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Hantavirus nucleocapsid protein: a multifunctional molecule with both housekeeping and ambassadorial duties

Brief Review

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Summary. In recent years important progress has been made studying the nucleocapsid (N) protein of hantaviruses. The N protein presents a good example of a multifunctional viral macromolecule. It is a major structural component of a virion that encapsidates viral RNA (vRNA). It also interacts with the virus polymerase (L protein) and one of the glycoproteins. On top of these "house keeping" duties, the N protein performs interactive "ambassadorial" functions interfering with important regulatory pathways in the infected cells.

Introduction

Genus *Hantavirus*, together with four other genera, *Orthobunyavirus, Phlebovirus, Nairovirus* and *Tospovirus* plus others comprise the family *Bunyaviridae* [18]. Rodents are the principle hosts of hantaviruses that can be transmitted via aerosolized excreta to humans, some causing hemorrhagic fever with renal syndrome (HFRS) or hantavirus pulmonary syndrome (HPS). Hantaviruses are the most widely distributed zoonotic viruses of rodents [42]. They are regarded as emerging pathogens since new viruses are discovered frequently; so far at least 22 hantavirus species have been identified [18].

Hantaviruses are enveloped, negative-sense RNA viruses. The hantaviral genome consists of small (S), medium (M) and large (L) segments encoding respectively the nucleocapsid (N) protein, two surface glycoproteins (Gn and Gc, formerly called G1 and G2), and the viral polymerase (L). The hantaviral particle is spherical or oval in shape with a diameter ranging from 80 to 120 nm [18]. The virus has an envelope formed by heterodimers of the glycoproteins Gn and Gc, and membrane lipids derived from the host cell. Within the envelope, three genomic RNA segments (vRNA) and the N proteins form three separate, filamentous,

2.5-nm wide RNPs with which the L protein is associated. As nucleoproteins of many negative-strand RNA viruses (NSRV), hantaviral N protein is a multifunctional molecule involved in various interactions during the life cycle of the virus. It has important functions in the viral RNA replication, encapsidation and also in the virus assembly. The current data suggest that the N protein undergoes oligomerization and the formation of a trimer is a crucial step in this process. It is thought that the trimerization is initiated by interactions between the coiled-coil motifs in the N-terminus and continued via helix protrusions in the C-terminus. The oligomerization also seems to be a prerequisite for the N protein targeting the sites of replication that occur in the perinuclear membranes. The N protein has been shown to interact with several cellular proteins, e.g. with Daxx- and SUMO-1-pathway constituents, and thus can modulate the metabolism in infected cells.

This review focuses on the structure-functional organization of the hantaviral N protein, particularly on the domains involved in the oligomerization, interactions with other viral components as well as cellular proteins. Relevant observations, which could be possible parallels, made on other bunyaviruses and also other negative strand RNA viruses (NSRV) with both segmented and nonsegmented genome are described as well.

Brief overview of a hantaviral replication cycle

Virus attachment and entry into cells are the first steps in the replication cycle. Hantaviruses infect many types of cells in culture including endothelial cells and monocyte/macrophages [80, 105]. Recently, dendritic cells were shown to be susceptible to hantaviral infection [85]. For cellular entry, integrins have been found to act as receptors for hantaviruses [25, 27]. Human pathogenic hantaviruses causing HFRS and HPS use β3-integrins and apathogenic viruses use β1-integrins [26]. Since β3-integrins are known to regulate vascular permeability and platelet activation, it has been speculated that hantaviral interactions with β3-integrins may contribute to viral pathogenesis [64]. Recently, a 30-kDa protein has been suggested as an additional receptor for Hantaan virus [51]. No further characterization of the 30-kDa protein has been published so far.

