Molecular characterization of the interaction between Tick-borne encephalitis virus NS5 protein and the Interferon α/β- and γ receptors

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ABSTRACT
Flaviviruses, family flaviviridae, are often associated with severe diseases and many of the viruses have been shown to affect the immune response. Interferons confer important ways of defending the host against viral infections because they provide a link between the innate and the adaptive immunity. Langat virus (LGTV), belonging to the Tick-Borne Encephalitis (TBE) complex of viruses, has recently been shown to provide interactions with interferon receptors and inhibit the interferon-mediated response. Similarly, it has been demonstrated that the TBE virus NS5 protein affects type 1 interferon signaling. In this study we have analyzed the interaction between the TBE NS5 protein and interferon receptors by using the yeast two-hybrid system. Our results support the idea that the inhibition of interferon signaling does not involve a direct interaction between TBEV NS5 and the interferon receptors IFNAR2, IFNGR1 and IFNGR2.

INTRODUCTION
Tick-borne encephalitis virus (TBEV) belongs to the TBE complex of viruses, family Flaviviridae, of the flavivirus genus. This genus contains nearly 80 viruses where the majority is arthropod-borne, using ticks or mosquitoes as vectors. The genus can be organized into three clusters; the mosquito-borne, the tick-borne and those with no known vector. The arthropod-borne viruses are in many cases responsible for severe diseases varying from encephalitis to hemorrhagic fever. They are often named after the disease that they cause, for example the dengue fever caused by the dengue virus, the yellow fever caused by the yellow fever virus and tick-borne encephalitis caused by TBEV (1, 2).

Their RNA genome is single stranded and plus-sensed, consisting of about 11 kilobases, with a type I cap in its 5’ end. They have an open reading frame of about 10 kilobases, which encodes a single polyprotein, and is flanked by a 5’- and 3’- non-coding region. The translated polyprotein is cleaved into three structural proteins; C (capsid), prM (membrane) and E (envelope), and seven nonstructural proteins; NS1, NS2A and B, NS3, NS4A and B and NS5 (1). Both the translation and the proteolytic processing are associated with the rough endoplasmic reticulum (Fig. 1) (3).
Translation of the RNA genome is generating a single polyprotein, which is processed in association with Rough endoplasmic reticulum, resulting in three structural (C, prM and E) and seven non-structural proteins (NS1-5).

The flaviviral NS5 protein is a multifunctional RNA dependent RNA polymerase with various functions beyond the C-terminal polymerase activity. It is a 903 amino acid long protein and has also been shown to contain a N-terminal methyltransferase activity (4, 5). For Dengue virus it has been shown that the NS5 protein also has an NLS-sequence (6) and it has recently been discovered that the NS5 protein of Langat virus (LGTV), belonging to the TBE complex of flaviviruses, interacts with interferon receptors and inhibits the Jak-Stat signaling (7).

In a screen for viral-host protein interactions, where TBEV NS5 protein was screened against a human brain cDNA library in a yeast two-hybrid analysis, it was found to interact with the human LAP protein hScrib. Interestingly, it was also shown that a double mutation in NS5 (Y222A/S223A) leading to a scribble binding-defective mutant affected the ability of TBEV NS5 to inhibit the interferon-mediated Jak-Stat response by the host (8).

Scribble is a scaffolding protein, belonging to the LAP family of proteins. It is localized to the cell membrane, due to its leucine-rich repeats (LRRs), where it has critical functions for various cellular activities (9, 10). It has four different PDZ domains, which are responsible for the protein-protein interactions characteristic of scaffolding proteins. The PDZ domains usually associate with a c-terminal consensus motif (S/T-X-V/L/I), but internal motifs has been described (11, 12, 13, 14).
NS5 was shown to interact with scribble by an internal motif, where Y222 and S223 were proved to be vital for the interaction (8).

The interferons can be of two types; type I (IFN-α/β) and type II (IFN-γ), and they signal to cells via related but distinct pathways. IFN α/β binds the Interferon alpha/beta receptors (IFNAR-1/2), while IFN-γ binds the Interferon gamma receptors (IFNGR-1/2). The two receptors come together upon interferon binding, activating preassembled Janus tyrosine-kinases (JAKs). The JAKs cross-phosphorylate each other and the cytoplasmic part of the receptor, providing a binding-site for STAT- (Signal transducers and activators of transcription) proteins. Upon binding of IFN-α/β to IFNAR-1/2, STAT-1 becomes phosphorylated, associates with phosphorylated STAT-2 and a non-STAT protein, p48. The complex localizes to the nucleus and activates ISRE elements (Fig. 2). IFN-γ binds to and activates IFNGR-1/2 receptors in a similar way as described for IFN-α/β. However, only STAT-1 homodimers are formed and translocate to the nucleus and hence, enable transcription of different genes coupled to GAS elements (Fig. 2). Very important responses mediated by the interferons are the antiviral activities including both the innate and the adaptive immune response, inhibition of cell growth and control of apoptosis (15, 16).

