Enzymatic characterization of a trypsin-like serine protease encoded by the genome of *Cell fusing agent virus*

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Abstract *Cell fusing agent virus* (CFAV) is a positive strand RNA insect virus first isolated from a mosquito cell line. Based on viral morphology, phenotypic and phylogenetic studies, CFAV had been tentatively assigned to the genus *Flavivirus* (family *Flaviviridae*). The determination of the CFAV polyprotein complete sequence showed a putative serine protease domain analogue to the flaviviral NS2B/NS3 complex. This complex had been extensively studied, because it represented one of the main targets for anti-flavivirus therapy development. We report herein the biochemical characterization of CFAV Δ NS2B-NS3pro protease complex. CFAV polyprotein sequence was computationally analysed to identify the amino-acid regions involved in protease activity. We designed, expressed and purified a catalytically active protease whose enzymatic properties were determined using fluorogenic substrates. Our results showed that, despite the low level of conservation of its amino-acid sequence, CFAV protease exhibited physico-chemical properties of other flaviviruses (high pH value requirement for optimal activity, inhibition by salt and preference for substrates featuring a basic residue at P₁ position).

Keywords *Cell fusing agent virus* · Flavivirin · *Flavivirus* · NS2B · NS3 · Trypsin-like serine protease

Introduction

Cell fusing agent virus (CFAV), an insect virus, was isolated from an *Aedes aegypti* mosquito cell line 30 years ago [1]. CFAV-infected *Aedes albopictus* cell lines formed a massive syncytium while vertebrate cells could not be infected [1]. This virus was also isolated from a natural mosquito pool in Puerto Rico [2]. Virions are enveloped, spherical of approximately 50 nm in diameter and contain a single, positive-sense single-stranded RNA genome. These characteristics suggested that CFAV was related to the *Flavivirus* genus (family *Flaviviridae*), in spite of the absence of any serological cross-reactivity with the other flaviviruses [3]. Complete genomic sequencing confirmed this hypothesis by revealing a single open reading frame that encodes a polyprotein containing the flaviviral enzymatic domains [4].

Phylogenetic studies showed that CFAV is distantly related to the other flaviviruses [5–7] and is part of a distinct cluster inside the genus [8]. The distant relationship suggests CFAV evolved separately from the flavivirus common ancestor.

Cammissa-Parks et al. have reported the identification of a putative protease motif contained in the N-terminal portion of CFAV NS3 protein [4]. Their sequence analysis suggested a CFAV protease cleavage specificity slightly different from the other flavivirus proteases [4]. The study of CFAV phenotypic characteristics and biochemical properties of its enzymes is of

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particular interest because of CFAV original position in the *Flavivirus* genus.

In previous works [9–11], we have focused on the flavivirus-encoded protease. This protease consists of two viral proteins: NS3, which contains the conserved catalytic triad of serine proteases [12, 13], and NS2B, a membrane-bound protein required as a co-factor for enzymatic activity [14, 15]. This binary complex is involved in catalysing several cleavages inside the viral polyprotein [16–22]. The essential role of the viral protease in flavivirus replication makes the protease an attractive target for antiviral drug development [23].

We report herein the biochemical characterization of CFAV protease. The CFAV polyprotein sequence was computationally analysed to identify the amino-acid regions involved in protease activity. Based on our results, we designed, expressed and purified a catalytically active protease whose enzymatic properties were determined using fluorogenic substrates.

Material and methods

Amino-acid sequences used in this work were available on GenBank, except those of Bussuquara virus (BSQV), St.-Louis encephalitis virus (SLEV) and Zika virus (ZIKV), provided by Gilda Grard (Laboratoire de virologie moléculaire, tropicale et transfusionnelle, Faculté de médecine de la Timone, Marseille, France) and *Yellow fever virus* (YFV), determined in our laboratory.

