Tick-borne encephalitis virus NS5 associates with membrane protein scribble and impairs interferon-stimulated JAK-STAT signalling

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Summary

Tick-borne encephalitis virus (TBEV) NS5 protein is a multifunctional RNA-dependent RNA polymerase that is indispensable for viral replication. TBEV is considered to be highly neurovirulent and can cause lethal encephalitis. In this study, we demonstrate a novel interaction between TBEV NS5 and the PDZ protein scribble (hScrib) affecting interferon (IFN) type I and II mediated JAK-STAT signalling. The sequence of TBEV NS5 interacting with hScrib was identified using extensive site-directed mutagenesis analysis. Two consecutive mutations in the methyltransferase (MTase) domain of NS5 were found to disrupt binding to hScrib. Colocalization studies with hScrib demonstrated that TBEV NS5 was present at the plasma membrane of mammalian cells. To address the role of viral interference with the IFN response, NS5 proteins were expressed in IFN-stimulated cells. While TBEV NS5 substantially blocked phosphorylation of STAT1, a mutated NS5 protein defective in hScrib binding failed to inhibit JAK-STAT binding correctly. Furthermore, hScrib knock-down resulted in relocalization of NS5 to intracellular locations and abrogated the impaired STAT1 phosphorylation. These results define the TBEV NS5 protein in concert with hScrib as an antagonist of the IFN response, by demonstrating a correlation between the association and JAK-STAT interference.

Introduction

In humans, infections by tick-borne flaviviruses (TBEV) may result in encephalitis, meningitis and haemorrhagic fevers with mortality rates as high as 20–30% (Pletnev and Men, 1998; Gritsun et al., 2003a,b; Charrel et al., 2004; Mandl, 2005). TBEV contains a positive sense RNA genome that encodes a single polyprotein and has significant similarity to mosquito-borne flaviviruses such as Dengue virus (DENV, type 1–4), West-Nile virus (WNV) and Japanese encephalitis virus (JEV). Co- and post-translational processing of the polyprotein yields three structural and seven non-structural (NS) proteins in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 (Chambers et al., 1990; Heinz and Mandl, 1993; Mandl, 2005). Flaviviral replication, which occurs at ER membrane-associated replication complexes (RCs), has been thoroughly investigated and involves several viral-encoded NS proteins (Kapoor et al., 1995; Li et al., 1999a; Mackenzie et al., 1999). The two major components of the RCs are the NS3 and NS5 proteins, providing vital enzymatic properties for RNA replication. NS3 contains protease/helicase/triphosphatase activities (Wengler and Wengler, 1993; Huyer et al., 1997; Li et al., 1999a; Xu et al., 2005) while RNA cap methyltransferase (MTase)/RNA-dependent RNA polymerase (RdRp) activities are coupled to the N-terminal and C-terminal of NS5 respectively (Koonin, 1993; Ackermann and Padmanabhan, 2001; Egloff et al., 2002; Malet et al., 2007; Yap et al., 2007).

Signal transducer and activator of transcription (STAT) proteins are transcription factors that are essential in mediating IFN type I signalling during innate immune responses, and are necessary for vertebrate control of viral infections. The STAT1 and STAT2 proteins exist as latent monomers in the cytoplasm and become activated by phosphorylation upon IFN-α/β stimulation of the IFNAR-1/2 receptor through tyrosine phosphorylations via the associated janus protein tyrosine kinases (JAK1/ JAK2) (Bromberg and Darnell, 2000; Shuai, 2000; Shuai and Liu, 2003). Phosphorylated STAT1 (pSTAT1) forms heterodimers with STAT2 and assembles with a non-STAT protein, p48 (IRF-9), forming the interferon-stimulated gene factor 3 (ISGF3) complex (Fu et al., 1990; Levy,
ISGF3 enters the nucleus and binds to a conserved DNA sequence known as the interferon-stimulated response element (ISRE). This results in expression of genes involved in antiviral activities, cell cycle progression and apoptosis (Schindler et al., 1992). Following stimulation of cells with IFN type II (IFN-γ), homodimers of pSTAT1 are formed that activate the IFN-γ-activated sequence GAS (Shuai et al., 1992). Cross-talk between these two pathways occurs as IFN-α/β-activated pSTAT1 homodimers also stimulate transcription of GAS (Theofilopoulos et al., 2005). STAT dimers are believed to be inactivated by protein tyrosine phosphatases (PTPs) in the cytoplasm or the nucleus, which relocate STAT1 to the cytoplasm (Darnell, 1997; Shuai and Liu, 2003).

In order to circumvent the IFN type I system tick- and mosquito-borne flaviviruses use different NS proteins to downregulate distinct signalling components of the JAK-STAT pathway. The NS5 protein of Langat virus (LGTV), a member of the TBEV complex, counteracts the IFN response by blocking phosphorylation of STAT1, STAT2, TYK2 and JAK1 (Best et al., 2005). Likewise, the JEV NS5 protein impairs nuclear translocation of STAT1 and phosphorylation of TYK2, possibly via a PTP-dependent mechanism (Lin et al., 2006). Other flavivirus proteins reported in IFN antagonism include DENV2 NS4B, WNV NS4B, and Kunjin virus (KUNV) NS2A, NS2b, NS3, NS4A and NS4B (Munoz-Jordan et al., 2003; 2005; Liu et al., 2005).

hScrib belongs to the LAP, leucine-rich repeats (LRRs) and PSD/Dlg/ZO-1 (PDZ), family of proteins that contain 16 LRRs and 4 non-identical PDZ domains (Bilder et al., 2000a; Santoni et al., 2002). hScrib is widely expressed at the membrane of polarized mammalian cells and is believed to stabilize cell-to-cell contacts through a mechanism involving E-cadherin (Navarro et al., 2005). A pivotal role in T cell polarity, which is essential for T cell activation and antigen presentation, has recently been attributed to hScrib (Ludford-Menting et al., 2005). In Drosophila, scribble has been reported to control neuronal synaptic plasticity, cell polarity and growth control (Bilder et al., 2000b; Roche et al., 2002). In mice, scribble knockouts display impaired neuronal tube development (Murdoch et al., 2003) while an association with the guanine nucleotide exchange factor βPIX suggests involvement in neuronal vesicle transport (Audebert et al., 2004).