**Figure 2.** A schematic of the Jak-Stat pathway. (1) IFN-α/β or γ binding to its cognate receptor, (2) Cross-phosphorylation and activation of JAKs, (3) generates further phosphorylation of the receptors, (4) which creates binding sites for STAT proteins. Phosphorylation of STATs by JAKs leading to, (5) dissociation from receptor subunits and dimerization of STATs. (5-6a) In the case of IFN-α/β
stimulation, STAT-1 associate with STAT-2 and forms a complex with the non-STAT protein p48 that translocate to the nucleus and activate target genes. (5-6b) Upon IFN-γ stimulation STAT-1 homodimers are formed that enter into the nucleus and start transcription of GAS elements.

AIM

As previously detected a subset of the TBEV NS5 protein localizes at the cell membrane due to its ability to bind the scaffolding protein hScrib (8). This interaction was also shown to influence the ability of TBEV NS5 to impair the IFN-α/β stimulated Jak-Stat pathway (7). Similarly, an interaction between the Langat virus and the IFNAR2 and IFNGR1 subunits was recently shown to affect the interferon-mediated response.

Thus, this project was aimed to study the possible physical interaction between the TBEV NS5 protein and the cytoplasmic part of the IFN-receptors. By using molecular biology tools combined with yeast two-hybrid (Y2H) analysis the interaction between full-length wt TBEV NS5 was tested against the different receptor subunits.

MATERIAL AND METHODS

PCR. The interferon α/β and γ receptor subunits were provided in plasmids AT40-H8 (IFNAR1), AU21-D1 (IFNAR29), AU42-H6 (IFNGR1) and AU5-G1 (IFNGR2), transformed into Escherichia coli cells. The plasmids were purified with a plasmid purification kit (QIAGEN) and primers targeting the cytoplasmic part of the receptor were designed (MWG) with NcoI- and EcoRI restriction sites (table 1). PCR was performed with Expand High Fidelity System (Roche) using 0,3 µM of respective primer and 0,6 µg/µl template together with 200 µM nucleotide-mix, 1x PCR-buffert and 3,5 units/µl Expand. The PCR products were separated on a 1% agarose gel and extracted with a gel extraction kit (QIAGEN).

<table>
<thead>
<tr>
<th>Target (nucleotide no.)</th>
<th>Primer sequence</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNAR1 (1442-1753)</td>
<td>CCATGGGGGTATGCTGCAGAAGTCTTCTT</td>
<td>GAATTCTCATACAAAGTCCTCTGTA</td>
</tr>
<tr>
<td>IFNAR2 (1164-1349)</td>
<td>CCATGGGGTGCTTAAAGAATTAGGCCTCCC</td>
<td>GAATTCTTAAACTCAGGGGCACAGGG</td>
</tr>
<tr>
<td>IFNGR1 (893-1552)</td>
<td>CCATGGGGGAAGAAATATACATTGAAG</td>
<td>GAATTCTCATGAAAATATTCTTTGGAAT</td>
</tr>
<tr>
<td>IFNGR2 (927-1130)</td>
<td>CCATGGGGCTGAATATAGAGTCCTGTAT</td>
<td>GAATTCTAAAGCGTTTGGAGAACAT</td>
</tr>
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Table 1. Primer sequences used to amplify the cytoplasmic parts of the receptors. Bases in italics were added to the primers and correspond to NcoI\textsuperscript{a} and EcoRI\textsuperscript{b} restriction sites. Bases in bold were added to the sequences to get the gene in frame with gene expression in the pACT2 vector.

**TOPO\textsuperscript{®}TA-cloning and restriction analysis.** The extracted PCR products were cloned into a TOPO\textsuperscript{®}-TA vector and 2 µl of the cloning product were transformed into a vial of chemically competent *E.coli* DH5α cells, according to the TOPO\textsuperscript{®}-TA Expression kit manual (Invitrogen). The transformed cells were plated on 1x LB-medium containing 100 µg/ml ampicillin and incubated overnight (o/n) in 37°C. Selected colonies were picked, DNA was purified and subsequently exposed to restriction analysis using 0,2 Units/µl of NcoI and 0,2 Units/µl EcoRI. The restriction products were analyzed on a 1% agarose gel and purified by gel extraction.