After attachment, hantaviruses are taken up by the receptor-mediated endocytosis using clathrin-coated vesicles that are targeted to early endosomes [41]. The exact site of uncoating is not known but it is believed that the acidic environment in endosomes induces fusion of virus membrane with the endosome membrane leading to release of the viral RNP to the cytoplasm. It is logical to assume that the L protein stays associated with the complex formed by the vRNA and N protein. Shortly after the RNP release, primary transcription, i.e. synthesis of mRNA, begins (for a recent review on hantaviral transcription and replication, see [55]). The L protein cleaves 7–18 nucleotides (nt)-long primers for virus mRNA synthesis from the 5′ termini of cellular mRNA molecules present in the cytoplasm. Transcription continues according to the "prime-and-realign" mechanism that ensures that the exact copy of the 3′ end of the vRNA is produced [24]. Each primer has a G nucleotide at the 3′ end that pairs with the C nucleotide at the position 3 on the vRNA template (3′AU**C**AUCAUC...). After short initial elongation of

the primer, the newly synthesized RNA shifts three nucleotides backwards and realigns with the template. It has been observed that the transcribed virus mRNA is shorter than vRNA and its exact complementary copy, the antigenomic cRNA, suggesting that the transcription is terminated before the L protein reaches the 5′ end of the genome RNA template [37]. The mechanism(s) of mRNA synthesis termination remain, however, largely unknown.

Immediately after the appearance of mRNAs, the translation of viral proteins begins. Upon an unknown mechanism, perhaps by reaching of a threshold level of viral proteins in the cytoplasm, viral genome replication begins. As a first step, cRNA is produced. Next, cRNA is used as a template for the synthesis of new vRNA. The accumulation of vRNA probably activates the secondary transcription which further increases both mRNA and virus protein production [43].

Although the exact location for hantaviral RNA synthesis is not known it is safe to assume that, similarly to other bunyaviruses [18, 89], it occurs in the cytoplasm. Recently hantaviral L and N proteins were found to be membrane-associated and localized to the perinuclear region [56, 86]. Hantaviral RNA replication could occur on cytoplasmic surfaces of cellular membranes (similarly to alphaviruses) or on surfaces of small vesicles (similarly to poliovirus) [92]. In addition, hantaviral N protein forms filamentous structures in infected cells. Specifically, the N protein of Black Creek Canal virus has been shown to interact with actin, and actin depolymerization reduced the virus production in cell culture [88] suggesting that cytoskeletal filaments might be somehow involved in the replication. It is possible that membrane-associated or perhaps actin filament-associated viral replication would ensure high local concentrations of the proteins and substrates involved and hence accelerate the process.

Encapsidation of cRNA and vRNA into RNPs seems to happen in a concerted fashion, i.e. as soon as RNA is synthesized, it is immediately encapsidated by the N protein. Encapsidation requires the newly synthesized N protein that appears by 2 h post-infection (p.i.) [46]. The fact that cRNA and vRNA molecules are complexed with the N protein presents a challenge for the virus replication machinery since the N protein has to detach (or be detached) from vRNA when the L protein synthesizes the new RNA strand.

Gn and Gc proteins are synthesized on the endoplasmic reticulum membrane where they are cotranslationally cleaved, glycosylated and transported to the Golgi complex. Accumulation of Gn and Gc proteins on the Golgi complex has been thought to determine the budding site for hantaviruses [82]. Since they do not have a matrix protein, the N protein likely has a crucial role in linking RNPs to envelope proteins. The three encapsidated RNA segments (S, M and L) are incorporated into virus particles. Details of this step remain largely unknown.

Progeny virions are transported in special vesicles from the Golgi to the plasma membrane where the first hantaviral particles are released by 24 h p.i. [46]. It may be that some hantaviruses, e.g., Black Creek Canal Virus and Sin Nombre Virus, mature not in the Golgi but at the plasma membrane [29, 87]. Although current data are limited only to these two hantaviral species, the appearance of Gn and Gc proteins at the plasma membrane suggests that cell surface maturation is possible for some hantaviruses [104].

General features of hantaviral N protein

The N protein of hantaviruses contains from 429 to 433 amino acid (aa) residues and has a molecular weight of approximately 50 kDa. It is the same size as the N protein of nairoviruses (48–50 kDa) and almost twice as long as the N protein in viruses of three other genera of *Bunyaviridae* (26–29 kDa) [18]. The functional implications of the observed size difference remain largely unknown.