In this report, we provide functional evidence for the interaction between TBEV NS5 and the mammalian protein hScrib. We show that the MTase domain of TBEV NS5 is essential for binding the fourth PDZ domain (PDZ4) of hScrib in vitro and in vivo. Furthermore, immunostaining and confocal laser scanning microscopy (CLSM) revealed that TBEV NS5, hScrib and E-cadherin colocalize at cell–cell contacts in mammalian cells. In a recent study, the LGTV NS5 protein was demonstrated to directly bind to the IFN receptor subunits (Best et al., 2005), suggesting an unknown host factor for proper targeting of NS5 to the plasma membrane. To evaluate whether hScrib is required for TBEV NS5 to prevent JAK-STAT signalling, we examined the combinatorial effects of IFN stimulation and hScrib knock-down in cells transfected with TBEV NS5. We found that the TBEV NS5 protein inhibits formation of pSTAT1 in response to IFN-α/β and -γ. However, hScrib depletion in cells containing NS5 or a binding defective mutant protein, NS5(Y222A/S223A), restored nuclear pSTAT1, indicating that the inhibition of IFN signalling by TBEV NS5 is linked to hScrib binding.

Results

An internal binding motif of TBEV NS5 is essential for association with the host factor hScrib

Although flaviviral replication seems to involve recruitment of host proteins (Blackwell and Brinton, 1997), only a few host proteins have been reported to bind flaviviral NS5 proteins outside this context (Johansson et al., 2001; Brooks et al., 2002; Best et al., 2005). Therefore, we sought to identify new binding partners for TBEV NS5, which may play a role in future drug target evaluation. Full-length NS5(1–903) of TBEV was used as bait in a Gal4-based yeast two-hybrid (Y2H) screen against a human brain cDNA library, which isolated a cDNA clone of the human LAP protein hScrib. The clone encoded the C-terminal hScrib region, 988–1630, including PDZ domains 3 and 4 (Fig. 1A and B, a). PDZ domains of LAP proteins are class I domains that adhere to the extreme C-terminus of proteins with the typical PDZ binding motif, S/T-X-L/V/I (Harris and Lim, 2001). Not unexpectedly, analysis of the C-terminal region of TBEV NS5 revealed the motif –SII, which fits the aforementioned consensus sequence. Comparison of other members of the flavivirus genus identified a –TVL motif in the NS5 protein of both WNV and KV, while the NS5 protein of JEV and DENV (serotypes 1–4) displayed atypical C-terminal sequences not likely to be involved in PDZ binding. The variability in these sequences led us to examine whether individual WNV NS5(1–905) or DENV2 NS5(1–900) protein could bind hScrib. Even though the three proteins expressed similarly in yeast (Fig. 2B, immunoblot), neither WNV nor DENV2 NS5 was capable of binding hScrib (Fig. 1B, b and c). The association of TBEV NS5 with hScrib was further verified reciprocally in the Y2H system (Fig. 1B, e and f). Thus, these results identify hScrib as a novel binding partner for TBEV NS5.

To determine whether binding involved the C-terminal –SII motif of TBEV NS5 we generated a series of mutations of residues critical for PDZ domain recognition. Surprisingly, we found by extensive directed mutagenesis as
well as deletion of the C-terminus that binding with hScrib was yet retained (Table S1). In order to identify the minimal region sufficient for binding, we generated a library of NS5 deletions that were evaluated in Y2H assays defining the region 219–223 as predominant for association (Fig. 1C, a–c) (Table S2). A comparison with the mosquito borne flaviviruses, unable to bind hScrib, suggested that the non-conserved residues Tyr222 and Ser223 were putative targets. As demonstrated in the Y2H assay, mutations at residue Y222A/S223A in NS5 totally impaired binding (Fig. 1C, d). Furthermore, individually expressed protein constructs NS5(Y222A) and NS5(S223A) showed loss of binding while NS5(Y222F) and NS5(S223A) demonstrated interaction with hScrib (Fig. 1C, e–h). These results were intriguing and indicated an internal PDZ binding site in NS5.

**TBEV NS5 interacts specifically with PDZ4 of hScrib**

To determine which region of the hScrib protein associates with TBEV NS5 we generated various host protein deletion fragments. The full-length hScrib(1–1630) was unable to interact with NS5 (data not shown). Inability of full-length hScrib to bind seemed contradictory on the basis of previous Y2H results. However, as LRR domains anchor LAP proteins at the cell cortex (Legouis et al., 2003), a functional LRR domain of hScrib would impair Y2H interactions in the nucleus. To verify this assumption we repeated the Y2H experiments with a construct lacking the LRRs, hScrib(615–1630), which restored association with NS5 as expected (Fig. 2A, a). Moreover, hScrib deletions encoding the far C-terminal region hScrib(1194–1630), the central PDZ1-2 region hScrib(615–833) and PDZ3(988–1100) were unable to bind NS5 (Fig. 2A, b–e), even though these fusions were more abundant than hScrib(615–1630) expressed in yeast (Fig. 2A, immunoblot). A fragment that expressed the 1100–1630 region containing PDZ4(1100–1630) bound readily (Fig. 2A, f) and indicated that PDZ4 is a major factor for hScrib targeting of NS5.

Structural studies show that PDZ ligands bind in a binding groove between the βBstrand containing a GLGF
motif and the αB-helix (Fig. 2B). Also, involvement of electrostatic interplay between charged residues of the ligand and the PDZ domain has been reported. Specifically, Arg and Lys residues flanking the GLGF repeat are likely to be involved in such electrostatic interactions (Doyle et al., 1996; Harris et al., 2003). Comparison of hScrib PDZ4 with PDZ1-3 suggested Lys/Glu/Arg to be residues for contribution of ionic interaction. Furthermore the length of the PDZ4 carboxylate-binding loop is unique compared with PDZ1-3 (Fig. 2B). Substitution of the predicted important residues in the binding pocket and K1105A, the carboxylate binding lysine of PDZ4, completely abolished NS5 binding (Fig. 2B, a and e). These substitutions were performed in the hScrib(988–1630) construct encoding both PDZ3-4 and the hScrib C-terminus and support that TBEV NS5 interacts specifically to PDZ4.