**Cloning into the yeast pACT2 vector.** The extracted products from IFNGR2, IFNAR2 and IFNGR1 were cloned into yeast pACT2 vector (Clontech) in 3:1, 5:1, 10:1 and 20:1 ratios using 0,1 units/µl T4 ligase in a cold room o/n. The ligation products were transformed into a vial of chemically competent *E.coli* DH5α cells and plated as above. The plasmids were purified from selected colonies and treated with 1 unit/µl NcoI and 1 unit/µl EcoRI for 1½ h to check for proper ligation. The pACT2 plasmids with correct inserts were chosen for transformation into *Saccharomyces cerevisiae*, strain AH109, together with the matching target plasmids (table 2). Control plasmids were provided from Clontech. An o/n culture of AH109 were divided into 1 ml aliquots, pelleted and resuspended in 100µl of filter sterilized One Step Buffer (0,2 M LiAc, 40% PEG 3350 and 100 mM DTT). 8µl of single stranded herring sperm DNA and 10µl of each plasmid in the proper combinations were added and the mixture was incubated in 45°C for 30 minutes. The heat-shocked cells were pelleted and resuspended in 100µl TE-buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7,5), plated on selective SD medium lacking the amino acids leucine and tryptophan (-leu/trp) and grown for five days in 30°C.

**Yeast two-hybrid analysis.** Colonies from each plate were picked and streaked onto full selection SD media lacking additional adenine and histidine (-leu/trp/ade/his). For every replicate a control-replicate was performed on SD -leu/trp media to verify presence of plasmids. All plates were incubated for at least four days in 30°C.
Table 2. The plasmid match set-up in the yeast two-hybrid system. Each receptor subunit was tested against wildtype TBEV NS5 and mutant TBEV NS5(Y222A/S223A). One positive and one negative control were used and every reaction was performed in duplicates.

<table>
<thead>
<tr>
<th>Plasmid 1 (Gal4 AD)</th>
<th>Plasmid 2 (Gal4 DNA-BD)</th>
<th>No of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a pACT2 – IFNAR2</td>
<td>pAS2-1 – NS5TBEmut</td>
<td>2</td>
</tr>
<tr>
<td>2a pACT2 – IFNGR1</td>
<td>pAS2-1 – NS5TBEmut</td>
<td>2</td>
</tr>
<tr>
<td>3a pACT2 – IFNGR2</td>
<td>pAS2-1 – NS5TBEmut</td>
<td>2</td>
</tr>
<tr>
<td>1b pACT2 – IFNAR2</td>
<td>pAS2-1 – NS5TBEmut</td>
<td>2</td>
</tr>
<tr>
<td>2b pACT2 – IFNGR1</td>
<td>pAS2-1 – NS5TBEmut</td>
<td>2</td>
</tr>
<tr>
<td>3b pACT2 – IFNGR2</td>
<td>pAS2-1 – NS5TBEmut</td>
<td>2</td>
</tr>
<tr>
<td>+ control pTD1 – SV40 T-antigen</td>
<td>pVA3-1 – p53</td>
<td>2</td>
</tr>
<tr>
<td>- control pTD1 – SV40 T-antigen</td>
<td>pLAM5'-1 – Lamin C</td>
<td>2</td>
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**RESULTS**

**PCR.** Electrophoretic analysis of the PCR products showed that fragments of proper size from each receptor subunit were generated. The fragments were retained after gel extraction from a 1% agarose gel (Fig. 3).

![Figure 3](image.png)

**Figure 3.** Purified PCR products analyzed on a 1% agarose gel. Fragments of correct size were generated for each receptor subunit, indicated with arrows; (a) IFNGR2 (203 bp), IFNAR2 (185 bp), IFNAR1 (311 bp) and (b) IFNGR1 (659 bp).

**Restriction analysis of TOPO®TA-cloning.** Electrophoretic analysis of restriction treatment of TOPO®TA cloning product with NcoI and EcoRI showed DNA fragments of correct size for IFNGR2, IFNAR2 and IFNGR1 (Fig. 4a). Restriction treatment did not,
however, generate a proper sized fragment for IFNAR1. Instead a restriction product of about 200 bp was generated (Fig. 4b). To check for PCR-induced restriction sites within the gene, a further restriction treatment was performed where the gene was treated with each of the restriction enzymes separately. However, the analysis did not show any cleavage at all within the gene fragment (data not shown), indicating that no additional restriction sites were generated.

**Figure 4.** Purified restriction products from the TOPO®TA cloning reaction. (a) NcoI- and EcoRI cleavage plasmids generated fragments of expected size for IFNGR2, IFNAR2 and IFNGR1, indicated with arrows. (b) The cleaved plasmid gave rise to an unexpected fragment of ~200 bp, indicated with an arrow, instead of the expected size of ~300 bp for IFNAR1.

