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BRIEF REPORT

Tula hantavirus NSs protein accumulates in the perinuclear area in infected and transfected cells

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Abstract The small RNA segment of some hantaviruses (family *Bunyaviridae*) encodes two proteins: the nucleocapsid protein and, in an overlapping reading frame, a nonstructural (NSs) protein. The hantavirus NSs protein, like those of orthobunya- and phleboviruses, counteracts host innate immunity. Here, for the first time, the NSs protein of a hantavirus (Tula virus) has been observed in infected cells and shown to localize in the perinuclear area. Transiently expressed NSs protein showed similar localization, although the kinetics was slightly different, suggesting that to reach its proper location in the infected cell, the NSs protein does not have to cooperate with other viral proteins.

Keywords Hantavirus (*Bunyaviridae*) · Tula virus · Nonstructural (NSs) protein

Hantaviruses (genus *Hantavirus,* family *Bunyaviridae*) are enveloped, negative-strand viruses with a tripartite RNA genome. The large (L) genome segment encodes the RNA-dependent RNA-polymerase (the L protein). The medium (M) segment encodes two surface glycoproteins, Gn and Gc, generated from a single glycoprotein precursor. The small (S) segment encodes the nucleocapsid (N) protein, which encapsidates the genome RNA into three viral segments [1]. In addition, the S segment of some hantaviruses carries an overlapping (+1) open reading frame (ORF) for the nonstructural protein, NSs [2]. In this respect, hantaviruses resemble orthobunyaviruses (genus *Orthobunyavirus*, family *Bunyaviridae*) with their S segment encoding, in an overlapping fashion, two proteins: N and NSs [1, 3]. The predicted length of the NSs protein in isolates of different hantavirus species varies from 95 amino acid (aa) residues in Topografov and Khabarovsk viruses to 52 aa residues in Rio Segundo virus. The NSs proteins of Tula (TULV)- and Puumala (PUUV)-like viruses are 88-90 aa residues long, and those of Sin Nombre-like viruses are 63 aa residues long [2].

Until recently, there was only suggestive evidence that the hantaviral NSs ORF is functional: the part of the S segment with double-coding potential appeared more conserved than the adjacent regions [4–6]. Our data on TULV and PUUV showed that the hantaviral NSs-ORF is indeed functional and the protein acts as an interferon (IFN) antagonist. Transiently expressed NSs proteins of both TULV and PUUV inhibited the activities of the IFN-beta promoter and NF-kB- and IRF-3-responsive promoters in COS-7 cells. The decline in the expression of IFN-beta mRNA was evident in TULV-infected MRC5 cells or those transiently expressing NSs protein [7]. The competitiveness of two TULV isolates that differ in the length of the NSs ORF, TULV/Lodz and TULV/Moravia, was evaluated in IFN-competent and IFN-deficient cells [8]. In Vero E6 cells (which are IFN-deficient), both isolates survived equally well. In contrast, in the IFN-competent MRC5 cells, the TULV/Lodz isolate, which possesses the NSs ORF for the full-length protein of 90 aa, survived for more successive passages than the TULV/Moravia isolate, which contains the ORF for a truncated NSs protein (66–67 aa).

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It was concluded that expression of a full-length NSs protein is beneficial for the survival of the virus and its competitiveness in IFN-competent cells, but it is not essential in IFN-deficient cells.

Taken together, the data suggest an involvement of the hantaviral NSs protein in counteracting the innate immune response of the host. This ability, however, appears to be much weaker than the corresponding activities of orthobunya- and phleboviruses. For example, the NSs protein of Bunyamwera virus (BUNV, genus *Orthobunyavirus*) is a potent inhibitor of both host transcription and the IFN response. This protein induces modifications to the C-terminal domain of RNA polymerase II and interacts with the MED8 component of the Mediator protein complex [9, 10]. The NSs protein of Rift Valley fever virus (RVFV, genus *Phlebovirus*) strongly inhibits cellular RNA synthesis by interacting with the p44 subunit of the basal cellular transcription factor TFIIH and antagonizes IFN-beta gene expression and IFN production [11, 12]. Perhaps, the hantaviral NSs protein is a genuinely weak IFN antagonist because hantaviruses are the only bunyaviruses that cause persistent, rather than acute, infection in their natural hosts [13]. The 5′ termini of their genomes do not activate RIG-I [14]. In addition, hantaviral proteins Gn and N have been demonstrated to inhibit the IFN response as well [15].

