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ORIGINAL ARTICLE

# Effect of protease and helicase mutations on HCV NS3 activity

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**Abstract** Hepatitis C virus (HCV) causes serious infections in the liver which may lead to liver cirrhosis and hepatocellular carcinoma. Non structural 3 (NS3) protein is one of the most important proteins of the virus which has protease and helicase activities. Protease activity has a crucial role in the replication and persistence of the virus. Site directed mutation was carried out in the protease region of one NS3 and another site directed mutation in the helicase region of another NS3. The expression of both mutated NS3 was compared with wild NS3. Expression of the three different NS3 types was confirmed by *in situ* staining and western blotting using an anti-NS3 antibody and correlated with a reduced antiviral response after treatment with interferon-α. Mutation analysis showed that the NS3 protease activity andnot the NS3 helicase was essential for the inhibition of the interferon-α response.



## 1. Introduction

Hepatitis C virus (HCV) was identified as the causative agent for most post-transfusion and sporadic non-A, non-B hepatitis cases in 1989 (Ramadori and Meier, 2001). According to recent estimates, about 170 million individuals worldwide are infected and 3–4 million people are newly infected each year (Cohen, 1999). It is a serious infection, affecting 1–2% of the population in most developed countries. Around ninety percent of HCV infections become chronic, up to 20% of these develop into liver cirrhosis, and 1–5% of the cases leads to hepatocellular carcinoma (Lanford et al., 2001). Although treatment of this infection using interferon-α and ribavirin has been effective in a minority of cases (about 40%), more than 60% of patients show no response to treatment.

HCV is a single stranded RNA with positive polarity. The HCV genome is approximately 9.6 Kb in length, shorter than that of Flaviviruses and consists of a 5′ NCR, a single open reading frame (ORF) encoding a polyprotein of between 3010 and 3033 amino acids, which contains both the structural proteins and non-structural proteins. The HCV ORF encodes a single polyprotein that is 3008–3037 aa in length and is post-translationally modified to produce at least ten different pro-

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teins: core, envelope proteins E1 and E2, p7, and nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The NS3 protein is approximately 70 kDa in size and has been shown to possess several important features for HCV replication and host cell interactions. This protein has protease activity in the N-terminal one third of the protein which is necessary for the cleavage of certain regions of the HCV polyprotein (Grakoui et al., 1993), helicase and NTPase activities in the C-terminal two thirds (Kanai et al., 1995; Satoh et al., 2000).

The catalytic domain of the NS3 protease has been mapped to the amino-terminal 181 amino acid (amino acids 1027–1207 of the polyprotein), with the minimal protease region being narrowed down to amino acids 1059–1204. The N-terminal amino acid region contains a characteristic serine protease catalytic triad (His-1083; Asp-1107; Ser-1165). The importance of these residues for polyprotein processing was later proven by several groups who showed that substitutions affecting these residues abolished the cleavage at the NS3/4A, NS4A/B, NS4B/5A and NS5A/B junctions but not at the other sites (Tomei et al., 1993). Deletions from the C-terminus of the NS3 181 protease domain completely destroy the enzymatic activity whereas removal of N-terminal sequence leads to a gradual loss of cleavage activity at various sites. While normal processing patterns are observed for NS3 proteinases that lack up to 14 residues at the N-terminus, deletion of 22 amino acid residues drastically reduces the cleavage at the NS4A/B site and blocks processing at the NS4B/5A junction, whereas cleavage at the NS5A/B site is poorly affected (Bartenschlager et al., 1994).

To investigate the possible roles of HCV NS3 protease and helicase activities in the inhibition of the mode of action of interferon alpha (IFN-α), it was necessary to obtain NS3 mutants that were inactive for the protease or the helicase. An NS3 expression construct (pcDA) which has mutations that result in almost complete inactivation of the helicase-ATPase was previously constructed in Dr. McGarvey's Laboratory (Errington et al., 1999) and was available for this work.