TBEV NS5 associates with hScrib in pull-down and co-immunoprecipitation assays

To further characterize the TBEV NS5–hScrib complex and test whether the observed interaction was direct,
Escherichia coli-expressed protein components were used in a GST pull-down experiment. The GSThScrib (988–1630) protein was partially purified (Fig. 3A, left). We found that the expression in E. coli of full-length hisTBEV NS5 protein was incomplete due to toxic effects by NS5 during protein isolation. Similar results have previously been observed for the DENV NS5 protein where protein breakdown during bacterial expression produced a 35 kDa fragment corresponding to the MTase domain (Ackermann and Padmanabhan, 2001; Egloff et al., 2002). Accordingly, we recovered an equivalent 32 kDa MTase fragment that was purified from TBEV NS5-induced cell extracts (Fig. 3A, middle). Next, the NS5(MTase) and the GST fusion proteins were mixed in equal amounts, pulled down under physiological conditions with GST binding beads and analysed by immunoblotting using an anti-his antibody. TBEV NS5(MTase) bound specifically to GSThScrib(988–1630) but not to excess amounts of DENV2 NS5(MTase) (Fig. 3A, right). These results correlated well with those from the Y2H assays and support the findings that the N-terminal domain of NS5 is the major region recognized by hScrib and that the interaction does not require additional cellular factors.

We next examined the association of hScrib with transfected full-length NS5 protein in mammalian cells. C-terminal V5 epitope-tag constructs of TBEV NS5, NS5(Y222A/S223A) and DENV2 NS5 were generated and transiently transfected in HeLa cells. After 24 h constructs were immunoprecipitated with anti-V5 antibodies and examined by Western blotting with either anti-V5 or anti-hScrib antibodies.
anti-hScrib antibodies. We found that endogenous hScrib co-precipitated with TBEV NS5, but not with NS5(Y222A/S223A) or DENV2 NS5 (Fig. 3B, top). We also analysed a fraction of the cell lysate by detecting NS5 and endogenous hScrib as a control for protein expression (Fig. 3B, bottom). These results confirm that intact Tyr222 and Ser223 residues of the TBEV NS5 protein are required for association with hScrib and suggest a physiological role for TBEV NS5–hScrib complex formation.

**hScrib-dependent recruitment of TBEV NS5 at the cell–cell contacts**

Previous reports have revealed that hScrib is associated at cell–cell contacts in polarized mammalian cells (Navarro et al., 2005). Thus, in order to examine the association further, MDCK cells transiently transfected with TBEV NS5 were fixed, permeabilized and co-stained with anti-V5 and anti-scrib antibody followed by Alexa Fluor-488 and Texas Red–conjugated antibody. Visualization revealed colocalization (yellow) of TBEV NS5 (green) and hScrib (red) at the membrane.

**Fig. 4.** TBEV NS5 colocalize with endogenous hScrib at the plasma membrane of contacting cells.

A. MDCK cells transfected with NS5 were fixed and co-stained with anti-V5 and anti-scrib antibody followed by Alexa Fluor-488 and Texas Red–conjugated antibody. Visualization revealed colocalization (yellow) of TBEV NS5 (green) and hScrib (red) at the membrane.

B. Cells were stained for NS5 as in (A) and with anti-E-cadherin antibody followed by Alexa Fluor-594-conjugated antibody (red). Arrows point to regions of marked TBEV NS5 staining; Y222A/S223A was found mainly in the cytoplasm and was absent from cell-cell contacts. Scale bar, 16 μm.
of NS5 and that hScrib plays a major role in targeting TBEV NS5 to the cellular membrane.

Knock-down of hScrib results in altered localization of NS5

To directly investigate the impact of hScrib on TBEV NS5 localization to the cell periphery, siRNA was used to reduce endogenous levels of hScrib in HeLa cells. For these studies, two different RNAi duplex sequences, siRNA#1 and siRNA#2, targeting hScrib expression, hScrib and α-Tubulin protein levels determined 72 h post transfection by Western blot.

A. Cells treated with CsiRNA or target siRNA were transfected at 48 h with TBEV NS5 and NS5(Y222A/S223A). Seventy-two hours post siRNA transfection cells were fixed, stained with Alexa Fluor-488 and processed for CLSM. Note the marked redirection of TBEV NS5 from the plasma membrane in hScrib knock-down cells. Scale bar, 16 μm.

Fig. 5. Downregulation of hScrib alters the localization of TBEV NS5.

TBEV NS5 antagonizes IFN-stimulated JAK-STAT signalling

A recent study showed that LGTV NS5 interacts with the IFN receptor subunits at the plasma membrane, resulting in impaired JAK-STAT signalling (Best et al., 2005). Given our observation that TBEV NS5 is partially localized at the cellular periphery we addressed the role of TBEV NS5 in IFN antagonism. Immunostaining of HeLa cells with an antibody specific for phospho-tyrosine 701 in STAT1 monitored nuclear pSTAT1 in INF-α-induced cells (Fig. 6A, top). Expression of NS5 in cells stimulated with IFN-α resulted in undetectable staining of pSTAT1 while faint accumulation was observed in the nucleus of a majority of cells expressing NS5(Y222A/S223A) (Fig. 6A, middle). Similar results were obtained in HEK293 cells, verifying the results in HeLa cells (Fig. 6A, bottom). In DENV NS5-transfected cells treated with IFN-α, pSTAT1 localized primarily in the nucleus, as previously reported (data not shown). These results suggest that blocking of STAT1 phosphorylation by TBEV NS5 is specific following IFN signalling.

