Hantavirus nucleocapsid protein interacts with the Fas-mediated apoptosis enhancer Daxx

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Hantaviruses cause two severe diseases, haemorrhagic fever with renal syndrome in Eurasia and hantavirus pulmonary syndrome in the Americas. To understand more about the molecular mechanisms that lead to these diseases, the associations of Puumala virus nucleocapsid protein (PUUV-N) with cellular proteins were studied by yeast two-hybrid screening. Daxx, known as an apoptosis enhancer, was identified from a HeLa cDNA library and its interaction with PUUV-N was confirmed by GST pull-down assay, co-immunoprecipitation and co-localization studies. Furthermore, domains of interaction were mapped to the carboxyl-terminal region of 142 amino acids in Daxx and the carboxyl-terminal 57 residues in PUUV-N, respectively. In pepscan assays, the binding sites of Daxx to PUUV-N were mapped further to two lysine-rich regions, of which one overlaps the sequence of the predicted nuclear localization signal of Daxx. These data suggest a direct link between host cell machinery and a hantavirus structural component.

Introduction

Hantaviruses, a genus of the family Bunyaviridae, are enveloped, spherical, negative-stranded RNA viruses with three genome segments, small (S), middle (M) and large (L), which encode the nucleocapsid protein (N), two envelope proteins (G1 and G2) and the RNA polymerase/ transcriptase (L protein), respectively (Schmaljohn et al., 1985; Schmaljohn, 1996; Plyusnin et al., 1996). Transcription and replication of viruses of the family Bunyaviridae are mediated by the L protein and directly co-ordinated with translation of the N protein (Jonsson & Schmaljohn, 2001).

Unlike other members of the family Bunyaviridae, which require arthropod vectors, hantaviruses are persistently maintained in the different species of rodents with which they have co-evolved (Schmaljohn et al., 1985; Schmaljohn, 1996; Plyusnin et al., 1996). Rodent carriers remain symptomless, whereas in man, hantaviruses cause two severe diseases, haemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) (Khan et al., 1996; Lee, 1996; Kanerva et al., 1998). Puumala virus (PUUV) is the causative agent of nephropathia epidemica, which, in general, is a milder form of HFRS (Brummer-Korvenkontio et al., 1980; Kanerva et al., 1998).

Apart from endothelial cells, monocyte/macrophages are considered to be the major targets of hantaviruses and presumably spread the virus via circulation to other parts of the body (Temonen et al., 1995). The prototype hantavirus, Hantaan virus (HTNV), has also been found in the B- and T-cells of HFRS patients. In particular, lung endothelium and kidney tubular cells are involved in HPS and HFRS, respectively. In addition, viral antigens have been detected in the brain, liver and heart and, to a lesser extent, in other organs or glands (Kanerva et al., 1998; Meyer & Schmaljohn, 2000). Although hantaviruses have a wide cell susceptibility, their growth is surprisingly slow even in the experimentally preferred Vero E6 cells, as well as in primary human kidney cells (Temonen et al., 1993). Hantaviruses do not cause any pronounced cytopathic effect; yet a more recent study (Kang et al., 1999) demonstrated that HTNV-infected Vero E6 cells slowly undergo apoptosis: the detailed mechanism of virus-induced apoptosis is still unclear.

There is as yet limited information on the biological functions of the hantavirus gene products to explain the molecular mechanisms that trigger and control the presumed
pathogenesis of HFRS and HPS in the infected tissues, in which, for example, elevated levels of T-cell inflammatory cytokines, such as TNF-α and IFN-γ, are considered to be important markers of pathogenesis (Peters et al., 1999). We thought that direct interactions of hantavirus gene products with host cell proteins could be central for both HFRS and HPS. The search for cellular proteins that interact with the nucleocapsid protein (N) of PUUV (PUUV-N) in particular was thus of interest to us. The N protein is the major structural component of hantaviruses, it is abundant in infected tissues and is a major target of the immune response (Vapalahti et al., 1992; Van Epps et al., 1999; de Carvalho Nicacio et al., 2001).

