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Characterization of the cysteine protease domain of Semliki Forest virus replicase protein nsP2 by in vitro mutagenesis

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Abstract The function of Semliki Forest Virus nsP2 protease was investigated by site-directed mutagenesis. Mutations were introduced in its protease domain, Pro39, and the mutated proteins were expressed in *Escherichia coli*, purified and their activity in vitro was compared to that of the wild type Pro39. Mutations M781T, A662T and G577R, found in temperature-sensitive virus strains, rendered the enzyme temperature-sensitive in vitro as well. Five conserved residues were required for the proteolytic activity of Pro39. Changes affecting Cys^{478}, His^{548}, and Trp^{549} resulted in complete inactivation of the enzyme, whereas the replacements N600D and N605D significantly impaired its activity. The importance of Trp^{549} for the proteolytic cleavage specificity is discussed and a new structural motif involved in substrate recognition by cysteine proteases is proposed.

Keywords: Alphavirus; Semliki Forest virus; nsP2 protease; Temperature-sensitive mutants; Glycine specificity motif; Cysteine protease

1. Introduction

Semliki Forest virus (SFV) is an enveloped positive-strand RNA virus belonging to the Alphavirus genus of the Togaviridae family. The structure and replication of alphaviruses have been studied in detail (reviewed in [1,2]). The virus has been used as an important tool in studies of protein folding [3,4], intracellular membrane transport and endocytosis [5,6] and viral pathogenesis [7]. The SFV based replicons have been used as expression vectors for the production of recombinant proteins in eukaryotic cells [8]. Attempts to use them for the production of vaccines and in gene and cancer therapies have also been reported [9].

Upon infection of the host cell, the 5′ two-thirds of the SFV 42S RNA genome is translated into a 2432 amino acid-long polyprotein, designated P1234, which is autocatalytically processed to yield non-structural proteins nsP1–nsP4. All these function as virus-specific components of the membrane-associated RNA polymerase [2,10]. The processing intermediates P123 plus nsP4 are needed for the synthesis of complementary 42S RNA early in infection [11,12], while the complete cleavage products are responsible for the synthesis of positive sense 42S RNA genomes and the subgenomic 26S mRNA [2,11,12].

The protease activity responsible for the non-structural polyprotein cleavage resides in the C-terminal domain of nsP2 protein [13–15]. It belongs to the papain-like peptidase type (C9 family of CA clan in the MEROPS database [16]). The papain-related proteases have little sequence similarity but they share some biochemical and structural properties [17]. We have recently isolated the protease domain of SFV nsP2 (Pro39) and tested its activity using model substrates containing short sequences from each of the P1234 polyprotein processing sites fused to thioredoxin protein (Trx12, Trx23 and Trx34) [15].

Several temperature sensitive (*ts*) mutants of SFV have previously been characterized in our laboratory [18,19]. Temperature-shift experiments performed with these mutants have provided invaluable information on the biology of SFV, particularly on RNA synthesis and the function of the individual non-structural proteins [20–23]. Sequence analysis of the cDNA derived from the genome of those *ts* mutants has shown that *ts4*, *ts6*, and *ts11* mutations are the result of single aminoacid changes in the protease domain of nsP2, M781T, A662T and G577R, respectively [23,24]. Despite these substitutions being located in poorly conserved regions of the nsP2 protease, they cause functional defects in virus propagation. The *ts4* mutation has been shown to result in the halt of viral RNA synthesis and processing of the non-structural polyprotein at the restrictive temperature of 39 °C [23,25,26]. The *ts6* and *ts11* mutants also displayed an RNA negative phenotype, failing to synthesize any viral RNA at 39 °C [18].

To obtain an insight on the mechanisms responsible for the virus *ts* phenotype as well as the role played by other residues of the SFV protease in the proteolytic reaction, we produced Pro39 variants with substitutions at key positions and analyzed their activity with Trx34 model substrate.

2. Materials and methods

2.1. Pro39 expression plasmids

The plasmid construct for the expression of *wt* Pro39 (residues 459–799 of SFV nsP2), tagged with the peptide LEHHHHHH at its C-terminus, has been described previously [15]. This plasmid was used as a template to obtain the various mutated protein forms. The point mutations were introduced using the Quick Change XL Site-Directed Mutagenesis kit (Stratagene) and verified by DNA sequencing.

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2.2. Expression and purification of Pro39

Wt and mutant Pro39 were expressed in *E. coli* and purified essentially as described previously [15] with some modifications in the isolation procedure. Briefly, the cell lysates in 20 mM sodium phosphate buffer, pH 7.4, 200 mM NaCl, 0.1% Tween 20 (buffer A), supplemented with 0.1 mM EDTA and 1 mM PMSF, were cleared by centrifugation at 15000 × g for 30 min at 4 °C. The supernatant was supplemented with 150 mM imidazole and loaded to a Hi-Trap Chelating HP column (Amersham Biosciences) charged with $NiSO_4$. After extensive washing, Pro39 was eluted with buffer A containing 500 mM imidazole. The resulting protein solution was supplemented with 2 mM EDTA and the buffer was changed to 20 mM HEPES, pH 7.4, containing 200 mM NaCl, 20% glycerol, 0.1% Tween 20, 1 mM DTT, and 1 mM EDTA using a PD10 gel filtration column (Amersham Biosciences). The final preparation was divided into aliquots, frozen and stored at −70 °C. Under these storage conditions, Pro39 retained its activity for at least one year.