## 2. Material and methods

### 2.1. Tissue culture materials and reagents

All tissue culture plasticwares were purchased from VWR. All tissue culture reagents were purchased from Invitrogen: RPMI 1640 and media supplements (FCS, glutamine, geneticin, penicillin and streptomycin). Cell lines were maintained in DMEM growth media supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS), 100 international units/ml penicillin and 100 μg/ml streptomycin.

### 2.2. Site-directed mutagenesis

A point mutation was introduced into the plasmid pcDNAS3 (Dr. Andrew Wardell, Department of Medicine, Imperial College, London) using a QuickChange® II Site-Directed Mutagenesis kit from Stratagene and standard PCR. All reaction buffers were provided with the kit. Five microliter of 10x reaction buffer, 1 μl of dsDNA template, 1.25 μl of primer $S_{1165}$A (TTG AAA GGC TCC GCG GGG GGT CCG C), 1.25 μl of primer $S_{1165}$A (GCG GAC CCC CCG CGG AGC CTT TCA A), 1 μl of dNTP mix, and 40.5 μl of $ddH_2O$. In addition, 1 μl of 2.5 U/μl PfuUltra HF DNA polymerase was added to the sample. The sample was placed in a PCR cycler. Following temperature cycling the reactions were placed on ice for 2 min to cool the reactions to 37 °C. Once the reaction had cooled down, 1 μl of 10 U/μl *Dpn* I restriction enzyme was added to the reaction. The reaction was mixed by pipetting the solution up and down several times. The reaction mixtures were centrifuged in a microcentrifuge for 1 minute and immediately incubated at 37 °C for 1 h to digest the non-mutated supercoiled parental dsDNA.

Fifty microliter of XL1-Blue supercompetent cells (provided with the kit) were gently thawed and transformed with 1 μl of the *Dpn* I restricted sample for 30 min on ice. The cells were heat-shocked for 45 s at 42 °C and then placed on ice for 2 min. $NZY^+$ (0.5 ml) was added to the transformation reaction which was incubated for 1 h at 37 °C with vigorous shaking. Hundred microliter, 150 μl and 250 μl were plated onto LB-Amp agar plates and incubated overnight at 37 °C. Single colonies were picked to inoculate 5 ml of LB-amp and incubated overnight at 37 °C with vigorous shaking. The mutated DNA was purified using a Qiagen Miniprep kit and sent for sequencing by using a primer for sequencing NS3 mutants (5′ CCC CGG TGT T) to confirm the mutation.

### 2.3. Immunofluorescent in situ staining

Glass cover slips were sterilised (dipped in alcohol and flamed) and placed in the bottom of the wells of Sterilin 6 well plates. HCV RNA replicon cells were seeded in half of the wells while the rest of the wells were seeded with Huh7 cells. The cells left to grow up to 80% confluence.

### 2.4. Western blot

Western blot analysis was performed on the total lysates from negative and positive controls and siRNA-transfected S1179I cells as described (Kapadia et al., 1999). The gels were transferred to Hybond-N membranes (Amersham Pharmacia), blocked, and incubated with antibodies to NS3 followed by incubation with a horseradish peroxidase-conjugated mouse anti-rabbit antibody. The blots were developed with (Hyperfilm ECL, Amersham Biosciences).

### 2.5. Interferon anti-viral assay

The standard antiviral assay used here is based on the fact that encephalomyocarditis virus (EMCV) (Generously provided by Dr. D. Watling from Cancer UK, London, UK) is sensitive to the antiviral effects of interferon and EMCV can replicate in the Huh7 cell line (Koev et al., 2002).

A 96 well plate was seeded with Huh7 cells which were allowed to grow at a density of $1 \times 10^4$ cells per well. These cells were then transfected with plasmids which expressed protease mutant NS3 (pcDNS3-SA 3), helicase mutant NS3 (pcDA) and wild-type NS3 as well as a negative control (pcDNA). The wells were treated with interferon-α at different concentrations (0, 0.3, 0.7, 1.5, 3.1, 6.3, 13.5, 25, 50, 100, 1000 U/ml) (18 IE/IU, Roche). All the cells were then infected with EMCV, except the virus negative control (C), for 1 h and then left to grow overnight. The cells were then stained with ethylene violet (Fig. 3a). The cells in the 96 well plate were then counted by using a cell reader at absorbance 540 nm (Fig. 3b). The absence of staining at pcDNA (at concentration of 100 U/ml IFN-α) and wild-type NS3-1B (at concentration

of 13.5 U/ml IFN-α) was probably due to an experimental error.

