Volume 328, number 1,2, 115–118 FEBS 12811 August 1993

Site-directed mutagenesis of the tick-borne encephalitis virus NS3 gene reveals the putative serine protease domain of the NS3 protein

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Received 9 June 1993

Several mutations were introduced into the putative serine protease domain of the tick-borne encephalitis virus NS3 protein and into a possible internal cleavage site within the protein. The influence of these mutations on proteolytic activity of NS3 protein and NS3′ protein formation was tested in vitro. It was found that NS3′ formation was not dependent on the activity of the NS3 N-terminal serine protease. Mutations affecting the Ser-138 residue of the NS3 protein prohibited cleavage between NS2B and NS3 proteins when the NS2B-NS3 part of the viral genome was expressed in vitro, suggesting the key role of Ser-138 in viral serine protease functioning.

Flavivirus; Nonstructural protein, Serine protease; Cleavage

1. INTRODUCTION

Tick-borne encephalitis virus (TBEV) belongs to the Flaviviridae, a family of positive-stranded RNA viruses the genomes of which contain a single long open reading frame [1]. Computer analysis of flavivirus polyproteins has revealed homology between trypsin-like serine proteases and the N-terminal 180 a.a. of the flaviviral nonstructural protein NS3 [2,3]. Previously we found in TBEV-infected mammalian cells in addition to the full-length NS3 protein (69 kDa) a short form of the TBEV NS3 protein (49 kDa; called NS3′) and proposed that NS3′ was formed due to autocatalytic internal cleavage of the NS3 protein by its own N-terminal serine protease at a potential internal cleavage site [4]. In this work, to prove the hypothesis that the N-terminus of the TBEV NS3 protein contains a serine protease and to check our proposal on NS3′ formation, we introduced several mutations into the putative serine protease domain of NS3 and the potential internal cleavage site of the protein and tested their influence on the proteolytic activity of NS3 and NS3′ protein formation in vitro.

2. MATERIALS AND METHODS

2.1. *Construction of initial recombinant plasmids*

Standard methods for in vitro DNA manipulations and plasmid DNA cloning were essentially as described [5]

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To introduce a termination codon into the 3′ end of the TBEV NS3 gene coding region, the polylinker *Bam*HI–*Hin*dIII part of the plasmid pGEM2-NS3 [4] was replaced by a synthetic DNA-fragment composed of oligonucleotides. 5′-GATCCTGTAATAAAAA and 5′-AGCTTTTTTATTACAG The resultant plasmid pS-NS3 was selected. In the same manner the termination codon was introduced into pGEM2-NS3* [4], and the plasmid pS-NS3* was selected.

Plasmid pG123, containing the NS1-NS2A-NS2B-NS3 portion of the TBEV genome (Dobrikova, unpublished), was used to obtain the TBEV NS2B-NS3 gene block. The *Nco*I#(4,185)–*Pst*I(5,345) fragment was cut from the pG123 plasmid (the *Nco*I site precedes the beginning of NS2B gene; sign '#' means that a marked 5′-overhanging end was made blunt with the Klenow fragment; numbering of the TBEV nucleotides and amino acid residues is according to [6]). This fragment was ligated with large *Sal*I#–*Pst*I fragment of the pS-NS3* plasmid, and the resulting pS-2B3 plasmid was selected which carries TBEV NS2B-NS3 gene block suitable for in vitro expression.

2.2 *Site-directed mutagenesis on the gapped duplex plasmid DNA*

The procedure used was a modified method designed for oligonucleotide-directed mutagenesis on the M13 phage DNA [7]. The modifications were: (i) pTZ18R plasmid vector was used (instead of M13 phage) to produce single-stranded (ss) target DNA (TBEV NS3 gene was cut from the pS-NS3 plasmid with *Eco*RI and *Hin*dIII and re-cloned into pTZ18R, yielding in pTZ18RNS3 plasmid), (ii) ss-target DNA was prepared using *E. coli* RZ1032 (ung^-, dut^-) cells [8] (helper phage was M13K07) Such ss-DNA is partially 'uracylated' and is quickly destroyed in the (ung^+, dut^+) *E. coli* strains.

Selection of the required mutants (plasmids pGTA, pGIP, pGST, pGAP, pHCTG and pQRSW; see Table I) was made by restriction analysis of plasmid DNAs and sequencing of respective plasmid regions according to [9].

Choice of the primers for mutagenesis (Table I) was made using the program 'DIROM' designed to facilitate the oligonucleotide-directed mutagenesis experiments [10] Primers were selected according to the following requirements: (i) they must introduce one of rare restriction sites into the DNA at the place of mutation, to facilitate mutant clones selection; (ii) mutation should lead to amino acid substitution(s); (iii) primers should be specific to the target DNA, providing high efficiency of annealing and low level of alternative attachment.