We performed yeast two-hybrid screening with a HeLa cDNA library and identified the protein Daxx, well known as a Fas death-domain adaptor protein, which transduces death signals through the Jun N-terminal kinase (JNK) pathway (Yang et al., 1997). To our knowledge, PUUV-N is the first viral protein proposed to interact with Daxx. In this report, we describe the interaction between Daxx and PUUV-N, as confirmed by GST pull-down assay, co-immunoprecipitation and co-localization studies.

**Methods**

- **Cell cultures and antibodies.** Human embryonic kidney (293-T) and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) and MEM, respectively, with 10% heat-inactivated foetal calf serum, glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO2. Generation of rabbit polyclonal antibodies against the amino-terminal two-thirds of PUUV-N has been described previously (Vapalahti et al., 1992). Monoclonal antibody (mAb) 3H9 against PUUV-N, which was produced in bank voles (a generous gift from Dr Åke Lundkvist, Swedish Institute for Infectious Disease Control), was characterized previously (Lundkvist & Niklasson, 1992). A rabbit polyclonal antibody against the carboxyl terminus of Daxx and a mAb against VP16 were purchased from Santa Cruz Biotechnology.

- **Virus infection.** The prototype of PUUV Sotkamo strain, adapted previously to Vero E6 cells, was used in this study. PUUV was produced in Vero E6 cells and its titre was determined as described (Kanerva et al., 1999). All processes of handling live virus were performed in a laboratory of biosafety level 3. For infection, an equal amount of virus (0.1 f.u. per cell) was added to each well of a 24-well plate in which Vero E6 monolayer cells had been transfected with pEBB-HA-mDaxx overnight. After absorption for 1 h at 37 °C, the virus inoculum was removed and replaced with complete medium. After 14 days, cells were fixed for immunofluorescence study.

- **Plasmid constructions and transfections.** The coding sequence of PUUV-N was obtained from the S segment of PUUV Sotkamo strain by RT–PCR, as described previously (Vapalahti et al., 1992), and then subcloned into the yeast and bacterial expression vector pGEX-4T (Pharmacia Biotech), as well as mammalian expression vector pAHC (Täiner et al., 1999), with appropriate restriction enzymes. In brief, pGEX-4T-1-E11, used for the production of GST–E11 fusion proteins, pAHC-E11, used in co-immunoprecipitation assays, and pVP16-E11, used in the mammalian two-hybrid assay, were constructed by subcloning with EcoRI and XhoI restriction enzymes. The E11-encoding sequence from pIG-E11 was obtained from library screening. Two deletion mutants of PUUV-N in bait plasmids (pEG202-PUUV-N5 and pEG202-PUUV-N7) were made using PCR cloning techniques. The template was pcdNA3-PUUV-N and the forward primer used for both reactions was 5’ «TCCCCGCGGTTACCATGACTGACCTGACAGAT 3’». The reverse primer for producing the amino-terminal 338 residues of PUUV-N (named PUUV-N5) was 5’ GGGCTCGAGTTATATGAAAAAAGCC 3’ and for the amino-terminal 214 residues (named PUUV-N7) was 5’ GGGCTGAGATGTATCAAGACTGATCTG 3’. The coding region of the S segment of PUUV from pGEM-3Z-PUUV-S was subcloned into either pcdNA3 to produce pcdNA3-PUUV-N, which was used in immunoprecipitation assays, or pM vector to produce pM-PUUV-N, which was used in the mammalian two-hybrid system. The deletion mutant encoding the carboxyl-terminal 57 residues of PUUV-N in pM (named pM-PUUV-N) was generated by direct subcloning with appropriate restriction enzymes. pEBB-HA-mDaxx, haemagglutinin-tagged murine Daxx, was kindly provided by Dr David Baltimore (California Institute of Technology). Interactions in yeast were characterized by β-galactosidase assays, according to the manufacturer’s instructions (Clontech). The FuGENE 6 Transfection reagent (Boehringer Mannheim) was used to transfect eukaryotic expression vectors into 293-T cells, according to the manufacturer's instructions.