To extend the newly defined role for TBEV NS5 in IFN antagonism, we used a stable HeLa cell line with a luciferase (luc) reporter construct regulated by the STAT1 response element GAS. A dose–response curve to IFN-γ and -α monitored the luc activity (data not shown) and established 100 ng ml⁻¹ as the optimal concentration used in subsequent experiments (Fig. 6B, top). Cells expressing NS5 or NS5(Y222A/S223A) were stimulated with IFNs for 5 h. Immunofluorescence and Western blotting confirmed equal NS5 expression in the HeLa-lucRep cells (data not shown). Cells expressing NS5 and stimulated with IFN-γ showed a significant 30% reduction of luc activity compared with cells treated only with IFN. Furthermore, NS5(Y222A/S223A) exhibited only a 43% reduction (Fig. 6B, middle). As functional activation of the GAS promoter also was observed upon induction with IFN-α (Fig. 6A), we monitored the IFN type I response in cells expressing NS5. Our results verified the data obtained with IFN-γ by demonstrating a 36% reduction in luc activity in cells transfected with NS5 whereas a significantly lower 55% reduction was observed in cells expressing NS5(Y222A/S223A) (Fig. 6B, bottom). These results suggest that NS5 is a potent inhibitor of both IFN type I and type II signalling while NS5(Y222A/S223A) displayed a reduced ability to block JAK-STAT signalling.
Fig. 6. Expression of NS5 inhibits IFN-α-stimulated STAT1 phosphorylation.

A. (Top) HeLa cells not treated (left) or stimulated with 1000 U ml$^{-1}$ IFN-α for 30 min at 37° (right). To detect pSTAT1, anti-phospho (pTyr701) STAT1 antibody and Alexa Fluor-594-conjugated antibody (red) was used. (Middle) HeLa cells transfected with TBEV NS5 or NS5(Y222A/S223A) were stained for pSTAT1 as in Fig. 5. Arrows point to NS5-positive and pSTAT1-negative cells. (Bottom) HEK 293 cells transfected with TBEV NS5 or NS5(Y222A/S223A) and treated with IFN as described for HeLa cells. Arrows point to NS5-positive and pSTAT1-negative cells.

B. (Top) HeLa-lucRep cells stimulated with either 100 ng ml$^{-1}$ INF-α or INF-γ for 5 h. (Middle) HeLa-lucRep cells transiently transfected with either NS5 or NS5(Y222A/S223A) and stimulated with 100 ng ml$^{-1}$ INF-γ. (Bottom) HeLa-lucRep cells transiently transfected with either wild-type NS5 or mutant Y222A/S223A and stimulated with 100 ng ml$^{-1}$ INF-α. Bars indicates standard error of means (SEM) for six experiments; Student’s t-test *$P$ < 0.05. Results are expressed as $n$-fold increase in luciferase activity over identical cultures not treated with cytokines or percentage luc activity related to IFN-induced non-transfected cells (100%).

C. (Top) Treatment with sodium orthovanadate does not affect inhibition of STAT1 phosphorylation by NS5. HeLa cells transfected with NS5 were treated with 25 μM sodium orthovanadate for 6 h and then stimulated with IFN-α followed by immunofluorescence processing as in (A). Scale bar, 16 μm. (Bottom) Quantitative measurements of pSTAT1 in HeLa-lucRep cells transfected with NS5 and treated with sodium orthovanadate. Bars indicates SEM for six experiments; Student’s t-test *$P$ < 0.05. Results are expressed as $n$-fold increase in luciferase activity of cells not treated with cytokines.

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The precise mechanism for inhibition of the JAK-STAT pathway by any flaviviral NS protein is presently unknown. A prior study suggested that the JEV NS5 protein interferes with the IFN response via PTPs (Lin et al., 2006). To examine whether TBEV NS5 shares this mechanism for inhibition we employed the protein phosphatase inhibitor sodium orthovanadate, which acts on receptor-associated kinases and phosphorylated STAT1 (Huyer et al., 1997). Transfected HeLa cells pre-treated with sodium orthovanadate followed by IFN-α stimulation were investigated both by immunostaining and CLSM and in the HeLa-lucRep cell line. Sodium orthovanadate had no effect on pSTAT1 inhibition by TBEV NS5, either in CLSM analysis or in pSTAT1 reporter system (Fig. 6C).

**Knock-down of hScrib reverses inhibition of pSTAT1 by NS5**

As knock-down of hScrib is predominantly responsible for relocalization of TBEV NS5 from the plasma membrane, we wanted to determine how this would affect phosphorylation of STAT1. Initially, we monitored pSTAT1 accumulation to test whether knock-down of hScrib in HEK293 or HeLa affected JAK-STAT signalling. Results show that although the overall levels of internal control (α-Tubulin) and hScrib were lower in HEK293 than in HeLa, phosphorylation of STAT1 appeared uniformly in all lanes and in both cell types (Figs 5A and 7A). The knock-down of hScrib expression using siRNA#1 and #2 was ~60% of non-treated or CsiRNA-treated cells, evaluated by densitometry of Western blots (data not shown). Thus, as both siRNAs depleted hScrib equally well, subsequent cells that were hScrib silenced were transfected with siRNA#1. To address the role of NS5 antagonism in HEK293 depleted of hScrib, cells were stimulated with IFN-α and processed for CLSM. Importantly, we found that depletion of hScrib affected phosphorylation of STAT1 by preventing the inhibition imposed by NS5 (Fig. 7B). Quantitative analysis (described in Experimental procedures) of the digital images confirmed this by showing that the decrease in pSTAT1 accumulation was significant with a 90% inhibition of nuclear pSTAT1 in cells with endogenous hScrib levels expressing NS5, compared with a 50% inhibition in silenced cells (Fig. 7C). Likewise, cells containing hScrib and expressing NS5(Y222A/S223A) demonstrated an 80% inhibition of accumulated nuclear pSTAT1, compared with 40% inhibition in knock-down cells, thus displaying a similar variation in blocking as that demonstrated for NS5. Consistent with reversed inhibition of pSTAT1 in HEK293, wild type and mutant NS5 lost their ability to inhibit pSTAT1 even further in HeLa cells, with marked differences between untreated and hScrib-depleted cells (Fig. 7C). Silencing of hScrib efficiently affected blocking of the IFN response by NS5, suggesting that functional host factor binding is a crucial requirement for IFN antagonism by TBEV NS5.