2.3. *Introduction of mutations into the NS2B-NS3 gene block*

(i) Into NS3 protease catalytical center of NS2B-NS3 gene block: the *Sac*I(4,910)–*Sal*I(5,117) fragment of the pS-2B3 plasmid insert was replaced by the same fragment but derived from the pGTA, pGIP, pGST, or pGAP. The plasmids pS-2B3/GTA, pS-2B3/GIP, pS-2B3/GST and pS-2B3/GAP were selected.

(ii) Into proposed internal NS3 cleavage site of the block, the *Bgl*I(5,956)–*Bgl*II(6,330) part of pS-2B3 insert was replaced by the ones cut from pHCTG or pQRSW. The plasmids pS-2B3/HCTG and pS-2B3/QRSW were selected

2.4 *In vitro transcription and translation*

These procedures as well as immunoprecipitation of ^{35}S-labeled translation products and their analysis in 7.5–25% SDS-PAGE were essentially as described previously [4].

3. RESULTS AND DISCUSSION

3.1. *NS3 N-terminal serine protease activity is not required for TBEV NS3′ protein formation*

To check our recent proposal [4] that TBEV NS3′ may be generated due to internal autocatalytic cleavage of the full-length NS3 by its putative N-terminal serine protease domain, site-directed mutagenesis of the TBEV NS3 gene was carried out. Mutagenic primers and mutations introduced into the predicted main pocket (136-**GTSGSP** [2,3]) of the NS3 serine protease catalytic center and the potential internal 459-**RRG**-cleavage site of the protein [4] are listed in Table I.

The RNAs transcribed from the pGTA, pGIP, pGST, pGAP, pHCTG, pQRSW and pTZ18R-NS3 plasmids (which are based on pTZ18R) by T7 RNA polymerase were not active in stimulating translation in rabbit reticulocyte lysates. Therefore the mutant variants of the TBEV NS3 gene were excised from pGTA, pGIP, pGST, pGAP, pHCTG and pQRSW plasmids with *Eco*RI and *Hin*dIII and recloned in a pGEM2 vector. The resulting plasmids pS-GTA, pS-GIP, pS-GST, pS-GAP, pS-HCTG and pS-QRSW were digested with *Hin*dIII and transcribed with SP6 RNA-polymerase. These RNAs were successfully translated in rabbit reticulocyte lysate (Fig. 1). Although the reason for such an observation is unknown, it is likely to be accounted for by the diversity of the 5′-ends of the TBEV NS3-mRNAs, generated by means of pTZ18R and pGEM2 vectors, which may influence secondary structure of the mRNAs and initiation of their translation on the eucaryotic ribosomes. For the pTZ18R-NS3 (inactive mRNA is produced with T7 RNA polymerase) and pS-NS3 (active mRNA under SP6 RNA-polymerase transcription), 5′-ends of the mRNAs are: 5′-cap-GG**GAATTC**CCCGATAA... and 5′-cap-GAATAC-ACG**GAATTC**CCCGATAA..., respectively (*Eco*RI-sites are in bold).

Fig. 1 shows that neither mutations in the putative NS3 N-terminal protease catalytic domain nor in the internal RRG-site influence the formation of NS3′ protein (49 kDa). Hence the TBEV NS3′ protein formation is not due to activity of the viral serine protease.

There may be two other possible mechanisms for TBEV NS3′ formation. (i) NS3′ may be formed in a similar manner to that shown for dengue virus polyprotein cleavage at the NS1/NS2A junction [11,12]. This proteolysis occurs autocatalytically, however no protease motifs have been found within the flavivirus NS1 and NS2A amino acid sequences. (ii) NS3′ may also be formed due to co- or post-translational proteolysis of the full-length NS3 by a cellular protease (E. Koonin, personal communication).

Table I

Oligonucleotides (mutagenic primers) used for site-directed mutagenesis of the TBEV NS3 gene and mutations in the NS3 protein induced by them.

Designation	Oligonucleotide structure	Mutation induced	Restriction site induced	Resulting plasmid designation
PI52	GGGACAGCTGGCAGTCC (4,999–5,015)	GTSGSP (1,627(138)) → GTAGSP	*Pvu*II	pS-GTA
P153	AGGGGATCCCTGGCAGTC (4,997–5,014)	GTSGSP → GIPGSP	*Bam*HI	pS-GIP
P154	GGCAAAGGGGTCGACTGGCAG (4,992–5,012)	GTSGSP → GSTGSP	*Sal*I	pS-GST
P155	GACATCTGGAGCTCCCATCC (5,001–5,020)	GTSGSP → GTSGAP	*Sac*I	pS-GAP
P156	GCGGCCCACTGCAGTGGGAGAG (5,962–5,983)	QRRG (1,948(459)) → HCTG	*Pst*I	pS-HCTG
P157	GGCCCAACGCAGCTGGAGAGTC (5,964–5,985)	QRRG → QRSW	*Pvu*II	pS-QRSW