- **Yeast two-hybrid system.** Yeast two-hybrid assays were carried out according to the instructions of Clontech. A HeLa cDNA library in pG4-5 (Clontech) was used for screening the interacting partners of PUUV-N in the bait plasmid pEG202 in yeast strain EGY48.

- **Mammalian two-hybrid system.** The reporter plasmid pG5luc (Promega) was co-transfected in combination with pM and pVP16 (both plasmids from Clontech) constructs, described above, into HeLa cells grown on 6-well plates. After 48 h, cell lysates were prepared and luciferase activity was determined following the instructions provided by Promega.

- **GST pull-down assay.** The procedures for expressing GST fusion proteins were done by following the manual from the manufacturer (Pharmacia Biotech). In short, Escherichia coli strain DH5α was transformed with pGEX-4T-1-E11, grown in LB broth containing 100 µg/ml ampicillin and induced for protein expression in 0.1 mM of IPTG at room temperature for 4 h. Bacterial cells were harvested, washed twice in cold PBS by centrifugation at 5000 r.p.m. and disrupted by sonication in lysis buffer (PBS with 1% Triton-X-100). Appropriate volumes of glutathione–Sepharose 4B beads were added to the supernatants and beads with bound recombinant proteins were used directly for in vitro-binding assays. baculovirus-expressed PUUV-N was prepared as described previously (Vapalahti et al., 1996). For in vitro-binding assays, different dilutions of recombinant PUUV-N were incubated with a constant volume of GST- and GST–E11-bound beads, respectively. Incubations were carried out for 2 h at 4 °C in E1A modified buffer (50 mM HEPES, pH 7–8, 50 mM NaCl, 10% glycerol, 0.1% NP-40 and 5 mM EDTA). The beads were then washed four times in the same buffer before SDS–PAGE. Polyclonal antibodies were subjected to both Coomassie blue staining and immunoblotting, in which complex formation between E11 and PUUV-N was monitored using the anti-PUUV-N mAb, 3H9.

- **Pepscan assay.** The carboxyl-terminal 243 amino acids of Daxx were synthesized as 18-mer overlapping peptides with a three-residue shift on a cellulose membrane by an Abimed Autospot Robot ASP 222. For interaction studies, the membrane was blocked overnight at 4 °C with 3% BSA in TBST (10 mM Tris, pH 7.4, 150 mM NaCl and 0.05%
Yeast two-hybrid screening of cellular proteins interacting with PUUV-N

The aim of our study was to identify cellular proteins that interact with PUUV-N. Over 1000000 clones from a HeLa cell cDNA library were screened using PUUV-N as bait and about 100 surviving clones were obtained. Restriction analysis of 42 clones gave eight groups and sequencing of group representatives showed that 12 of the clones (28%) were derived from the open reading frame of Daxx, a Fas-mediated apoptosis enhancer (Yang et al., 1997). Another protein identified in screening was the ubiquitin-like protein SUMO-1 (Ishov et al., 1999). The Daxx clones represented the carboxyl-terminal part of Daxx and had two different sizes, 243 (represented by E11) and 142 (represented by A10) residues (Fig. 1). Interactions were confirmed by retransformation. In β-galactosidase assays, both SUMO-1 and Daxx clones gave positive signals with full-length PUUV-N (Table 1).

Determination of the interacting region on PUUV-N with Daxx

To confirm that the interaction between Daxx and PUUV-N is specific, we performed a series of experiments using PUUV-N deletion mutants in both yeast and mammalian two-hybrid systems. Two PUUV-N gene deletion constructs, which lacked the carboxyl-terminal 95 residues (PUUV-N2; Δ339–433) and 219 residues (PUUV-N3; Δ214–433), respectively, were made (Fig. 1). In yeast two-hybrid assays, both PUUV-N2 and PUUV-N3 in the DNA-binding hybrid abolished its interaction with the two Daxx clones A10 and E11 in activation domain vectors (Table 1), indicating that the carboxyl terminus of PUUV-N is essential for this interaction. The N protein of Tula virus (TULV; an apathogenic hantavirus), which shares more than 80% identity with PUUV-N, also interacted with both A10 and E11 in the yeast two-hybrid system (data not shown).