Identification of NS2B and NS3pro encoding sequences was performed by alignment of the amino-acid sequences using Clustal_X 1.81 software [24]. Hydrophobicity profile of NS2B was generated using Vector NTI Suite v.7 (InforMax Inc., Bethesda, United States). Transmembrane helix was predicted using different web servers (Table 1).

Table 1 Web servers used to predict transmembrane helices in CFAV NS2B

Name	Weblink
HMMTop	http://www.enzim.hu/hmmtop/index.html
PRED-TMR	http://www.athina.biol.uoa.gr/PRED-TMR/
SOSUI	http://www.sosui.proteome.bio.tuat.ac.jp/sosui_submit.html
TMAP	http://www.bioweb.pasteur.fr/seqanal/interfaces/tmap.html
TMHMM	http://www.cbs.dtu.dk/services/TMHMM/
TMpred	http://www.ch.embnet.org/software/TMPRED_form.html
waveTM	http://www.athina.biol.uoa.gr/bioinformatics/waveTM/input.html

The CFAV cDNA encompassing NS2B-NS3 region was provided by Xavier de Lamballerie (Laboratoire de virologie moléculaire, tropicale et transfusionnelle). The vectors encoding CFAV Δ NS2B-NS3pro and Δ NS2B-NS3proHDA were obtained following the methods previously described [9] (primer sequences are available on request). Recombinant proteins were expressed and purified and enzymatic assays were carried out as previously reported [9]. Five substrates were commercially available (Orpegen Pharma, Heidelberg, Germany) and six were synthesized by JPT Peptide Technologies GmbH (Berlin, Germany). All but one substrates had a purity higher than 95 %. Experimental initial velocities (V_i) were determined using SoftMax Pro software (Molecular Devices, Saint-Grégoire, France).

Results and discussion

In our alignment, the CFAV NS2B D¹³⁰³ residue was found aligned with the N-terminal residue of NS2B protein of the other flaviviruses, whereas CFAV NS2B R¹⁴⁵¹ was aligned with C-terminal residue. The N-terminal limit of CFAV NS2B protein was found different from Cammisa-Parks et al. proposition (D¹³⁰³ instead of H¹³²⁸). Based on the same alignment, CFAV NS3 protein sequence was identified from S¹⁴⁵² through R²⁰³⁸.

The hydrophobicity profile of CFAV NS2B protein showed a central ~50 amino-acid residue-long hydrophilic domain surrounded by hydrophobic regions (Fig. 1A). Similar hydrophobicity plots have been observed in other flaviviruses [9, 25, 26].

In silico analysis predicted three different models in which CFAV NS2B was a membrane-bound protein (Fig. 1B). The TMAP software predicted 2 membrane helix, HMMTop, TM-PRED and waveTM predicted 3 helix while PRED-TMR, SOSUI and TMHMM predicted a 4-helix model. Depending on the used software, the residues Y⁴⁴, M⁴⁵, Q⁴⁷ or V⁵⁰ were predicted to be the N-terminal limit of the soluble domain. In contrast, the C-terminal limit was unclear, regarding the divergent results obtained (near residue 85 using PRED-TMR, SOSUI and TMHMM, near residue 100 with HMMTop, TMAP, TMPred and waveTM). These variable results can be explained by the different methods used to predict protein structure in the different software [27, 28]. The CFAV V⁵⁰–S⁹⁶ region was found aligned with the Δ NS2B sequences designed in our previous works [9, 10] (data not shown), and was cloned as CFAV Δ NS2B in this work.

The CFAV NS3 protein sequence analysis showed the four boxes conserved amongst the serine protease

Fig. 1 Hydrophobicity profile (A) and schematic representation of the organisation of CFAV NS2B protein predicted using different web servers (a: HMMTop, b: PRED-TMR, c: SOSUI, d: TMAP, e: TMHMM, f: TMPred, g: waveTM) (B). The solid boxes indicate the predicted transmembrane domains of NS2B. The lower scheme shows the hydrophilic domain cloned as Δ NS2B

family [10, 13, 29] and particularly the H, D and S residues that form the catalytic triad of serine proteases (Fig. 2).