Within a given hantavirus type, the primary structure of the N protein is highly conserved, with only a few nonhomologous substitutions having been registered. Between all hantaviruses, even those with differences in the N protein sequences as high as 39% (Plyusnin, 2002), most of the charged aa residues, as well as cysteins, glycines, prolines and tryptophans are well conserved. The overall pattern of the sequence is well conserved, also: three more conserved domains are separated by two more variable regions spanning aa residues from appr. 50 to 80 and from 230 to 310 (Fig. 1). The central conserved domain contains a large cluster of 15 lysines/arginines located between pos. 136 and 213. Notably, all 15 positive charges are absolutely conserved in all known hantaviruses. This cluster overlaps with the RNA-binding domain (see below).

As mentioned above, the hantaviral N protein is the most abundant viral component in both virions and infected cells where it is located in the cytoplasm and forms inclusion bodies and filamentous structures [18]. The N protein of hantaviruses has not been found to be modified posttranslationally, e.g. phosphorylated like some of the NPs of NSRV [81, 109]. The 3-D structure of the N protein has not been solved yet. First data on EM-negative staining suggested a curved shape for the N protein molecule, with the dimensions of approximately 8 by 3 nm [48, 57].

The N protein has many functions, including RNA packaging; these multiple functions of the N protein are discussed below (Fig. 2).

RNA binding and RNP assembly

A primary function of the viral nucleocapsid proteins (NP) is to protect the RNA genome. As in other NSRVs, the functional templates for transcription and replication of the hantaviral genome are RNPs. The mechanism of hantaviral RNA encapsidation is not well understood. The RNP formation depends on the N protein interactions with the viral RNA and with other N protein molecules previously bound to the RNA. The N protein most probably interacts also with the L protein, which is thought attached to the panhandle-forming terminal sequences of vRNA.

During encapsidation, viral RNA molecules must be selected and distinguished from cellular RNA molecules; the discrimination is conceivably based on the elements of primary or higher structures that deliver an encapsidation signal. In the hantaviral replication cycle, both vRNA and cRNA are encapsidated while viral mRNA remains naked. Terminal, non-coding regions of the vRNA molecule have been shown to possess a unique binding region for the N protein [73, 98]. In the proposed model, nt 1 to 39 at the 5′ end of the vRNA form a stem-loop structure with a large single-stranded loop. The encapsidation starts when the N protein