The medium was removed and the cells were incubated with EMCV at 1 pfu/cell (in 2% RPMI 1640). After 1 h the medium was removed and replaced with supplemented RPMI 1640. Approximately 16–24 h later, cell viability was determined by staining with crystal violet and the absorbance was measured at 570 nm using a plate reader (Dynatech Immunoassay System). Assays were usually performed in duplicate, data were plotted and a dose-response curve was constructed.

## 3. Results

### *3.1. Site-directed mutagenesis of NS3 protease*

Primers were designed that would introduce mutations into amino acids that were known to be essential for protease activity (i.e. the catalytic triad) and studies have shown that mutations at this position result in the loss of protease activity (Grakoui et al., 1993).

Site-directed mutagenesis using these primers was performed, using a QuickChange® II Site-Directed Mutagenesis Kit. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to restriction enzyme *Dpn* I digestion. Therefore after site-directed mutagenesis, the DNA was digested with *Dpn* I and transformed into XL 1-Blue supercompetent cells. The *Dpn* I cleaved the parental DNA template and consequently selected the newly synthesized DNA that contains that target mutation. The transformed bacteria were plated onto an agar plate containing ampicillin. Six colonies were picked and grown in 5 ml of LB media overnight. The plasmid DNA was then purified from the cultures using a Qiagen Miniprep Kit. The purified plasmids DNA were run on a 1% agarose gel.

A single nucleotide change corresponding to the target site, nucleotide 1165 was observed in the DNA sequence of two of these plasmid clones: pcDNS3-SA2 and pcDNS3-SA3. The mutation resulted in the change of serine (TCG) at position 1165 to alanine (GCG) and was thus called $S_{1165}A$ (Table 1).

To confirm that the correct mutation had been introduced, the clones were sequenced. The sequence data confirmed that a point mutation was introduced, a T to a G, at the correct position in pcDNS3-SA clones 2 and 3. No other changes in the coding sequence of NS3 were found with pcDNS3-SA3, however, an additional (unpredicted) mutation was found with pcDNS3-SA2 (Table 1). Consequently pcDNS3-SA3 was used

**Table 1** Position of mutated nucleotides in sequencing data and additional mutation was detected in one of the clones.

| Clone | Nucleotide position of the desired mutant | Additional mutations |
|---|---|---|
| pcDNS3-SA2 | 1165 | 1158: G to A |
| pcDNS3-SA3 | 1165 | None detected |

