above inhibition of PC4 by CRES protein.

The formation of fragment at m/z 9,226 in the mass spectrum (Fig. 11) due to the cleavage of rec-mCRES at **SRSNCKK**<sup>97</sup> $\downarrow$ **PL** probably indicated that, in our rec-mCRES, at least one Cys residue namely **Cys**<sup>95</sup> is present in free state, rather than linked to another Cys

residue, as physiologically present. This may suggest that our rec-mCRES did not contain the expected S-S linkages and this may explain the observed modest inhibition of PC4 activity by our *E coli* produced rec-CRES protein. It is possible that with the proper S-S linkages and glycosylation as found in physiological condition, CRES may inhibit PC4 activity with altered efficiency. Further studies are in progress.

# **CRES** Aggregation

Our study confirmed the previous report that CRES is prone to aggregation. However herein we first demonstrated that CRES can produce even up to heptamer with dimer being the major stable oligomeric form that inhibits PC4 activity more potently than the monomer. Aggregation of serpins (serpinopathy) has been reported in the literature [38-41] that is relevant to diseases such as neonatal hepatitis, cirrhosis, and emphysema. However their detail effects on protease inhibition have not been fully explored. Whether CRES dimerization or aggregation in vivo leads to enhanced inhibition of PC4 activity remains to be seen. Interestingly, our cell culture study indicated that CRES monomer inhibits primarily the PC4-mediated cleavage of intermediate-IGF-2 into mature IGF-2. On the other hand CRES dimer inhibits both PC4-mediated cleavages of proIGF-2 and intermediate IGF-2 into mature IGF-2. So far based on Dixon plot and measured IC<sub>50</sub> values at various concentrations against the substrate Boc-RVRR-MCA, it appears likely that PC4 inhibition by CRES is largely competitive in nature despite the cleavage. The measured K<sub>i</sub> value from Dixon plot and the value calculated from Cheng and Prusoff equation [33] were found to be relatively close to one another.

# CRES (Cst-/-) Knock Out and Our Data

Recently CRES knock out (KO) mice has been generated and the data on phenotype has been published [27]. It indicated an overall reduction in fertilization. In addition, morphological abnormalities and differences have been noticed for KO mice lysosomes in principal cells and immature germ cells in epididymal lumen. Besides, dense granular bodies of different shapes and sizes were observed abundantly both above and below nucleus. Altered testicular and epididymal histology of KO mice reflects a cumulative effect of the loss of CRES and support a role for CRES in maintaining the normal integrity and function of the testis and epididymis. The KO mice in comparison to wild type (WT) showed decrease in the size of the testis, disrupted sertoli cells resulting in premature sloughing of germ cells leading to reduced number of spermatozoa in epididymal cauda and defective binding ability of sperm to egg zona pellucida (ZP). In addition it was noted that, after 60-90 mins of capacitation, the KO spermatozoa underwent reduced acrosome stimulation of reaction than WT spermatozoa. Another point to note is protein Tyrosine phosphorylation which is an important downstream event for the cAMP/PKA mediated signaling pathways

that is vital for sperm capacitation. Thus overall it was concluded that the defect in signaling pathway in KO mice led to impaired capacitation and fertilization [27]. Our present data showing PC4-inhibitory activity of CRES particularly its dimer is not in conflict with the above KO-results. We hypothesize that in the complete absence of CRES in KO mice, PC4 activity remained high in early epididymal compartment leading to premature activation of sperm proteins including the pro-ADAMs. This may partly explain the loss of fertility in CRES KO mice.

# CONCLUSION

The epididymal serpin CRES in dimeric state is a modest inhibitor of PC4 enzyme in contrast to the monomer which is a poor inhibitor when tested in vitro against the fluorogenic substrate Boc-RVRR-MCA. However both mono and dimeric CRES blocked PC4 mediated pro-IGF-2 processing into its mature form in a dose dependent manner in human tropoblast placenta cell line. Interestingly while the dimer blocked PC4 mediated cleavages of both proIGF-2 and intermediate IGF-2 into mature IGF-2, the monomer inhibited only the cleavage of intermediate IGF-2 into mature IGF-2. It is likely that inhibition of PC4 by CRES may ultimately define sperm's ability to interact with and fertilize an egg. This study is likely to reveal some of the unexplained infertility in male patients and may lead to new mechanism of PC4 activity regulation in vivo.

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#### CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

### SUPPLEMENTARY MATERIAL

Supplementary material is available on the Publisher's web site along with the published article.

## ABBREVIATIONS

aa	= Amino acid
ACN	= Acetontrile

ADAMs	=	A Disintegrin And Metalloproteinase	
BTMD	=	Before transmembrane domain	
CRES	=	Cystatin related epididymal spermatogenic	
ER	=	Endoplasmic reticulum	
h	=	Human	
IGF	=	Insulin-like Growth Factor	
m	=	Mouse	
MALDI-TOF	=	Matrix-Assisted Laser Desorption time of flight	
MES	=	2 (N-morpholino) ethane sulphonic acid	
MS	=	Mass spectrum	
MW	=	Molecular weight	
pAb	=	Polyclonal antibody	
PC	=	Proprotein Convertase	
PCSK	=	Proprotein Convertase Subtilisin Kexin	
PACAP	=	Pituitary Adenylate Cyclase-Activating Polypeptide	
r	=	Rat	
RFU	=	Relative Fluorescence Unit	
RSL	=	Reactive site loop	
Serpin	=	Serine proteinase inhibitor	
TGN	=	Trans-golgi network	
TRIS	=	Tris hydroxyl methyl amino methane hydrochloride	
SELDI-TOE	=	Surface Enhanced Laser Desorption	

- SELDI-TOF = Surface Enhanced Laser Desorption Ionization time-of-flight
- WT = Wild-type

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