2.3. Protease assay

An 18 kDa substrate, comprising 19 (nsP3) and 18 (nsP4) residues of the SFV nsP3–nsP4 junction fused to 115 residues of thioredoxin, was used to assess the activity of the purified proteins [15]. The protease activity of the various Pro39 proteins was assayed in 50 mM HEPES, pH 7.4, containing 50 mM NaCl, 1 mM DTT, 2 mM spermidine and 10% glycerol, at a substrate concentration of 0.8 mg/ml. For time-course experiments, enzyme and substrate were mixed in pre-warmed buffer. At the indicated time points, 5 μl aliquots were withdrawn, mixed with electrophoresis sample buffer and boiled immediately. In experiments aimed at determining and comparing enzymatic activities, Pro39 was pre-incubated in buffer for 2 min at the desired temperature prior to the addition of the substrate. The proteolytic products of the reaction were analyzed by electrophoresis in 17% polyacrylamide gels.

2.4. General analytical methods

SDS–PAGE was carried out according to Laemmli [27]. Gels were stained with Coomassie Brilliant Blue and destained in 10% acetic acid before being dried. For the quantification of the proteolytic activity of the proteins, gels were scanned and analyzed by densitometry using the Tina 2.0 program. Care was taken not to overload the gels and the linearity in the signal detection was checked in gels containing known amounts of Pro39 or Trx34. Protein concentrations were determined with the Bradford assay [28] using bovine serum albumin as the standard.

3. Results

3.1. Expression and purification of mutant Pro39

In order to study the role of individual residues in activity of SFV protease, point mutations were introduced in the protease domain of nsP2 by site-directed mutagenesis to obtain eight Pro39 mutants (M781T (*ts4*), A662T (*ts6*), G577R (*ts11*), C478A, H548A, W549A, N600D, N605D). The proteins were expressed in *E. coli* and purified by metal affinity chromatography (Fig. 1). In all cases, the conditions for expression and purification were reproduced as precisely as possible to facilitate comparison of the properties of the mutant enzymes. The expression of the N600D and N605D mutants had a strong negative effect of the host cell, resulting in a slower growth rate and thus smaller bacterial mass as well as in a reduced expression level of the corresponding soluble Pro39. This, in turn, resulted in preparates with lower degree of purity, as can be seen in Fig. 1. Since the two mutant proteins showed a tendency to aggregate, particularly when submitted to the changes of ionic strength associated to many chromatography techniques, further purification steps were not successful and the shown preparates were used for the experiments reported here.

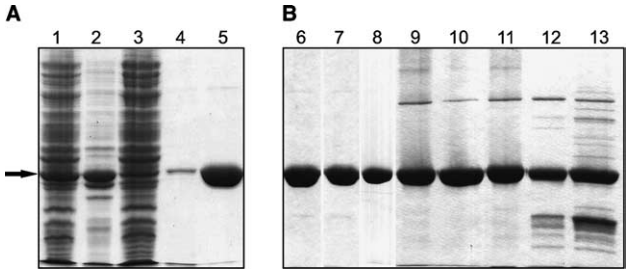

Fig. 1. Purification of Pro39 by immobilized Ni affinity chromatography. Panel A: Purification of *wt* Pro39. Lanes (1) soluble protein fraction after cell lysis and clearing at 15000 × g; (2) insoluble protein fraction; (3) flow through of the chromatographic run; (4) 150 mM imidazole column wash; (5) final Pro39 preparation. Panel B: Samples of final preparations of purified Pro39 point mutants. Lanes (6) *ts4* (M781T); (7) *ts6* (A662T); (8) *ts11* (G577R); (9) C478A; (10) H548A; (11) W549A; (12) N600D; (13) N605D. The numbering of the residues corresponds to their position in SFV nsP2. The gel has been overloaded to give an impression of the purity of the preparations used in this study.

3.2. Effect of temperature on the activity of Pro39

It has been shown previously that Pro39 readily cleaves the site between nsP3 and nsP4 in the synthetic substrate Trx34 [15]. The proteolytic reaction results in the appearance of a large fragment, L, of 14 kDa that can easily be detected and quantified after electrophoresis (Fig. 2). A smaller fragment, S, with a molecular mass of 4.1 kDa often appears as a broad and rather diffuse band at the bottom of the gel.

To determine if the mutations causing the *ts* phenotype in the virus would also render the protease temperature sensitive in vitro, *wt* and *ts* Pro39 were mixed with the Trx34 substrate

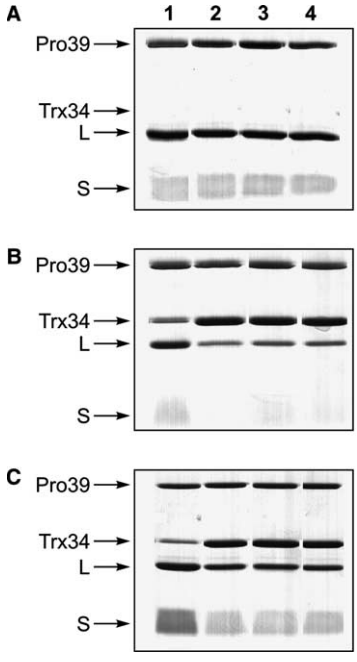

Fig. 2. Effect of temperature on Pro39 activity. The proteolytic activity of the *wt* enzyme (lane 1) and the *ts4* (lane 2), *ts6* (lane 3), and *ts11* (lane 4) mutants was assayed as described in Section 2 at 28 °C (panel A), 39 °C (B) and 28 °C after a 10 min pre-incubation at 39 °C (C). The positions of Pro39, Trx34, and the cleavage products L and S are indicated with arrows.