

# EXAMINATION OF THE REGULATORY ROLE AND N-GLYCOSYLATION OF CHYMOTRYPSIN C

PhD theses

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## INTRODUCTION

Chymotrypsin C (CTRC) is a serine protease secreted by acinar cells of the pancreas. Beyond its digestive function, human CTRC also facilitates activation and degradation of human cationic trypsinogen. The trypsin-degrading activity of the CTRC probably has an important protective function against intrapancreatic trypsin activation which can easily lead to pancreatitis. This hypothesis has been recently supported by genetic evidence demonstrating that loss-of-function variants of CTRC increase the risk of chronic pancreatitis. The mutations cause decreased enzyme activity, misfolding and diminished secretion of the protein. In addition, misfolding of CTRC mutants can cause endoplasmic reticulum (ER) stress, which may contribute to acinar cell damage through induction of apoptosis.

Prompted by these observations, we initiated a systematic investigation into novel regulatory roles of CTRC in digestive enzyme activation and degradation. The aim of the present study was to explore the effect of CTRC on procarboxypeptidase activation. We also examined the glycosylation state of human CTRC and characterized the role of N-glycosylation in CTRC function.

Pancreatic carboxypeptidases are metalloproteases with exopeptidase activity responsible for removal of C-terminal residues from their dietary protein and peptide substrates. Human digestive carboxypeptidases CPA1, CPA2 and CPB1 are secreted by the pancreas as inactive proenzymes. Procarboxypeptidases contain a 94-96 amino-acid long N-terminal propeptide which acts as a strong inhibitor of the enzymes thereby maintaining their zymogen state. The propeptide consists of an inhibitory globular domain linked

through an  $\alpha$ -helical connecting segment to the enzyme core. Activation of procarboxypeptidases is initiated by tryptic cleavage at the C terminus of the connecting segment. This cleavage destabilizes the  $\alpha_3$  helix and leads to the dissociation of the inhibitory domain. In addition to the critical role of trypsin, chymotrypsin and elastase were also shown in several studies to catalyze procarboxypeptidase activation to varying degrees; however, neither the mechanism nor cleavage sites involved in these alternative activation pathways have been clarified yet.

The majority of secretory proteins undergo N-linked glycosylation during their endoplasmic reticulum transit. In the case of N-linked glycosylation the preassembled core oligosaccharide binds to the Asn residue of the Asn-Xxx-Ser/Thr motif (sequon) of the nascent polipeptide chain. The oligosaccharide promotes protein folding, quality control and intracellular trafficking within the secretory pathway. The glycosyl residue has direct effect on protein folding and it also mediates association with the lectin-type chaperones calnexin and calreticulin. The oligosaccharide can also influence the stability of folded, mature proteins and may modify their specific functions. Although the human and other mammalian CTRC sequences contain one or more potential N-linked glycosylation sites, it is yet unknown whether these sites are indeed glycosylated or not.

## AIMS

The aims of the theses were as follows:

### **1. Investigating the role of CTRC in procarboxypeptidase activation**

1.1. Determining the effect of CTRC on proCPA1, proCPA2 and proCPB1 activation

1.2. Determining the effect of CTRC on the activity of trypsin-activated CPA1 and CPA2 and CPB1.

1.3. Exploring the mechanism of procarboxypeptidase activation and determining the cleavage sites of the propeptide digested by trypsin and CTRC.

### **2. Exploring the N-glycosylation of CTRC**

2.1. Examining the glycosylation state of human CTRC: determining whether the three potential N-glycosylation sites are occupied by glycosyl residues or not.

2.2. Determining the role of glycosyl residue in CTRC folding and secretion.

2.3. Determining the role of glycosyl residue in CTRC activity and inhibitor binding.

2.4. Comparative study of N-glycosylation of CTRC from another species.

2.5. Determining whether the position of the N-linked glycan has an effect on the optimal secretion and activity of CTRC.

## **METHODS**

### **Plasmid construction and transfection**

The human embryonic kidney cells (HEK 293T) were transfected with eukaryotic expression vectors. The AR42J rat pancreatic acinar cell line was transduced via adenoviral infection. Sequence variants were generated by site-directed PCR mutagenesis.

### **Expression and purification of proteins**

Proenzymes were expressed in transiently transfected HEK 293T cells. The chymotrypsinogen C was purified from conditioned media with ecotin affinity chromatography. The conditioned media containing procarboxypeptidase was dialyzed against Tris-HCl (pH 8,0) and it was purified by MonoQ anion-exchange chromatography.