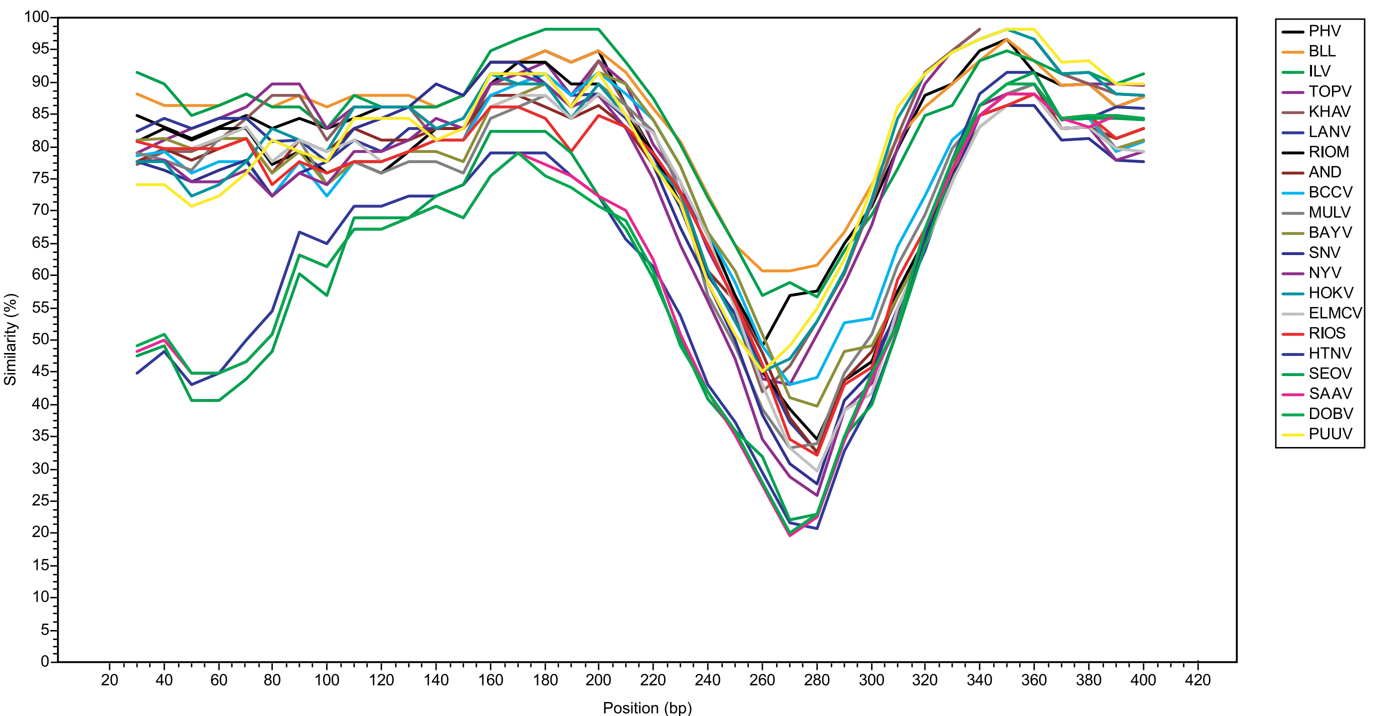

Fig. 1. Plots of similarity (generated by Stuart Ray's SimPlot2.5) between hantavirus N proteins. The curves are comparisons between the query sequence, Tula virus (TULV), and reference sequences: Prospect Hill virus (PHV), Blood Land Lake virus (BLLV), Isla Vista virus (ILV), Topografov virus (TOPV), Khabarovsk virus (KHAV), Laguna Negra virus (LANV), Rio Mamore virus (RIOMV), Andes virus (ANDV), Black Creek Canal virus (BCCV), Muleshoe virus (MULV), Bayou virus (BAYV), Sin Nombre virus (SNV), New York virus (NYV), Hokkaido virus (HOKV, Puumala-like hantavirus hosted by *C. rufocanus*), El Moro Canyao virus (ELMCV), Rio Segundo virus (RIOSV), Hantaan virus (HTNV), Seoul virus (SEOV), Saaremaa virus (SAAV), Dobrava virus (DOBV), and Puumala virus (PUUV). Each point plotted is the percent amino acid identity within a sliding window 60 aa wide, with a step of 10 aa. Note that, since the scan is done "eastward", the curves are slightly shifted to the left. The first variable region (aa 50 to 80) is seen more clearly with Murinae-associated hantaviruses (HTNV, SEOV, DOBV, and SAAV)