To provide direct evidence for the carboxyl-terminal interaction of PUUV-N and Daxx, the construct PUUV-N4 (Fig. 1), encoding 57 carboxyl-terminal residues of PUUV-N, was created. The mammalian two-hybrid assay results show that this very short carboxyl-terminal region of PUUV-N is sufficient for Daxx interaction, since the luciferase activity of the fragment (pM-PUUV-N-N4 + pVP16-E11) was well above vector background and twofold higher than the corresponding activity with full-length PUUV-N (pM-PUUV-N+pVP16-E11) (Fig. 2). Interestingly, this region of the N protein is free of antigenic activity but seems to be crucial for N–N self-interaction (Kaukinen et al., 2001).

Interaction of PUUV-N and Daxx in vitro

To see whether the interaction between Daxx and PUUV-N is direct in the absence of protein mediators, we performed a GST pull-down assay using recombinant GST–E11 and baculovirus-expressed, purified PUUV-N. As shown in Fig. 3, PUUV-N co-purified with the GST–E11 fusion protein but not with GST, indicating a direct physical association of PUUV-N and E11. The shorter Daxx construct, GST–A10, gave exactly the same results as that of GST–E11 (data not shown). These experiments also suggest that the carboxyl-terminal 142 residues of Daxx, which includes the Fas-binding domain, is sufficient for its interaction with PUUV-N.

Two lysine-rich regions in the carboxyl-terminal part of Daxx are the binding sites with PUUV-N

To identify further the binding region of Daxx to PUUV-N, we carried out pepscan analysis of 243 carboxyl-terminal residues of Daxx. Two lysine-rich regions of Daxx reacted
strongly with baculovirus-expressed PUUV-N. One region contained the motif PPCKKSRR, which, by prediction, is a nuclear localization signal (NLS) of Daxx (Pluta et al., 1998); the other binding region contained the peptide KNKKKI. This indicates that basic amino acid residues are essential for binding of PUUV-N (Fig. 4a, b). Taken together, we conclude that PUUV-N can associate with the two carboxy-terminal lysine-rich regions of Daxx, one of which is the predicted NLS motif.

**PUUV-N associates with Daxx in mammalian cells**

To study the interaction in vivo, we performed co-immunoprecipitation assays. Since full-length Daxx in our experiments was toxic to cells, we turned to use the E11 mammalian expression construct (pAHC-E11), which proved to give better expression levels. When 293-T cells were co-transfected with PUUV-N and E11 constructs, immunocom-

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**Table 1. Determination of the interacting region of PUUV-N with Daxx and SUMO-1 in the yeast two-hybrid system**

The DNA binding (pEG202) and activation (pG4–5) vectors were used as negative controls. PUUV-N, PUUV-N² and PUUV-N³ (as LexA-fusions) and A10 and E11 (as activation hybrids) were re-transformed into yeast. Interactions were assessed by filter assays. Interactions were scored according to the rate at which colonies turned blue as follows: < 2 h, ++; after 2–6 h, ++; overnight incubation and still white, − .

<table>
<thead>
<tr>
<th>DNA-binding hybrid</th>
<th>Activation hybrid</th>
<th>β-Galactosidase assay</th>
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<tr>
<td>pEG202</td>
<td>E11</td>
<td>−</td>
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<tr>
<td>pEG202</td>
<td>SUMO-A</td>
<td>−</td>
</tr>
<tr>
<td>LexA-PUUV-N</td>
<td>pG4–5</td>
<td>−</td>
</tr>
<tr>
<td>LexA-PUUV-N</td>
<td>SUMO-1</td>
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<td>LexA-PUUV-N</td>
<td>A10</td>
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<td>LexA-PUUV-N</td>
<td>E11</td>
<td>++ ++</td>
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<tr>
<td>LexA-PUUV-N²</td>
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<td>−</td>
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<tr>
<td>LexA-PUUV-N³</td>
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**Fig. 1. Schematic diagram of deletion mutants for PUUV-N (PUUV-N², PUUV-N³ and PUUV-N⁴) and Daxx (A10 and E11). The known functional domains of Daxx are illustrated.**