Among numerous genes that are induced by IFNs are several members of proteins that contain GTPase activity, including the guanylate-binding proteins (GBPs). Based on its antiviral activity (Andersson et al., 1999), and that gbp contain both GAS and ISRE in its promoter (Decker et al., 1989), we addressed whether TBEV NS5 had any effect on GBP1. Our results demonstrate that while both TBEV NS5 and NS5(Y222A/S223A) blocked GBP1 protein expression, the activity of NS5(Y222A/S223A) appeared to be reduced as defined by the intensity of GBP1 blots applied by densitometry (Fig. 7D). In contrast, treatment of cells with siRNA did result in reversed GBP1 block at equal levels for both NS5 proteins (Fig. 7D). These data conclude that TBEV NS5 is a general inhibitor of downstream antiviral factors during IFN type I and II mediated signalling.

**Discussion**

In this study we used several experimental approaches to investigate the hScrib–TBEV NS5 complex and demonstrate the critical role of this association for IFN-stimulated JAK-STAT antagonism. We found that host factor binding correlated with the ability to block STAT1 phosphorylation, the transcriptional response of a GAS reporter and the gbp1 gene in mammalian cells. However, knock-down of hScrib reversed the inhibition by TBEV NS5, phenocopying the effects of a NS5 protein defective in binding hScrib.

It is evident that flaviviruses express abundant amounts of non-structural proteins that are not otherwise involved in virus growth (Ferlenghi et al., 2001; Kuhn et al., 2002; Quinkert et al., 2005). Diverse members of the flavivirus genus have evolved distinct strategies to counteract the IFN response. While non-structural proteins other than NS5 for WNV, DENV and KUNV have been implicated in JAK-STAT interference, NS5 is the main virulence factor for JEV and LGTV (Munoz-Jordan et al., 2003; Best et al., 2005; Liu et al., 2005; Lin et al., 2006). However, the precise mechanism behind viral inhibition of signalling components of the JAK-STAT pathway has not yet been determined. Our observation that the TBEV NS5 protein localizes partially at cell–cell contacts indicated that the hScrib association could have a functional implication for IFN signalling. Re-localization of NS5 in cells depleted of hScrib and the inability of NS5(Y222A/S223A) to accumulate at the membrane supported this notion. Indeed, TBEV NS5 completely blocked phosphorylation of STAT1 upon IFN stimulation in HeLa cells, which demonstrate that the protein shares the common IFN-antagonistic activity implicated for other NS5 proteins (Best et al., 2005; Lin et al., 2006). Under conditions in which TBEV NS5
blocks STAT1 phosphorylation, IFN-γ induction of NS5-transfected cells affected expression of GBP1 directly or indirectly and depended on hScrib. As promoters of human gbp genes contain both GAS and ISRE (Decker et al., 1991), our results probably reflect a general effect of TBEV NS5 on IFN type I and II mediated signalling.

We also tested the possibility that TBEV NS5 acts by means of phosphatase activation, as shown for JEV NS5 in a prior report (Lin et al., 2006), by treating cells with the phosphatase inhibitor, sodium orthovanadate (Blackwell and Brinton, 1997). However, NS5 could not revert the block of STAT1 phosphorylation. Because of non-specific side-effects we were not able to prime HeLa cells with sodium orthovanadate beyond 6 h. Pre-treatment with the agent for 6 h followed by IFN stimulation did not restore STAT1 phosphorylation in NS5-transfected cells. In this context it should be noted that Vero cells were treated with sodium orthovanadate for 16 h before JEV NS5 transfection and IFN induction (Lin et al., 2006). While it remains to be determined whether long-term exposure to orthovanadate reverses the actions of TBEV NS5 our preliminary data point to targets undeniably important for this protein in inhibition. It is not known whether JEV NS5 interacts with IFN receptors; however, Lin et al. (2006) observed that treatment with TYK2 PTP inhibitors did not reverse inhibition of JAK-STAT signalling. This suggests that the JEV NS5 protein may act downstream of the IFN receptors. Conversely, LGTV NS5 interacted physically with IFNAR2 and IFNγR1, implying that inhibition of STAT1 phosphorylation occurs proximal to the receptors (Best et al., 2005). Taking this into account together with our findings, it might be postulated that membrane residing TBEV NS5 protein suppresses STAT1 phosphorylation early in the JAK-STAT pathway and possibly by interfering directly with IFN receptor complexes or receptor-associated JAKs. As further evidence for JAK-STAT antagonism, NS5 reduced both IFN-γ- and IFN-α-induced STAT1 phosphorylation whereas inhibition by NS5(Y222A/S223A) was impaired in HeLa-lucRep assays.