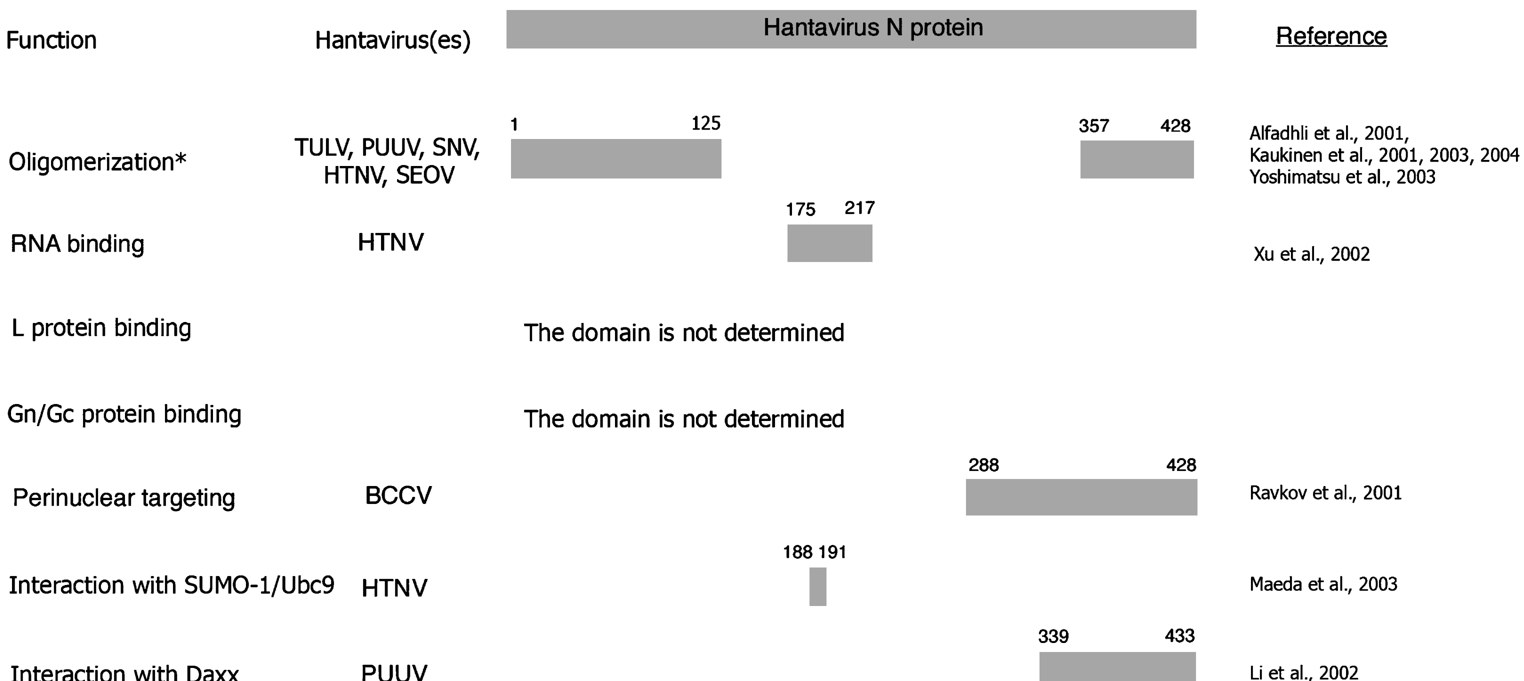

Fig. 2. Schematic representation of the functional domains in the hantavirus N protein

binds specifically to the loop; this is followed by the subsequent N–N interaction that encapsidates the remaining part of vRNA in a non-specific fashion [99]. Similarly, secondary structures formed near the 5′ terminus of vRNA have been suggested for encapsidation in Bunyamwera virus (BUNV), an orthobunyavirus [77]. The RNA-binding domain of the HTNV N protein has been localized in the middle part of the protein, within aa 175 to 217 [118]. Since this domain is highly conserved among different hantavirus species (up to 86% of aa sequence similarity) the authors concluded that the corresponding region in the N protein of other hantaviruses likely serves the same function.

Computer modeling of the secondary structure of hantaviral S RNA suggested extensive base pairing throughout the molecule, which is folded into a major backbone structure with several stem-loop structures [43]. On the contrary, electron microscopy (EM) revealed that hantaviral RNPs look like long helical filaments [11, 29]. EM data suggest that encapsidation prevents the folding of hantaviral RNA, like in the case of the Influenza A virus, another segmented NSRV, vRNA encapsidation. The binding of Influenza A virus NP to RNA removes secondary structures and keeps the RNA single-stranded [10]. The binding of the polymerase complex to the vRNA termini gives the RNP molecule a closed circular conformation [52]. Yet, the general structure of Influenza A virus RNP is not circular but linear [35]. The structure is dependent on the length of vRNA; the RNPs with an RNA longer than 400 nt were linear due to supercoiling of the RNP molecule, i.e. helical strands wound back on themselves [52, 76]. Circular or elliptic structures were seen only when the length of the RNA was not sufficient (<400 nt) to allow supercoiling [67, 76].