**Fig. 2. Association between Daxx and PUUV-N in the mammalian two-hybrid system. pM-PUUV-N and pM-PUUV-N⁴ were co-transfected with pVP16-E11 into HEK cells, respectively. The pM and pVP16 empty vectors were used as negative controls. Luciferase activity reflects interactive activity.**

**Fig. 3. GST–Daxx pull-down assay. Increasing amount of recombinant PUUV-N bound to the same amount of GST–E11 Sepharose beads (lanes 4–6) but not to GST (lanes 1–3). The equal loading of GST and GST–E11 was confirmed by Coomassie Staining (data not shown).**
Hantavirus nucleocapsid protein binds to Daxx

Fig. 4. Mapping of the PUUV-N binding site in Daxx by pepscan assay. (a) All peptides containing the residues PCKKSRK were bound to PUUV-N. (b) Peptides containing the residues KNKSKI were bound to PUUV-N.

Fig. 5. Association of PUUV-N and Daxx. 293-T cells were transiently transfected with empty vector (lanes 1 and 4), PUUV-N (lanes 2 and 5) alone and PUUV-N together with the Daxx deletion mutant E11 (lanes 3 and 6). Cell lysates were subjected to immunoprecipitation with polyclonal antibodies to Daxx (lanes 4–6). Immunocomplexes were detected using the anti-PUUV-N mAb 3H9. Expression of Daxx was confirmed by immunostaining.

plexes of these two proteins could be precipitated using a polyclonal antibody against Daxx (Fig. 5). This indicated that PUUV-N is able to associate with Daxx in mammalian cells.

To visualize further where PUUV-N binds to Daxx in the cell, we overexpressed full-length murine Daxx (mDaxx) in Vero E6 cells infected with PUUV and HeLa cells co-transfected with PUUV-N (Fig. 6). In some PUUV-N transfected and PUUV-infected cells alone, the N protein remained in the cytoplasm and perinuclear regions, where it formed granular or fibre-like structures (Yanagihara & Silverman, 1990). The overexpressed mDaxx was predominantly nuclear with a relatively diffuse distribution, while endogenous Daxx in Vero E6 cells was weakly expressed with a pattern of a few dots. However, when mDaxx and PUUV-N were co-expressed in the same transfected or infected cells, N-protein structures were localized in the nucleus in about 10% of such co-expressing cells in three independent experiments in which 200 cells (altogether) were counted. Both the endogenous and...
transfected mDaxx form nuclear dots consistent with nuclear bodies. The dot-like nuclear staining of PUUV-N in mDaxx-transfected cells indicates their co-localization with PML oncogenic dots (PODs). Endogenous Daxx was not sufficient to recruit PUUV-N to the nucleus, at least not to any visible extent. Next, we thought that the natural presence of different compartments of Daxx (in the nucleus) and PUUV-N (in the cytoplasm) limits their contact. We therefore created two constructs, VP16-TULV-N (data not shown) and VP16-PUUV-N (VP16; a 44 residue polypeptide containing an NLS signal). After transfection with mDaxx, almost 100% nuclear co-localization of the N protein with Daxx in all co-expressing cells was observed (Fig. 7).

Discussion

In this report, we provide the first evidence of interaction between PUUV-N and Daxx, a protein identified originally as a Fas-mediated apoptosis enhancer (Yang et al., 1997; Chang et al., 1998, 1999; Hollenbach et al., 1999).