We also show that the inhibitory effect on JAK-STAT signalling by NS5 was partially reversed during siRNA knock-down of hScrib. Expression of NS5 resulted in nuclear pSTAT1 regained to 50%, or higher in cells expressing NS5(Y222A/S223A), of the levels of siRNA-treated cells without NS5. Likewise, the reversed GBP-1 inhibition coincided with hScrib depletion. However, some variations were observed in pSTAT1 blocking between HEK293 and HeLa, indicating that NS5 might act by slightly different means in distinct cell types. Recent reports reveal apparently conflicting roles for the inhibition of JAK-STAT signalling by flavivirus NS5 proteins. Different lines of evidence suggested that IFN-induced JAK-STAT antagonism by the LGTV NS5 protein is conferred by the RdRp domain alone (Park et al., 2007). On the other hand, Lin et al. (2008) showed that neither the MTase nor the RdRp domains alone of JEV NS5 are capable of antagonizing the JAK-STAT pathway, indicating that the major part of the protein was needed for interference. These apparent discrepancies are further complicated by the fact that multiple signalling components involved in the JAK-STAT pathway are putative NS5 targets. Because LGTV NS5 also has been shown to interact with the IFN receptors (Best et al., 2005), it might be suggested that receptor-complex interaction and RdRp activity are mutually exclusive for inhibition. Retained JAK-STAT inhibition demonstrated by NS5(Y222A/S223A) in this study is not completely clear, although some possibilities exist. Given that TBEV NS5 interferes through direct association with receptor subunits, one explanation is that NS5(Y222A/S223A) is not constrained at IFN receptors for optimal blocking. Alternatively, protein folding and subsequent high-affinity binding to receptor-bound JAKs or other host factor(s) are impaired. A second possibility is that TBEV NS5 inhibits multiple signalling components simultaneously, for instance, by steric hindrance of receptors and by directly binding activated STATs and/or inhibiting nuclear translocation (Her et al., 1997; Miller et al., 1999; Ulane and Horvath, 2002; Palosaari et al., 2003; Chee and Roizman, 2004). Differences between our data and those of Park et al. (2007) are more difficult to reconcile considering the close relationship between LGTV and TBEV but may reflect inherent differences between the two species. TBEV NS5 share 88% identity with LGTV NS5; however, LGTV NS5 contains Phe at both 222 and 903, which indicate suboptimal PDZ motifs. Sequence comparison of NS5 proteins across the TBE complex of viruses showed that these residues are altered only in LGTV NS5 (data not shown). Yet, Tyr-to-Phe substitution in TBEV NS5 had no apparent effect on the binding capacity for hScrib (Fig. 1C), which may point to the possibility that LGTV NS5 interacts with hScrib. While a general ability for JAK-STAT antagonism among the tick-borne flaviviruses is likely to depend on C-terminal NS5 modulation, the overall efficiency to accomplish this may coincide with competence in binding hScrib. Given that an exact sequence is a prerequisite for PDZ binding (Thomas et al., 2001), it might be speculated that LGTV NS5 antagonize the IFN response suboptimally. This is feasible considering that the ability of LGTV to provoke an innate antiviral immune response is low as the virus is attenuated for humans (Pletnev and Men, 1998; Gritsun et al., 2003a). However, due to the complex nature of JAK-STAT signal transduction (redundancies and cross-talk between signal transduction pathways) inhibition by tick-borne flaviviruses probably involves interplay with other factors. These are likely to include other non-structural and structural proteins (Mandl, 2005; Evans and Seeger, 2007), as well as...
unknown host factor(s). Thus, we speculate that TBEV NS5 requires both the MTase and RdRp domains for JAK-STAT antagonism. The hScrib protein alone, or in concert with unknown cellular proteins, may confer proper plasma membrane localization of NS5 crucial for positioning of the RdRp domain proximal to the IFN receptor complexes.

It has been shown that scribble and other LAP family proteins assemble into macromolecular scaffolds, regulating localization of membrane receptors and stabilizing cell adhesion molecules (Apperson et al., 1996; Borg et al., 2000; Mathew et al., 2002; Metais et al., 2005; Qin et al., 2005; Kallay et al., 2006). While all members of this protein family share LRRs and LAP-specific
domains α/b, only scribble contains four C-terminal copies of the PDZ domain (Santoni et al., 2002). Thus, it may easily be envisaged that individual PDZ domains may selectively recognize a range of distinct C-terminal motifs, thereby offering a means to prevent non-specific recognition. However, a majority of hScrib-interacting ligands share a similar C-terminal class I PDZ-binding motif by which they interact (Nakagawa and Huibregtse, 2000; Mathew et al., 2002; Audebert et al., 2004; Takizawa et al., 2006). Our data suggest an altered binding pattern; even though a C-terminal PDZ motif exists in NS5, internal residues of NS5 were revealed to be crucial for binding to hScrib. While one explanation for this may arise from specificity of interacting residues in host and viral proteins, another possibility may be related to the length of individual hScrib PDZ domains. A longer sequence might be more flexible thus favouring internal binding. Also, comparing PDZ domains 1–4 of hScrib shows that PDZ4 overall contains additional residues in conserved regions as well as in the consensus binding pocket. Internal recognition is rare although PDZ domain-containing proteins are numerous in metazoans (Schultz et al., 1998; 2000). Nevertheless, accumulating evidence suggests that this might be a common mechanism for PDZ domain binding (Gee et al., 1998; Hillier et al., 1999; Hung and Sheng, 2002; Penkert et al., 2004). Thus, we speculate that this mode of PDZ targeting is more common than previously thought, applying to other viral proteins as well. Prediction of internal PDZ recognition has suggested exposure of binding residues within a secondary turn structure (Harris and Lim, 2001). We found that Tyr222 and Ser223 of TBEV NS5 are positioned adjacent to a loop region between subdomain 2 and 3 according to the DENV2 MTase crystal structure (Egloff et al., 2002). The additional mutations generated suggest that a structural framework is the basis for the association, as Tyr-to-Phe substitution did not alter the binding capacity for hScrib. In contrast, a Ser-to-Asp substitution, producing a phosphomimetic amino acid, was unable to confer binding to hScrib suggesting that phosphorylation might have a role in regulating this type of internal PDZ recognition.

Studies have shown that different PDZ proteins are substrates for individual viral proteins (Gardiol et al., 1999; Gaunsinger et al., 2000). However, human papilloma virus (HPV) E6 oncoprotein is the only viral protein hitherto identified to interact with hScrib (Nakagawa and Huibregtse, 2000). It is noteworthy that an independent study has demonstrated that HPV E6 inhibits JAK-STAT signalling (Li et al., 1999b). Whether the two events correlate for HPV E6 is unknown but plausible, as various examples of individual viral proteins interfering with unrelated parts of the cellular machinery have been reported (Lee et al., 2000; Alcom et al., 2001; Latorre et al., 2005; Munoz-Jordan et al., 2005; Sharma-Walia et al., 2005).