Changed

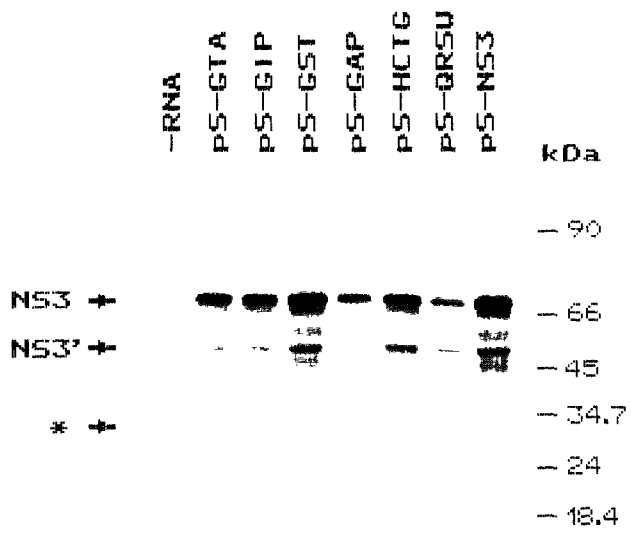


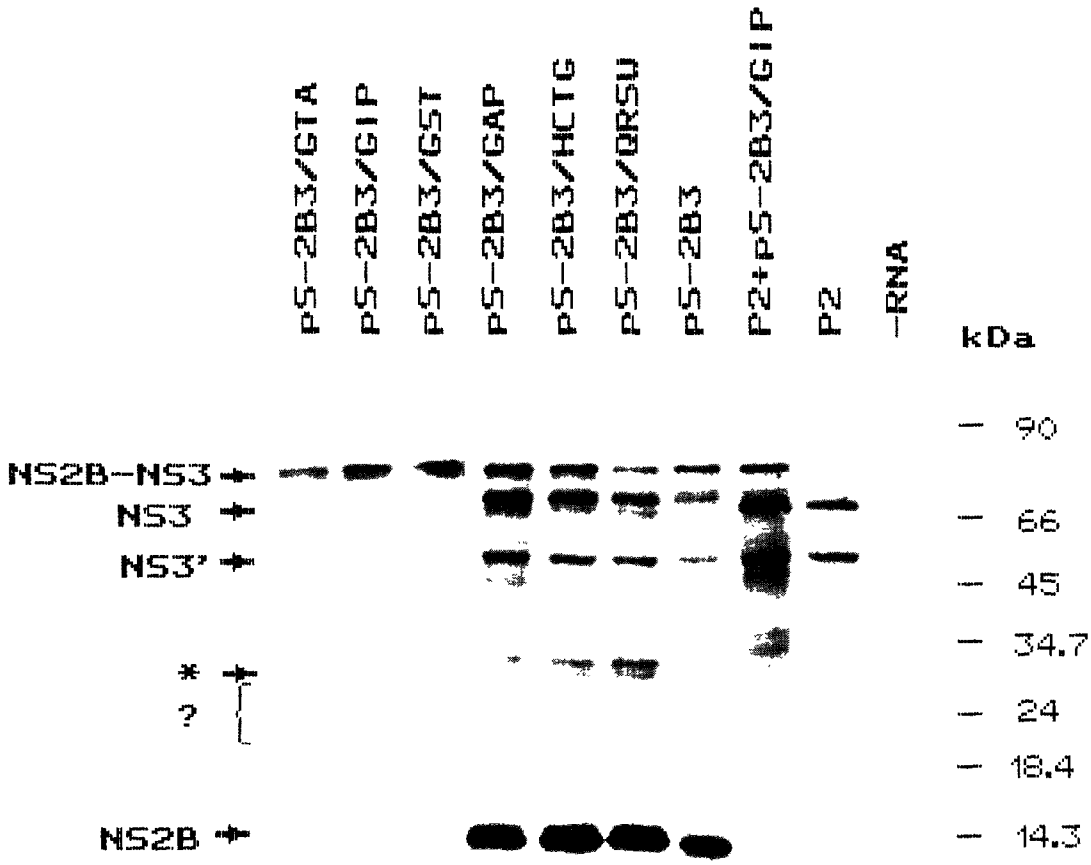
Fig. 1. Expression of the TBEV NS3 gene and its mutant forms in vitro The autoradiogram of an SDS-PAGE displays immunoprecipitated protein patterns from in vitro translation mixtures programmed with mRNAs produced from the plasmid templates indicated above each line. In the '−RNA' lane no mRNA was added The asterisk indicates a background band localization visible on the '−RNA' line too upon much longer exposure. Positions of molecular weight markers are indicated.