### **Assaying activation process of carboxypeptidase**

CPA1, CPA2 and CPB1 were activated with trypsin and CTRC. Carboxypeptidase activity was determined by photometry using a chromogenic substrate. The activation process of proCPA was also followed on SDS-PAGE. The cleavage sites of trypsin and CTRC were determined by A. Carpentieri and C. E. Costello using mass spectrometry at the Boston University Medical Center.

### **Assay of CTRC secretion**

HEK 293T and AR42J cells were transiently transfected with constructs carrying wild type and mutant CTRC cDNA. The conditioned media were harvested after 48 hour incubation. The secreted CTRC protein amount was determined by SDS-PAGE and by CTRC activity measurements.

### **Assaying transcription of ER stress genes**

AR42J cells transfected with CTRC variants were harvested 24 hours after transfection. Total RNA from cell extracts was prepared and reverse transcribed into cDNA. XBP1 mRNA levels were assayed with semi-quantitative PCR. The mRNA levels of BiP, calnexin and calreticulin chaperons were determined with real-time PCR.

### **Deglycosylation of CTRC**

The conditioned media of HEK 293T cells transfected with CTRC variants were incubated with PNGaseF and EndoH glycosidase enzymes. Samples were analyzed by SDS-PAGE and Coomassie blue staining. Lack of glycosyl residue causes decrease of molecular mass which is easily detectable on SDS-PAGE.

### **Assaying of CTRC enzyme functions**

For enzyme kinetic analysis purified and activated CTRC was reacted with increasing substrate concentrations. The time courses of chromogenic substrate cleavage were followed by photometry. The activity data (mOD/min) were plotted as a function of substrate concentration. After hyperbolic fit, kinetic parameters ( $V_{\max}$ ,  $K_M$ ) and  $k_{\text{cat}}/K_M$  specificity constant were determined.

Catalytic activity of CTRC was also determined using  $\beta$ -casein and human cationic trypsinogen which are physiological substrates of CTRC. Digestion of substrates were followed on SDS-PAGE.

The  $K_i$  value of *Schistocerca gregaria* proteinase inhibitor (SGCI) and CTRC was determined according to the method of the Laskowski laboratory.

## RESULTS

**1.1.** Activation of proCPA1 and proCPA2 with human cationic trypsin was visualized by SDS-PAGE. Our results revealed that trypsin cleaves the propeptide of both procarboxypeptidase isoforms. The cleaved propeptide was stable during the reaction. Mass spectrometry of the propeptides showed that trypsin cleaves in a sequential manner after the arginin amino acids near to the C-terminal end of the propeptide.

**1.2.** According to our results CTRC alone has no effect on carboxypeptidase activity. However CTRC induces a nearly 10-fold increase in the activity of trypsin-activated CPA1 and CPA2, whereas CPB1 activity was unaffected. Other human pancreatic proteases such as chymotrypsin B1, chymotrypsin B2, chymotrypsin-like enzyme-1, elastase 2A, elastase 3A or elastase 3B are inactive or markedly less effective at promoting procarboxypeptidase activation.

**1.3.** Examining the trypsin and CTRC mediated activation of proCPA1/proCPA2 on SDS-PAGE revealed that addition of CTRC induces rapid degradation of the propeptide. Mass spectrometry analysis showed that CTRC first cleaved the propeptide within the C-terminal third of helix  $\alpha_3$ , followed by cleavages at the N-terminal end of the helix. Finally CTRC, trypsin and the active CPA digest the globular domain of the propeptide to small peptides.

**2.1.** We found that human CTRC contains a single N-linked glycan on Asn52, because mutation of Asn52 (N52S) decreased the molecular weight of the protein. The observed gel shift of N52S CTRC is probably caused by the lack of glycosyl residue, because after deglycosylation with PNGaseF and EndoH glycosydase enzymes,

wild type and N52S mutant CTRC exhibited identical mobility on the SDS-PAGE.

**2.2.** Mutation of Asn52 (N52S) significantly reduced the CTRC secretion from HEK 293T and AR42J cells. We found that overexpression of the N52S mutant elicited ER stress in AR42J acinar cells as BiP and calreticulin chaperones and the spliced form of XBP1 mRNA were significantly upregulated in cells expressing the N52S mutant, relative to cells transfected with wild type CTRC.

**2.3.** Catalytic activities of glycosylated wild-type CTRC and the N52S mutant were essentially identical when measured on the small peptide substrate or by digestion of  $\beta$ -casein and human cationic trypsinogen. Binding of the chymotrypsin inhibitor SGCI to wild-type and N52S mutant CTRC was also comparable.