The intracellular localization of BUNV NSs protein remains unknown due to the lack of specific antibodies (Abs). The NSs protein of RVFV forms filamentous structures in the nuclei of infected cells [16]. The NSs protein of another phlebovirus, Uukuniemi virus, shows granular cytoplasmic staining [17]. In this paper, to gain more insight on hantaviral NSs protein functions, we study its distribution in a host cell. Earlier, Abs raised against TULV NSs peptides showed high background reactivity (our unpublished observations). Therefore, in this paper, polyclonal Abs raised against recombinant GST-fused NSs protein were used for the study of NSs protein localization in infected cells. In addition, FLAG-tagged NSs was transiently expressed and detected with anti-FLAG antibodies.

TULV-NSs-GST fusion protein was expressed in competent *E. coli* cells by subcloning the NSs ORF of TULV (strain Moravia02, wild-type, encoding a protein of 90 aa [7]) into the pGEX2T expression plasmid (GE Healthcare, Waukesha, WI). NSs-GST fusion protein was purified using glutathione Sepharose 4B (GE Healthcare) followed by concentration by Amicon Ultra-4 10 kDa filter centrifugation (Millipore, Billerica, MA). As expected, in SDS-PAGE, the fusion protein appeared as a band of approximately 37 kDa (Fig. 1a). Polyclonal antibodies raised in rabbits (Eurogentec, Liege, Belgium) recognized the fusion protein in immunoblotting (Fig. 1b). In infected cell culture and tissue samples of infected *Microtus arvalis* (European common vole, a natural host for TULV), the antibodies also recognized a protein of the expected size (data not shown). Unfortunately, a somewhat weaker, cross-reacting band in this region was also seen with both mock-infected cells and non-infected rodents, thus hampering further progress in this direction.

The kinetics of the NSs protein expression and its distribution in TULV-infected cells was studied using immunofluorescent techniques. For these experiments, IFN-competent human umbilical vein endothelial cells (HUVEC) were grown in Endothelial Cell Basal Medium with supplement pack (containing FCS, ECGS/H-2, hEGF-5 and HC-500) (PromoCell, Heidelberg, Germany), gentamycin (50 μg/ml) and amphotericin (0.5 μg/ml). Cells were infected with TULV strain Lodz (which carried the full-length NSs-ORF [8]) on coverslips using an MOI of 0.2 FFU/cell. At 12 h, 1d, 2d, 4d, and 8d postinfection (p.i.), cells were fixed and stained for the NSs protein with rabbit Abs. FITC-labelled swine anti-rabbit Abs (1:40) were used as secondary Abs. Staining for the N protein using rabbit anti N-GST-Abs [18] served as a control (Fig. 2a). Both proteins were seen already at 12 h p.i., and expression was sustained for the whole observation period of 8 days, when it reached maximum intensity (Fig. 2b). In general, the NSs protein appeared simultaneously with the N protein but was not restricted to N-positive cells, suggesting that, in some cells, the NSs protein might be expressed even earlier (Fig. 2c). The NSs protein displayed a punctate distribution within the cytoplasm and seemed to accumulate in the perinuclear area (Fig. 2a, b).

Notably, the intracellular distribution of transiently expressed TULV NSs protein was essentially the same as in infected cells, although the kinetics of its accumulation in the perinuclear area was slightly different. Since the NSs antibodies were much less efficient in detection of the

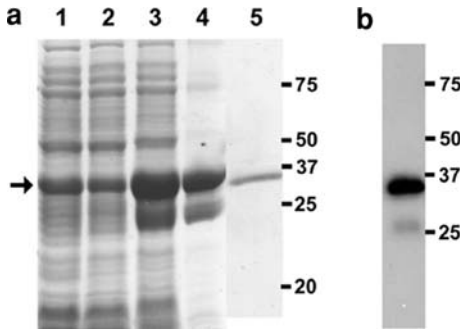

Fig. 1 **a** Expression and purification of TULV-NSs-GST fusion protein (Coomassie blue staining). Lanes: *1* soluble fraction of a cell lysate, *2* flow-through, *3* TULV-NSs-GST protein bound to Sepharose beads before washing, *4* TULV-NSs-GST bound to Sepharose beads after washing, *5* TULV-NSs-GST protein recovered from agarose gel and concentrated. **b** Immunoblot with rabbit polyclonal Abs raised against TULV-NSs-GST protein