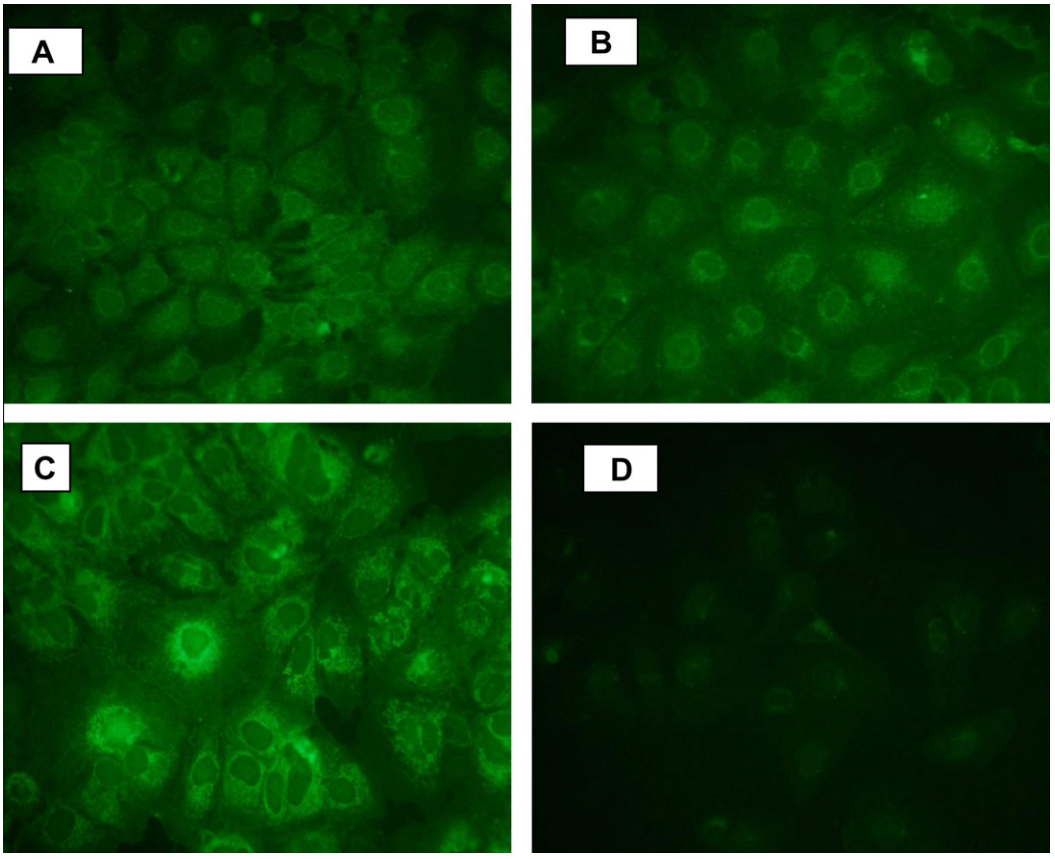


**Figure 1** Expression of NS3 protease and helicase mutants. NS3-7 antibody was used to stain Huh7 cells transfected with (A) pcDNS3 (Wild NS3), (B) pcDA (helicase mutant), (C) pcDNS3-SA 3 (protease mutant), and (D) pcDNA.

as a protease knock-out expression construct in subsequent experiments.

### 3.2. Expression of NS3 protease and helicase mutations: in situ staining

To investigate the effects of the site-directed mutagenesis of the protease and helicase regions of HCV-1a in the replication of the virus and their effect on the cell in the presence of IFN-α, the expression of wild type NS3, the protease mutant NS3 (pcDNS3-SA3) and the helicase mutant (pcDA) were compared. *In situ* staining for NS3 was carried out using the NS3-7 antibody and visualized by a fluorescent microscope.

Cells which had been transfected with constructs corresponding to all the different types of NS3 (wild-type, protease mutant and helicase mutant) were stained with NS3-7 showing that mutations in NS3 do not affect its expression in Huh7 cells. The negative control which contains pcDNA does not show any staining (Fig. 1).

Additional evidence that the mutations in the protease and helicase regions result in the same level of NS3 expression as with wild type NS3, Western blot analysis of Huh7 cells transfected with these constructs was carried out. It was clearly shown that NS3 was present at similar levels in the cells which expressed the protease mutant (pcDNS3-SA3), the helicase mutant (pcDA), and both wild-type NS3 1a and 1b by the presence of a 70 kDa band. No band was seen for the negative control, pcDNA (Fig. 2).

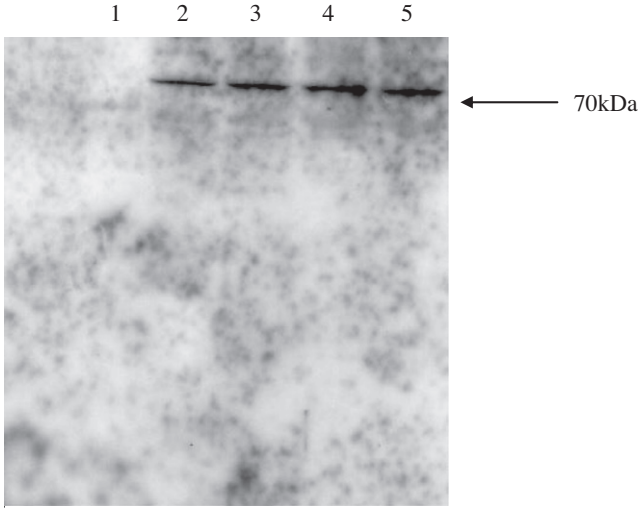


**Figure 2** Expression of NS3 protease and helicase mutants by Western blotting using NS3-7 antibody: (1) pcDNA, (2) wild-type NS3 1b, (3) wild-type NS3 1a, (4) helicase mutant NS3 (pcDA) and (5) protease mutant NS3 pcDNS3-SA3.