**Cloning into yeast pACT2 vector.** Positive results were obtained when ligation was performed in a 20:1 relationship between insert and vector. All the colonies picked for analysis showed to contain plasmids with inserts of proper size (Fig 5).

Transformation of the plasmid-pairs (Table 2) into yeast AH109 cells went successfully, with colonies present on all the plates (data not shown).
Figure 5. The restriction pattern of pACT2 plasmids after treatment with NcoI and EcoRI. All three plasmids were shown to contain inserts of proper size, indicated with arrows.

Yeast two-hybrid analysis. Surprisingly, none of the interactions tested were positive, except for the positive control (fig 6). All three receptors were sequenced to check for possible PCR induced mutations. IFNAR2 and IFNGR2 sequences were correct, while IFNGR1 had a base substitution, which generated a Glycine instead of a Serine at position 269.

Figure 6. The results of the yeast two-hybrid analysis. The control plate (-Leu/Trp) shows correct transformation of both plasmids in all four analyses. The full selection plate (-Leu/Trp/Ade/His) shows only growth on the positive control (b), while no growth can be observed when NS5 is tested against the interferon receptors (a, c and d).
DISCUSSION
LGTV and TBEV both belong to the TBE complex of viruses and have a high degree of sequence similarity. The open reading frame (ORF) on the amino acid level share ~85% similarity and the highly conserved NS5 protein has an amino acid sequence similarity as high as 88% (17). Still there are differences likely to have important consequences for the difference in pathogenicity.

The most striking difference between these two viruses is the fact that LGTV is attenuated to humans, while TBEV is the cause of severe human encephalitis. For this purpose LGTV has been used for immunization and is a potential target for the production of vaccines against TBEV infections (23). Their difference in pathogenicity can well correspond to their distinctive ability to affect the immune response in advantageous ways.

The type I-interferons induce transcription of multiple genes, highly involved in the defense mechanisms against viruses. They are extremely important because they confer an early response to infections. The ability of viruses to inhibit the interferon response at such an early state as signal induction greatly enhances the ability to replicate and therefore the ability to cause disease. Many viruses of the flavivirus genus have been shown to affect interferon responses by reducing the levels of phosphorylated STATs (18, 19, 20, 21, 22). LGTV NS5 showed to physically interact with IFNAR2 and IFNGR1 thereby inhibiting the phosphorylation of Jak-1 and Tyk-2 and in turn STAT-1 and STAT-2 phosphorylation. These interactions affect both the IFN-α/β and IFN-γ signaling. In contrast, our results indicate that TBEV NS5 does not physically interact with any of the receptors tested. This could be an interesting difference that might have implications on their respective ability to cause disease. The fact that TBEV NS5 affects phosphorylation of STAT-1 without directly interacting with the IFN-receptors could mean that other signaling pathways involving STAT-1 are also affected. This could have a large impact on the difference in pathogenicity for the two viruses.

Another difference is the ability of TBEV NS5 to interact with the scaffolding protein hScrib. This interaction makes it possible for the NS5 protein to locate at the cell membrane, which has been shown to generate advantages in its ability to inhibit host cell signaling (8). It would be of great interest to evaluate the precise mechanism of how TBEV NS5 reduces the rate of phosphorylated STAT-1.

Even if the analysis showed no interaction between TBEV NS5 and the interferon receptors it is important to keep in mind that a possible interaction could still occur in mammalian cells. The interaction could be indirect, meaning that other, mammalian proteins are mediating the interaction. However, an indirect interaction may be lost in the Y2H analysis. The fact that
only the cytoplasmic parts of the receptors were tested against TBEV NS5 could also have an impact on the results. Proper folding might be dependent on other components, not included in this study. Another factor that could have affected the results is the PCR-induced mutation of IFNGR1 leading to the S269G substitution. This mutation could have silenced an actual interaction.

The IFNAR1 receptor could not be tested due to difficulties with the cloning. It is hard to speculate why, but one reason could be that the PCR induced further restriction sites. But the fact that individual treatment of the PCR product with the two restriction enzymes showed no cleavage disagrees with that. To be sure, it would have been interesting to sequence the PCR product and perform the PCR and the cloning again. Though it remains to be seen whether an interaction with IFNAR1 and IFNGR1 occurs, it seems likely that none of the IFN-receptors is actually interacting directly with TBEV NS5. The NS5-mediated reduction of phosphorylated STAT-1 is likely to involve other components coupled to interferon-mediated signaling.

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