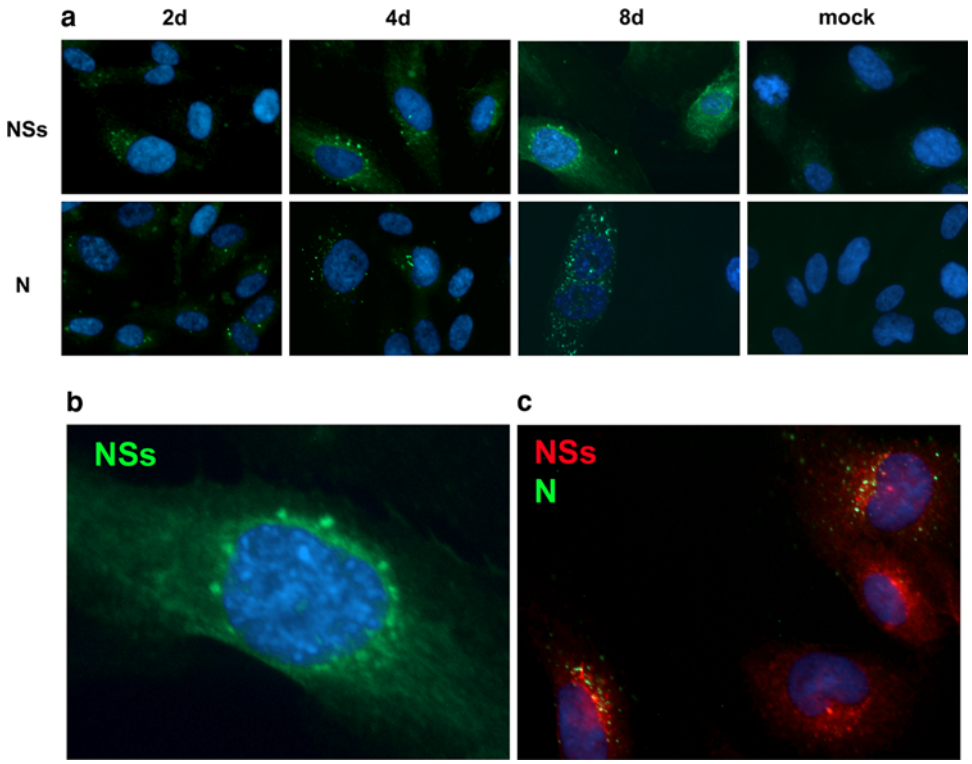

Fig. 2 **a** NSs and N proteins in TULV-infected HUVEC. Granular staining of NSs was observed in the cytoplasm of infected cells at 2d and 4d p.i. At 8d p.i., NSs staining was seen in the perinuclear area. **b** Higher magnification of a single NSs-positive cell at 8d p.i., showing accumulation of NSs outside the nucleus. **c** Double staining for the N and NSs proteins in TULV-infected HUVEC at 4d p.i. The NSs protein was seen in N-protein-positive as well as N-protein-negative cells, suggesting that the NSs protein might be expressed earlier after infection than the N protein