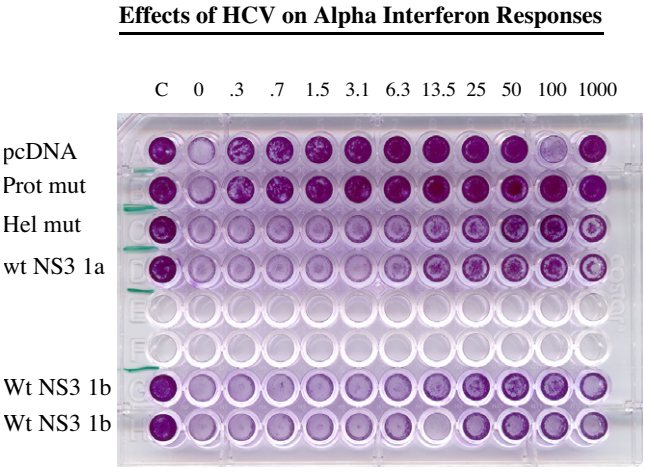


**Figure 3a** Effects of HCV NS3 on the presence of EMCV infected Huh7 cells to α-interferon treatment.

### 3.3. Effect of different HCV NS3 mutants on IFN-α responses

The results showed that cells transfected with pcDNA 3.1 had a similar resistance profile to EMCV infection when treated with increasing concentration of IFN-α, compared to the cells transfected with the protease mutant (pcDNS3-SA3). By contrast, cells transfected with pcDNS3 (wild-type NS3) had a similar resistance profile compared to those of EMCV infection when treated with increasing concentration of IFN-α as the cells transfected with the helicase mutant (pcDA). The cells transfected with pcDNA or the protease mutant have more resistance to EMCV (less cytopathic effect) than those transfected with the helicase mutant or wild-type NS3 which have less resistance to EMCV and more cytopathic effects. The results also showed that as IFN-α concentration increased, fewer cells died. However, increasing IFN-α concentration in the cells transfected with wild-type NS3 (pcDNS3) or helicase mutation showed more death in those cells (Fig. 3a).

## 4. Discussion

To try to determine if either the protease or helicase functions of NS3 were essential for the activity of NS3 in inhibiting IFN-α, mutants which were non-functional for one of these enzymatic activities were obtained. A non-functional helicase mutant was available in Dr. McGarvey's Laboratory (Errington et al., 1999), however, it was necessary to make a non-functional NS3 protease mutant expression construct. Before carrying out mutagenesis of the protease domain, it was important to choose an amino acid which was known to be critical for the function of the protease activity. The three amino acids that constitute the catalytic triad (His-57, Asp-81, Ser-139) have been shown to be crucial for the protease activity and are highly conserved among different HCV strains (Grakoui et al., 1993). Consequently site-directed mutagenesis was carried out to alter the serine residue (Ser-139) of the catalytic triad to alanine and subsequent DNA sequence analysis confirmed that the site directed mutagenesis had been successful, without any additional mutations being introduced into this construct.