**2.4.** Despite its critical role, Asn52 is not conserved among the otherwise highly homologous mammalian CTRC sequences. We found that rat CTRC is N-glycosylated on Asn90 and this modification is required for the efficient secretion of the rat proenzyme but it has no impact on enzyme functions. In contrast to human and rat CTRC, we found that bovine CTRC is not glycosylated.

**2.5.** To examine the functional importance of the position of glycosyl residue, we introduced the rat Asn90 glycosylation site into a non-glycosylated N52S human CTRC mutant. The newly created site was fully glycosylated and it partially restored the secretion of the N52S mutant CTRC to about 40% of wild-type levels.

## CONCLUSIONS

In this work we demonstrated that CTRC is a physiological co-activator of proCPA1 and proCPA2. CTRC alone has no effect on carboxypeptidase activity however it induces a nearly 10-fold increase in the activity of trypsin-activated CPA1 and CPA2. In contrast, activation of proCPB1 does not require CTRC and most likely depends on trypsin only.

According to our study, the human type A procarboxypeptidases are activated by the sequential action of trypsin and CTRC. Activation of proCPA is initiated by proteolytic cleavage at the C-terminal end of the propeptide by trypsin. This propeptide is still inhibitory; however, binding is already compromised as evidenced by the appearance of about 10% carboxypeptidase activity. The tryptic cleavage destabilizes helix  $\alpha_3$  and allows the further cleavage by CTRC. CTRC first cleaves within the C-terminal third of helix  $\alpha_3$ , followed by cleavages at the N-terminal end of the helix. These cleavages destabilize the helix  $\alpha_3$  which leads to dissociation of the globular inhibitory domain. The dissociated globular domain is then cleaved by CTRC, trypsin and the active CPA. After degradation of the propeptide, CPA1/CPA2 are relieved of inhibition, resulting in full carboxypeptidase activity.

Finally, our results confirm and extend the notion that CTRC is a unique digestive protease that, beyond its digestive function, also plays an important role in regulating the activation and degradation of other digestive enzymes. This unique regulatory function of CTRC is probably due to its specificity as other human pancreatic

chymotrypsins and elastases are ineffective at cleaving the CTRC-specific regulatory sites.

The primary amino acid sequence of human CTRC contains three potential N-glycosylation sites. We demonstrated that human CTRC undergoes N-linked glycosylation on a single site, on amino-acid residue Asn52.

Furthermore, we found that the N-linked glycan is required for efficient secretion of CTRC. Overexpression of the unglycosylated N52S mutant CTRC elicited endoplasmic reticulum stress which is probably due to misfolding of the protein. Accordingly, N-glycosylation is required for proper folding of human CTRC.

Although in human CTRC Asn52 is located in a surface loop that participates in substrate binding, the N-glycan has no effect on enzyme activity, substrate specificity or inhibitor binding.

Our comparative studies revealed that occurrence and position of glycosyl residue vary among the otherwise highly homologous mammalian CTRC sequences.

Furthermore, we demonstrated that the position of N-linked glycan is critical for optimal folding and secretion of human CTRC.

## PUBLICATIONS

### **Publications included in the thesis:**

**Bence M**, Sahin-Toth M. Asparagine-linked glycosylation of human chymotrypsin C (CTRC) is required for folding and secretion but not for enzyme activity. *FEBS J.* 2011; 278:4338-4350. (IF: 3,129)

Szmola R\*, **Bence M\***, Carpentieri A, Szabo A, Costello CE, Samuelson J, Sahin-Toth M. Chymotrypsin C is a co-activator of human pancreatic procarboxypeptidases A1 and A2. *J. Biol. Chem.* 2011; 286:1819-27. (IF: 5,328) \* contributed equally

### **Further publications**

**Bence M**, Koller J, Sasvari-Szekely M, Keszler G. Modulation of monoaminergic neurotransmission by inhibition of histone deacetylases. *J Neural Transm.* 2012;119:17-24. (IF: 2,597)

**Bence M**, Kereszturi E, Mozes V, Sasvari-Szekely M, Keszler G. Hypoxia-induced transcription of dopamine D3 and D4 receptors in human neuroblastoma and astrocytoma cells. *BMC Neurosci.* 2009;10:92. (IF: 2,744)

Mózes V, **Bence M**, Sasvári-Székely M, Keszler G. Dopamine D4 receptor hypoxia sensitivity and child psychiatric disorders. *Neuropsychopharmacol Hung.* 2010 Mar 12(1):289-93. (IF:0,0)

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