The apoptotic function of Daxx appears to be directed either by nuclear Daxx as a transcriptional regulator (Torii et al., 1999; Zhong et al., 2000a) or by cytoplasmic Daxx as a Fas receptor-associated protein that mediates the activation of JNK and programmed cell death by Fas (H. Li et al., 2000; Zhong et al., 2000b; Perlman et al., 2001). Some similarity, both in the primary and secondary structure (data not shown), was observed between the Daxx-interacting domains of PUUV-N and ETS-1-pointed domain region, which has been reported to interact with Daxx (R. Li et al., 2000). The possible significance of this relatively distant homology is also that the PUUV-N and Daxx interaction could be important for host defence.

HTNV infection of Vero E6 cells leads gradually to apoptosis and has been linked with post-translational degradation of Bel-2 (Kang et al., 1999). Furthermore, Bcl-2 is transcriptionally regulated by p51-ETS-1 and p42-ETS-1 (R. Li et al., 2000). It is therefore possible that hantaviruses may interfere with the apoptotic pathway at the post-translational level and could use Daxx as a mediator.

In the nucleus, Daxx is bound to chromatin or to the nuclear bodies called ND10 or PODs (Everett et al., 1999; Ishov et al., 1999). In the absence of sumoylated PML, Daxx is located in the condensed chromatin or centromeres and is recruited under cell cycle-dependent regulation to PODs together with the ubiquitin-like protein SUMO-1 (Ishov et al., 1999; Maul et al., 2000; Zhong et al., 2000a). It has been shown that the transcription factors of DNA viruses can cause disruption of nuclear PODs, which, in the case of herpes simplex virus immediate early Vmw110 protein, is mediated by proteasome-type degradation of the SUMO-1-modified POD proteins PML and SP100 (Everett et al., 1998). The responsiveness of POD structures and their dynamic counterparts, centromeres, to virus infections and other stress factors is functionally relevant for viruses. POD proteins can be either antiviral or they may assist virus replication, transcription and assembly (Chelbi-Alix et al., 1998; Bell et al., 2000). Recently, it was reported that PUUV-N could be found at perinuclear membranes in infected Vero E6 cells (Ravkov & Compans, 2001). We observed that overexpression of Daxx leads to the accumulation of PUUV-N in the nuclei. Our co-localization data implicate that hantaviruses have the potential to interfere with POD structures through interaction with Daxx. It is tempting to speculate that the interaction of PUUV-N with Daxx may be transient and takes place either in the cytoplasm or in the nucleus, for example, prior to nucleocapsid assembly and/or under stimulation of stress factors like apoptotic stimuli, Fas ligands or TNF-α.

Two recent reports propose that translocation of Daxx to the cytoplasm is mediated by its association with the apoptosis signal regulating kinase 1, Ask1 (Charette et al., 2001; Ko et al., 2001). Daxx contains two predicted NLSs (Pluta et al., 1998). One of the Daxx NLSs was found to bind to PUUV-N in pepscan assays. Additionally, another lysine-rich region was found also to interact with PUUV-N. It has been reported that cytosolic proteins interacting with NLS-containing proteins act as receptors for nuclear import (Adam & Gerace, 1991). Our present findings indicate that PUUV-N may be involved in nuclear events through interactions with NLS-containing proteins such as Daxx. The detailed mechanisms on how Daxx would regulate PUUV-N translocation and what role of PUUV-N is in the nucleus deserve further studies. It is also of interest to us whether the TNF-α-responsive Ask-1 activation would lead to a cytoplasmic interaction of hantavirus N and Daxx proteins.

Unravelling protein–protein interactions can provide new insight into both the replication and pathogenesis of hantaviruses. The main focus of our future studies is, on the one hand, on the nuclear association of PUUV-N and, on the other

![Fig. 7. Co-localization of VP16-PUUV-N with mDaxx in HeLa cells. The expression constructs for VP16 and VP16-PUUV-N combined with mDaxx were transiently co-transfected and co-immunostained with anti-VP16 and anti-Daxx antibodies. Nuclei were visualized by Hoechst 33342 staining (data not shown).](image-url)
hand, on the effects that Daxx, Fas and TNF-α may have on hantavirus infection and pathogenesis.

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