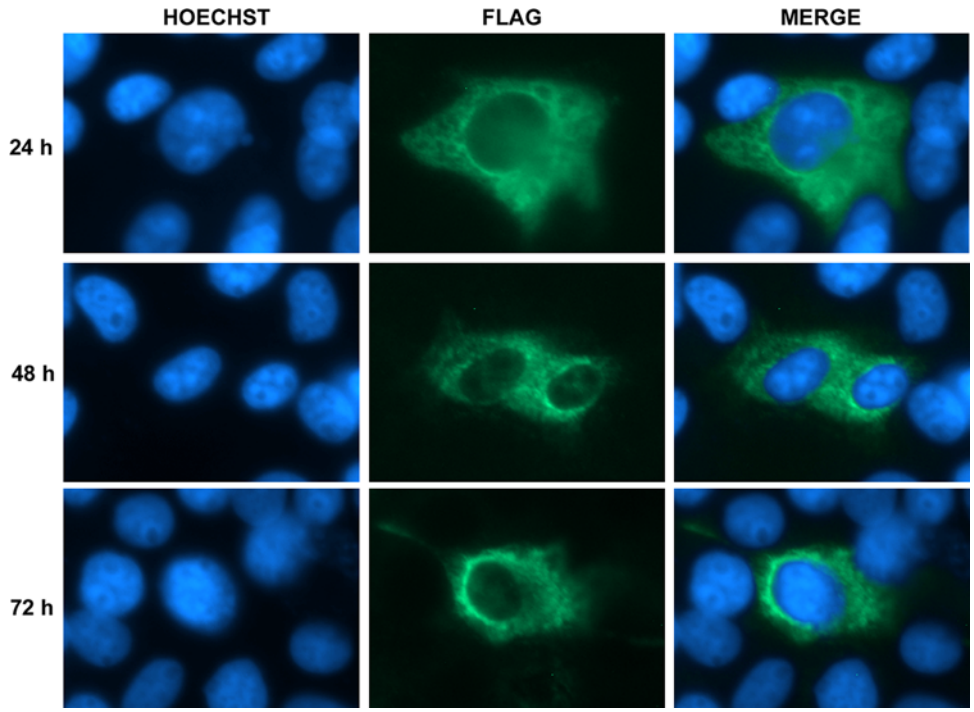

Fig. 3 Transiently expressed TULV (wt strain TUL/Moravia/5302 Ma/94 encoding full-length 90-aa NSs) NSs protein in COS-7 cells. After 24, 48 and 72 h, transfected cells grown on coverslips were fixed with ice-cold methanol and stained with mouse anti-FLAG Abs (Sigma–Aldrich, St. Louis, MO) diluted 1:1,000. At 48 h p.t., NSs protein started to translocate to the perinuclear area and showed some aggregation. At 72 h p.t., NSs protein was predominantly located around the nuclei

protein in transfected cells (data not shown), the FLAG-based approach [19] was chosen. COS-7 cells were grown on coverslips and transfected with pFLAG-cDNA + 3.1-TULwtNSs plasmid using Fugene 6 transfection reagent. At 24 h post-transfection (p.t.), the NSs protein appeared to be distributed throughout the cytoplasm. At time points 48 and 72 h, it had translocated to the perinuclear area but seemed not to enter the nuclei (Fig. 3). Staining in the perinuclear area was intense and mostly punctate or even granular.

Thus, for the first time, the NSs protein of a hantavirus has been observed in infected cells and shown to localize in

the perinuclear area. Transiently expressed NSs protein showed a similar localization. Two important conclusions could be drawn from these observations. First, it seems that, to find its proper location in the infected cell, the NSs protein does not have to cooperate with other viral protein(s). Second, since the hantaviral NSs protein is rather small (11 kDa, for TULV), it could diffuse through nuclear pores freely, not requiring assistance from specific nuclear transport mechanisms [20, 21]. As it accumulates in the perinuclear area but stays firmly outside the nuclei, one would assume that the NSs protein either forms oligomers or is involved in some specific interaction with yet unknown cellular partner(s). Recent yeast two-hybrid screening of a mouse cDNA library identified several promising candidates, including transcription factors (our unpublished data). Studies of NSs proteins of BUNV and RVFV demonstrated that bunyaviruses of distinct genera counteract the host innate immunity response by different means [12, 22 and references therein]. Interestingly, NSs proteins of TULV and PUUV failed to interact in the Y2H-assay with the p44 subunit of the transcription factor TFIIH complex (M. Bouloy and N. Le May, personal communication), suggesting that hantaviruses must differ from RVFV in the mechanisms they employ to antagonize the IFN response.

It would be interesting to see if the hantaviral NSs protein co-localizes with specific subcellular structures, e.g. P-bodies, stress granules, etc. Some of the NSs protein is colocalized with the N protein (Fig. 2c), which, in turn, is found abundantly in P-bodies [23]. Further investigations are on the way.

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