3.2. *Influence of the mutations in NS3 gene on the TBEV NS2B-NS3 block processing*

As was shown for dengue and yellow fever viruses, the N-terminal domain of NS3 is necessary for cleavage at the NS2B/NS3 junction as well as at the NS2A/NS2B and NS3/NS4A junctions [13–15]. NS2B is also required for these cleavages [14]. With these viruses, the NS3 serine protease acts only in *cis* when performing the hydrolysis between NS2B/NS3. Thus it was of interest to investigate how the TBEV NS2B-NS3 block would be processed in vitro and what effect on this process would be caused by mutations in the NS3 gene.

↓

Fig. 2. Expression of the intact and mutant TBEV NS2B/NS3 gene block in vitro. Plasmid templates for respective mRNAs that were translated are indicated above each line. The P2 mRNA encoding the C-terminally truncated form of the TBEV NS3 protein (65 kDa) was prepared as described previously [4]. The 'P2+pS-2B3/GIP' line shows the result of simultaneous translation of P2 mRNA and the one produced from the pS-2B3/GIP plasmid In the '−RNA' lane no mRNA was added. The asterisk indicates a background band localization visible on the '−RNA' line too upon much longer exposure. '?' denotes additional bands observed the nature of which is not clear. Positions of molecular weight markers are indicated.



The plasmids pS-2B3/GTA, pS-2B3/GIP, pS-2B3/GST, pS-2B3/GAP, pS-2B3/HCTG and pS-2B3/QRSW (see section 2) as well as the initial pS-2B3 plasmid were linearized with *Hin*dIII and used as templates for in vitro transcription with SP6 RNA-polymerase.

Translation of the original NS2B/NS3 TBEV gene block (plasmid pS-2B3) led to the appearance of labelled products with molecular weights of 69, 49 and 14 kDa (presumably NS3, NS3′ and NS2B). Some amount of unprocessed precursor (83 kDa) was also visible (Fig. 2). The same pattern was observed with pS-2B3/GAP, pS-2B3/HCTG and pS-2B3/QRSW. Only unprocessed 83 kDa product was produced when mRNAs transcribed from the pS-2B3/GTA, pS-2B3/GIP and pS-2B3/GST were translated. Summing up, the following conclusions may be made:

(i) The NS2B-NS3 region of the TBEV polyprotein is processed in vitro and individual NS2B, NS3 and NS3′ are formed. The mutations GTS → GTA, GTS → GIP and GTS → GST affecting the Ser-138 residue of NS3 fully inhibit the NS2B-NS3 block processing. Therefore the N-terminal part of TBEV NS3 represents a domain of the virus serine protease which is responsible for the cleavage at the NS2B/NS3 junction and Ser-138 plays a key role in the enzyme functioning.

(ii) Rearrangement of the NS3 Thr-137 and Ser-138 residues inhibits the virus protease activity suggesting that a correct order of amino acids in the catalytic center is essential for the enzyme to function.

(iii) The point mutation Ser-140 → Ala does not influence activity of the TBEV NS3 protease. Consequently Ser-140 is not required for protease activity. This residue cannot perform a compensatory function in the case of mutations affecting Ser-138 because a relatively gentle substitution Ser-138 → Ala [16] or rearrangement of Thr-137 and Ser-138 block the protease activity completely.

(iv) As was demonstrated when NS3 and NS3 mutants were expressed individually, the formation of NS3′ cannot be attributed to the activity of the NS3 serine protease. However, the mutations in the enzyme catalytic center, preventing the cleavage between NS2B and NS3 (when the NS2B-NS3 gene block is expressed), inhibit NS3′ formation. Therefore in the course of the NS2B-NS3 part of the TBEV polyprotein processing, NS2B must be removed to yield a free NS3 protein N-terminus after which formation of NS3′ can occur. Such an order of processing events observed in vitro may reflect the real course of events in TBEV-infected cells.

(v) The N-terminal serine protease of TBEV NS3 is most probably *cis*-acting when it cleaves the NS2B/NS3 junction because simultaneous translation of the mutant NS2B-NS3-mRNA (pS-2B3/GIP) and mRNA P2 produced as described previously [4] (the translation product may serve as a *trans*-acting protease) did not lead to the appearance of individual NS3 and NS2B (Fig. 2). The P2 mRNA used encodes a product of approximately 65 kDa which is a deletion mutant of NS3 protein truncated from the C-terminus.

Acknowledgements The authors would like to thank V.V. Gorn for the synthesis of oligonucleotides, M. Saparbaev for helpful discussions and Prof T.K Frey (Georgia State University, USA) for critically reading of this manuscript and discussions

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