The third box was found to contain a D¹³² residue located six positions upstream the catalytic triad-involved S residue, which could be the D¹⁸⁹ trypsin analogue. This pattern, observed in all NS3 flavivirus [10, 12, 13, 30] except in *Tamania bat virus* [31], was shown to be responsible for the trypsin-like proteases specificity towards basic substrates [32, 33]. The involvement of this D residue in substrate binding has been experimentally demonstrated for the WNV protease [34]. These results suggested that the CFAV protease targeted natural cleavage sites displaying a basic residue at P₁ position (according to Schechter and Berger's nomenclature [35]).

The sequence alignment showed several conserved residues amongst the flavivirus, except in CFAV and in KRV, two viruses phylogenetically close together [36]. Some of these positions (see exclamation marks in Fig. 2) are particularly outstanding such as F¹⁵¹ and F¹⁵⁴ in the fourth homology box, known to contain substrate binding residues [36]. All flavivirus proteases boxes contained a GLYGNG conserved sequence.

However, the CFAV NS3 protein displayed an unusual GFYGF_G sequence, which is also observed in the KRV NS3 protein. Moreover, CFAV and KRV proteases featured a GSSGSP in the serine-protease conserved motif GxSGxP (box 3) while the other flavivirus proteases exhibited a GTSGSP [12]. Surprisingly, this GSSGSP pattern was also found in the BSQV sequence, a virus phylogenetically far away from CFAV and KRV. These changes likely imply differences in the CFAV protease enzymatic properties.

The CFAV and KRV protein sequences showed a G¹⁶⁸ instead of the conserved Q residue found to be the last residue conserved in the flavivirus minimal domain [37]. The CFAV K¹⁷⁰ residue, located two positions downstream, was the C-terminal limit for cloning, according to the previously reported strategy [9, 10].

The CFAV protease complex was expressed as a single protein according to the expression strategy previously reported [9, 10, 38–43]. The NS3pro sequence was fused with Δ NS2B via the C-terminal residues of NS2B. To avoid a NS2B-NS3 the *cis*-cleavage of the NS2B-NS3 junction, the last NS2B residue was substituted by an A. A similar non-cleavable construct

grey. The catalytic triad residues are marked with hashes (#). Asterisks (*) indicate the residues conserved in all the aligned sequences; exclamation marks (!) indicate the residues conserved in all the aligned sequences except in CFAV and KRV ones. Residues of human trypsin (GenBank accession no. NP_002760) that belong to the similarity boxes are also displayed

methylcoumarine-labelled tripeptides. This assay had previously been used to study the activity of flavivirus proteases in vitro [10, 40, 46, 47].

No activity was detected using the catalytically inactive Δ NS2B-NS3proHDA; this result demonstrate the absence of contaminating bacterial serine protease in the final protein preparation.

The pH, ionic strength and temperature dependence of CFAV Δ NS2B-NS3pro activity was studied using *t*-butyloxycarbonyl-glycyl-*L*-lysyl-*L*-arginine 4-methylcoumaryl-7-amide (GKR-MCA) 100 μ M. A pH optimum was found at 10.5 (Fig. 4A): the enzyme was about 1.5-fold more active in cleaving GKR-MCA at this pH than at a pH of 10.0 or 11.0. This result was consistent with the high pH required for maximal *in vitro* Δ NS2B-NS3pro-synthetic substrates processing [9, 10, 38, 42, 48]. However, the optimal value observed for CFAV protease (10.5) is higher than for other flaviviral protease previously reported (8.5–9.5, depending on the virus). The CFAV protease activity was inhibited by NaCl (Fig. 4B): its activity dropped down approximately twofold at NaCl 250 mM compared to NaCl 10 mM. This result, already reported for other flavivirus proteases [9, 10, 38, 42, 44], can be explained by the importance of the electrostatic interactions in serine protease substrate binding. Over the examined range, temperature optimum was determined to be 28°C (Fig. 4C). This optimum could be related with the ability of this virus to infect mosquito cells, whose optimal temperature for *in vitro* culture is also 28°C.