In summary, we have defined a new mechanism for IFN antagonism by the TBEV NS5 protein in transfected mammalian cell culture. Several lines of evidence show that this mechanism involves association with the host factor hScrib. Because of well-defined binding sites, multiscaffolding PDZ domain proteins might represent a putative target for the design of antiviral candidates (Harris and Lim, 2001; Dev, 2004). In this context our findings may add insight for future studies of the TBEV life cycle and flavivirus pathogenesis.

**Experimental procedures**

**Cell culture and transfection**

Madin–Darby canine kidney-I (MDCK-I), human embryonic kidney 293 (HEK293) and cervical epithelia HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, 2 mM L-Glutamine and 10% fetal bovine serum (FBS, Gibco). The HeLa STAT1-1 luc reporter cell line (HeLa-lucRep) (Panomics) was grown as above but supplemented with 100 μg ml⁻¹ Hygromycin (Roche) after 48 h. Cells used in IFN-α/β stimulation assays, immunoprecipitation or colocalization experiments were seeded onto 6-well, 12-well or 10 cm plates, to grow for 24 h. HEK293 and HeLa cells were transfected using Fugene 6 (Roche) following manufacturer’s instructions, using 0.5–2 μg of NS5 DNA plasmid. MDCK cells (5 x 10⁵) were transfected in suspension by electroporation (Amaza), adding 5 μg of DNA. The cells were cultured at 37°C in a humidified 5% CO₂ incubator and used for immunoblotting or immunostaining assays 24–48 h post transfection.
Antibodies, chemicals and cytokines

Antibodies in this study were as follows: mouse monoclonal anti-His (Amersham Biosciences), mouse monoclonal anti-V5 (Invitrogen), rabbit polyclonal anti-pStat1 (Tyr701), rabbit polyclonal anti-α-Tubulin, rabbit polyclonal anti-E-cadherin (Cell Signalling) and goat polyclonal anti-scrib (C-20, Santa Cruz Biotechnology); a rabbit polyclonal anti-scrib antibody was generated against GSThScrib(988–1630) and used for immunoprecipitation through the following procedure: injection of 200 μg of GSThScrib(988–1630) emulsified in Freund’s complete adjuvant were followed by three boost injections with 100 μg of protein emulsified in Freund’s incomplete adjuvant over a 12-weeks period. Antisera was verified by ELISA and immunoblotting tests and collected with a final bleed after 14 weeks (Agrisera). Sodium orthovanadate and protease inhibitor cocktail (Sigma) and recombinant human IFN-α/β and IFN-γ were purchased from R&D systems (UK).

Luciferase reporter assay

A total of 6 × 10^5 cells per well were seeded in 12-well plates and allowed to grow for 24 h before transient transfection with appropriate plasmids. Twenty to 34 h post transfection the medium was changed to FBS-free DMEM and where indicated supplemented with 25 μM Sodium orthovanadate (Na3VO4) and incubated for 1 h at 37°C. The cells were supplemented with IFN-γ or IFN-α (100 ng ml^-1 final concentration) and incubated for 5 h at 37°C. After a wash in phosphate-buffered saline (PBS) the cells were lysed with 1× Cell Culture Lysis Reagent (Promega). Luciferase assays were conducted using the luciferase assay system (Promega) following manufacturer’s instructions and monitored in a LUMIstar 96-well plate reader (BMG labTechnologies GmbH).

Preparation and transfection of siRNAs

Target and non-targeting control siRNA were designed by Ambion (USA). The two double-stranded hScrib-targeting siRNAs have been previously reported (Takizawa et al., 2006), and were as follows: #1: 5′-CAGGATGAGTGTCAATGAAACA-3′ and #2: 5′-CCGCGAGGGAGGATGGAGAA-3′. All siRNAs were provided freeze-dried, pre-annealed and high-performance liquid chromatography (HPLC)-purified from Ambion, re-suspended as a stock solution at a concentration of 100 μM and stored at −20°C until required. Twenty-four hours before each experiment HeLa or HEK293 cells were seeded in six-well plates at a density of 1 × 10^5 cells. For the transfection of siRNAs, 2 μM of each siRNA (final concentration 10 nM) was mixed with 12 μl of HiPerfect (Qiagen), diluted in Opti-MEM (Invitrogen) and added to cell cultures grown at 37°C for 72 h under antibiotic-free conditions. Forty-eight hours post siRNA transfection, cells were transfected with NS5 DNA plasmid as described.

Plasmids and mutagenesis

All recombinant DNA techniques and cloning procedures were carried out by standard procedures (Sambrook et al., 1989). Complete or truncated versions of genes encoding TBEV NS5 (HYPR, Accession No. U39292), DENV NS5 (TSV01 serotype 2, AY037116), WNV NS5 (M12294) or hScrib (NM_182706) were amplified by PCR introducing suitable endonuclease restriction sites (Table S3). Bait and prey constructs were cloned into pAS2-1 or pACT2 (Clontech) generating yeast two-hybrid plasmids expressing proteins or truncated polypeptides fused in frame with the BD or AD domains of GAL4 respectively. TBEV and DENV NS5 genes were cloned into pET-15b (Novagen) generating expression plasmids for E. coli production of NS5 proteins with an N-terminal polyhistidine sequence. The 3′ end of hScrib(nt 2964–4893) was cloned into pGEX-6P-3 (Amerham biosciences) for E. coli expression and purification of GST-hScrib(988–1630). For mammalian cell culture transfection, TBEV and DENV NS5 were cloned into expression vector pCDNA3.1/V5-His-TOPO (Invitrogen). Site-directed mutated proteins were generated using synthetic oligonucleotide primers, containing appropriate base changes (Table S3) and using the QuikChange XL-Site-Directed Mutagenesis Kit (Stratagene) following the manufacturers’ protocol. To verify clones of introduced deletions and mutations, constructs were sequenced at MWG-Biotech (Ebersberg, Germany).