The plasmid constructs that expressed the helicase and protease mutants as well as wild-type NS3, as a positive control, and pcDNA, as a negative control, were used to transfect Huh7 cells. As expected, *in situ* staining of the transfected cells with anti-NS3 antibody was positive for the wild-type NS3 while pcDNA showed no staining. Western blotting also showed bands of 70kD with the NS3-1a and NS3-1b positive controls as well as the helicase and protease mutants whereas

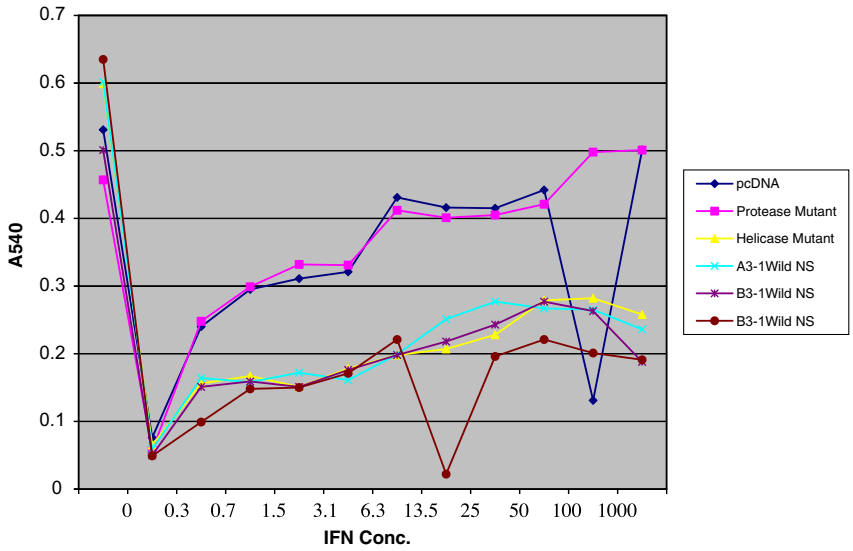


**Figure 3b** Effects of HCV NS3 on alpha interferon responses. The absorbance readings of the stained cells (shown in 5.3A) were measured at 540 nm and presented in graphic form.

no band was detected in the pcDNA transfected cells. The detection of similar levels of the helicase and protease mutants compared to wild-type NS3 using *in situ* staining and Western blotting means that these mutations have no effect on the expression of NS3 in Huh7 cells (Gorbalenya et al., 1989; Koonin, 1991).

Having established that the NS3 constructs could express the different NS3 proteins in the transfection system it was then possible to examine their effects on altering the responses of the cells to IFN-α treatment. The EMCV interferon sensitivity assay was used to compare the effects of the expression of the protease and helicase mutants to the wild-type NS3 and pcDNA transfected Huh7 cells. With pcDNA more cells were protected from EMCV as the concentration of IFN-α was increased compared to wild-type NS3-1a and 1b expressing cells, where more cells were killed by EMCV due to the inhibition of IFN-α by NS3. With the NS3 protease mutant, little sign of IFN-α inhibition was observed as the pattern of EMCV infection was similar to that of the pcDNA 3.1 transfected cells. However, the mutation in the NS3 helicase did not affect the ability of NS3 to inhibit IFN-α activity since the pattern of NS3 helicase mutant expressing cells killed by the EMCV was very similar to those of 1a and 1b wild-type NS3.

These results show that the NS3 protease domain is crucial in the inhibition of IFN-α activity whereas an active helicase domain is not necessary for this inhibition. The likely reason for this is that the protease targets one or more cellular proteins, which results in the inhibition of IFN-α activity. These findings are consistent with recently published results, which showed that NS3/4A protease activity inhibited the phosphorylation of IRF-3, a key transcriptional regulator of the IFN-α response (Wardell et al., 1999; Foy et al., 2003; Breiman et al., 2005). NS3/4A protease has also been shown to be able to disrupt retinoic acid-inducible gene I (RIG-I) signaling which interrupts an IFN-α signaling pathway (Foy et al., 2005). In addition, the NS3/NS4A serine protease caused proteolysis of a Toll-IL-1 receptor domain-containing an adaptor protein linking TLR3 to a kinase responsible for activating IRF-3 (Li et al., 2005). Therefore, there appears to be a number of cellular targets for NS3/NS4A protease which results in the inhibition of IFN-α signaling. In the present study NS3 on its own (i.e. without NS4A) was shown to be capable of inhibiting IFN-α activity. It will be interesting to see if NS3 targets the same substrates as NS3/NS4A.

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