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Fig. 3 Analysis by SDS-PAGE and Western blotting of ΔNS2B-NS3pro (A) and ΔNS2B-NS3proHDA (B) recombinant proteins after immobilized-metal affinity chromatography

However, our previous work showed that temperature optima determined for the other flaviviral proteases did not correlate with neither viral phenotypic properties nor the conditions used to propagate the viruses [10].

Among the tested substrates, the higher protease activity was observed using GKR-MCA (Fig. 4D). The use of different substrates featuring basic residues at P_2 and P_1 positions showed a modulation of protease activity. The protease activity was greatest when the P_2 position was taken up by a lysine residue (compare GKR/GRR and LKR/LRR). The P_3 and P_1 residues were also found to influence the protease activity (compare GKR/LKR, GRR/LRR, GRR/QRR and GRR/GRK). No activity was observed using AGA-MCA and AGG-MCA, two substrates that did not contain any basic residue.

Cammisa-Parks et al. predicted CFAV polypeptide cleavage sites. Some of the suggested sites, such as KA, NR or RA sequences at P_2 - P_1 positions were unusual. Based on our own sequence analysis, we propose that the NS4B-NS5 cleavage occurs between R and A residues of the predicted site (FR-A, positions P_2 - P_1 - P_1'). In an attempt to test the ability of CFAV ΔNS2B-NS3pro to cleave substrates featuring FR and NR sequence at P_2 - P_1 positions, the substrates GFR-MCA and GNR-MCA were synthesized. Our result using GFR-MCA provided evidence that CFAV protease was able to cleave this substrate, in spite of the lack of P_2 basic residue. Unfortunately, GNR-MCA purity was too low (<70%) to allow its use in our study.

Fig. 4 Enzymatic characterization of CFAV ΔNS2B-NS3pro recombinant protein. (A) pH profile; for each data point, relative activity was defined as the ratio $(100 \times V_i)/(V_i)_{pH 10.5}$. (B) Effect of ionic strength, observed in buffers containing CAPS 100 mM pH 10.5 and varying concentration of NaCl; for each data point, relative activity was defined as the ratio $(100 \times V_i)/(V_i)_{10 \text{ mM}}$. (C)

Temperature profile, performed in CAPS 100 mM pH 10.5, NaCl 10 mM; for each data point, relative activity was defined as the ratio $(100 \times V_i)/(V_i)_{28^\circ\text{C}}$. (D) Substrate preference assay, carried out in CAPS 100 mM pH 10.5, NaCl 10 mM at 28°C. Substrate concentration was 100 μM; for each data point, relative activity was defined as the ratio $(100 \times V_i)/(V_i)_{\text{GKR}}$. Error bars indicate $\pm$ SD

Taken together, these results demonstrate the requirement of a basic residue at P₁ position. As discussed above, this could be explained by the role of D¹³² in substrate binding. The absence of protease activity towards GQK-MCA suggests that the presence of a basic residue at P₁ position is necessary but not sufficient for CFAV protease activity. The involvement of the P₃ and P₂ sites in substrate specificity was previously established for DENV proteases [41, 43].

In this work, we confirmed that the CFAV genome encodes a trypsin-like serine protease that shares many features with other flavivirus proteases despite a low level of amino-acid sequence conservation. Our results suggest that the CFAV ΔNS2B-NS3pro protease functions optimally in an elevated pH environment, is inhibited by high salt concentrations and demonstrates a preference for substrates that feature basic residues at the P₁ position. These features are common to many other flavivirus proteases.

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