Gal4 yeast two-hybrid screen and assays

TBEV NS5 fused in frame with the BD of Gal4 was transformed into the Saccharomyces cerevisiae MATa strain AH109 and introduced to a 37-year-old male Caucasian human brain cDNA library (Clontech) pre-transformed into the MATα strain Y187 by using yeast mating techniques. Diploid yeast cells were selected by growth on synthetic dropout media lacking leucine, tryptophan, histidine and adenine (SD-leu/trp/his/ade). Positive interactions were verified with a second reporter, using the β-galactosidase filter lift assay described previously (Johansson et al., 2001). pACT2/cDNA plasmids were isolated with the YEASTMAKER yeast plasmid isolation kit (Clontech), transformed into E. coli KC8 cells and selected on M9 thiamine-HCl plates lacking leucine but supplemented with ampicillin (100 μg ml^-1). Protein–protein interactions were analysed in the Gal4 yeast system with pairs of pAS2-1 and pACT2 fusion constructs according to the manufacturers’ protocol (Clontech). Briefly, bait and prey vectors were co-transformed into the S. cerevisiae strain AH109, and transformants were selected for on SD-leu/trp and re-streaked onto SD-leu/trp/His/ade medium, followed by 4–6 days of incubation at 30°C for confirmation of interacting proteins.

Protein expression and purification

HisTBEV NS5, HisDENV NS5 and GSThScrib(988–1630) were expressed in E. coli BL21-Codon Plus (DE3) (Novagen) by induction with 1 mM isopropyl β-D-thiogalactoside (IPTG) at 30°C for 3 h. Soluble proteins were extracted using Bugbuster Protein Extraction Reagent (Novagen) according to manufacturers’ instructions. HisTBEV NS5 and HisDENV NS5 were purified on HiTrap Chelating HP columns whereas GSThScrib(988–1630) and GST were purified on GSTrap FF columns using the ‘Akta’ prime protein purification system (Amerham Biosciences).

GST pull-downs, immunoprecipitations and Western blot analysis

For pull-down experiments, glutathione-sepharose 4B (Amersham) was pre-washed with pull-down buffer (PB) 20 mM...
Tris-HCl/140 mM NaCl/20 mM MgCl2/0.1% Triton X-100/1 mM β-mercaptoethanol pH 7.4. The beads were supplemented with 40 ng ml\(^{-1}\) GSThScrib or 40 ng ml\(^{-1}\) GST and with 4 ng ml\(^{-1}\) HisTBEV NS5 or 40 ng ml\(^{-1}\) HisDENV NS5 (final concentrations), respectively, and incubated for 3 h at 4°C with gentle agitation. The beads were washed five times with PB and eluted with 20 mM reduced Glutathione (Sigma). The protein fractions were separated on 12% SDS-PAGE, transferred to nitrocellulose membranes, detected with monoclonal anti-His antibody (1:3000) and visualized using secondary horseradish peroxidase (HRP)-conjugated sheep anti-mouse antibody (1:5000) and ECL reagents (Amersham).

For co-immunoprecipitation, HeLa cells transiently transfected with V5-tagged TBEV NS5, TBEV NS5(Y222A/S223A) and DENV2 NS5 were lysed and collected on ice for 30 min in 500 µl of lysis buffer (LB) 1% Triton X-100/50 mM Tris, pH 8.0/150 mM NaCl/5 mM EDTA/15 mM MgCl\(_2\)/2 mM Dithiothreitol/0.02% NaN\(_3\)/1 µg ml\(^{-1}\) Aprotinin/1 µg ml\(^{-1}\) Pepstatin/50 µg ml\(^{-1}\) PMSF. After 30 min centrifugation, lysates were incubated with 1–2 µg of monoclonal anti-V5 antibody (Invitrogen), incubated for 4 h at 4°C and recovered by binding to protein-A (Sigma) bead suspension (10% v/v in LB) for 2 h. Immunocomplexes were washed five times in LB, re-suspended in 40 µl of SDS sample buffer (Laemmli) and resolved on 10% SDS-PAGE. Precipitated proteins were transferred to nitrocellulose membranes and incubated with polyclonal anti-hScrib(988–1630) (1:10000) or monoclonal anti-V5 antibody (1:5000) and visualized as described.

For immunoblots of siRNA-treated cells, cells were lysed with LB 72 h post transfection and resolved on 8% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and incubated with goat polyclonal anti-Scrib (1: 2000), rabbit anti-α-Tubulin (1:1000) or anti-phospho-Stat1 (1:1000) and visualized as described.

For detection of proteins expressed in yeast cells transformed with plasmids encoding the hybrid protein were grown in LB, re-suspended in 40 µl of SDS sample buffer (Laemmli) and resolved on 10% SDS-PAGE. Precipitated proteins were transferred to nitrocellulose membranes and incubated with polyclonal anti-hScrib(988–1630) (1:10000) or monoclonal anti-V5 antibody (1:5000) and visualized as described.

Quantification of hScrib knock-down and pStat1 nuclear location

To determine the downregulation of hScrib, Western blots were quantified by densitometry analysis of n = 3 samples with means ± SD. For estimation of pSTAT1 levels during hScrib knock-down and NS5 transfection, the pStat1 fluorescence (F) intensity in the nucleus (Fn) and the cytoplasm (Fc) within individual cells was calculated according to Fn/Fc. The data (n = 22) were normalized by log-transform and presented as per cent mean ± SEM relative to controls: IFN-α or IFN-α+siRNA. Data processing was performed on images using Image J (NIH image software) and the statistical significance was determined using Student’s t-test with a two-tailed distribution where differences between the mean values of each group of samples were considered significant at a level of *P < 0.05.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Recognition of NS5 by hScrib bypasses requirement for a C-terminal motif.

Table S2. hScrib targets the 212–223 region of TBEV NS5.

Table S3. Primers for cloning and mutagenesis. The position of the primers are relative to respective gene with non-coding sequences (e.g. introduced restriction sites) which are in lower case. Indicated mutations are in bold and underlined.

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