The vRNA genomes of NSRV are organized into RNPs in a way that NPs are bound to RNA at regular intervals. The length of an RNA molecule determines how many NP molecules are needed for the encapsidation. In all NSRVs nt:nucleoprotein (NP) ratio is a multiple of three [70]. The "rule of six" in the family *Paramyxoviridae* states that the virus genome is copied efficiently only if its length in nucleotides is a multiple of six, i.e., each NP monomer associates with exactly six nt [113]. The ratio determined for Influenza A virus (family *Orthomyxoviridae*) was 24 nt/NP [76], for vesiculo- and lyssaviruses (family *Rhabdoviridae*) 9 nt/NP [38, 107] and for Lake Victoria marburgvirus (family *Filoviridae*) 12 or 15 nt/NP [70]. Due to the lack of high-resolution images, the stoichiometry of hantaviral RNPs is not known yet [11, 29]. One reason is the flexibility and heterogeneity of hantaviral recombinant and viral RNPs. Mini-RNPs (i.e., small NP-RNA rings) are more regular than full-length viral RNPs. After EM analysis and 3-D reconstruction, the first 3-D structural models were obtained for Influenza A and Rabies viruses RNPs [67, 95]. These methods could be applied to study the stoichiometry of the hantaviral RNPs.

Oligomerization

A characteristic feature of the NPs of NSRV is that they oligomerize into helical nucleocapsid-like particles in the absence of viral RNA and any other viral proteins

[22, 38, 54, 103]. The self-assembly of viral NPs is a prerequisite for the assembly of the functionally active nucleocapsid. Oligomerization of the NPs can alter the NP conformation making it more disposed to vRNA binding, as suggested for the NP of porcine reproductive and respiratory syndrome virus (family *Arteriviridae*) [117].

The assembly of helical nucleocapsids of NSRV usually occurs without defined intermediate structures. Until recently, the oligomerization capacity of the N proteins of hantaviruses and other bunyaviruses has been largely unknown. The analysis of NSRV NPs, however, has revealed the existence of multimers, dimers in the case of influenza virus [76] and mostly trimers in the case of hantaviruses [3, 4, 47]. The trimers exist both in viral particles and infected cells. N protein trimers have been observed in other bunyaviruses such as Bunyamwera virus [77] and Uukuniemi virus (R. Pettersson, pers. commun.). Recently, N protein trimerization was found to play an important role to discriminate between viral and non-viral RNA molecules [73].

The interaction between N protein monomers is probably non-covalent and electrostatic, possibly occurring via hydrogen bonding of amino acid side chains exposed to the aqueous environment. Divalent cations (Ca^{2+}, Mn^{2+}, Mg^{2+} and Ba^{2+}) that are important for maintaining the structural stability of several viruses, including bovine papillomavirus type 1 [78], SV40 [93] and polyomaviruses [34], enhanced the interaction between hantaviral N proteins [47]. Recent studies have further characterized the interacting domains and pinpointed aa residues that are crucial for the interaction [4, 49, 119].

The N-terminal 75 aa residues were predicted to form a coiled-coil structure [4]. The coiled-coil is composed of α-helices wound around each other as a multistranded helix [116]. The homotypic interaction region was also mapped to this region [3, 49]. Another oligomerization domain has been identified in the C-terminal regions of different hantaviral N proteins [4, 49, 119]. Notably, also, the interaction regions of the N protein of tomato spotted wilt virus (genus *Tospovirus*) were defined to the first 39 aa and the last 16 aa [110]. The involvement of N- and C- terminal regions in the homotypic interaction could be a characteristic feature of all bunyavirus N proteins.

The analysis of the C-terminal interaction region has provided new insights into the mechanism of N protein oligomerization at molecular level [48]. The model developed suggests that the N protein trimerization is initiated by packing together the N-terminal coiled-coils of adjacent molecules. The interaction between C-termini is probably established via two α-helices between the molecules. A shared hydrophobic space is formed between these helices stabilizing the trimer structure. Both the preservation of the helix structure and the formation of a shared hydrophobic space are crucial for the interaction. The 3-D reconstruction from electron microscopy images showed that the recombinant N proteins assemble into trimers in which individual N proteins form a curved structure (Fig. 3). In the model, the viral RNA would be placed inside the trimer. When the trimer is bound to the viral RNA, the multimerization of trimers is the next step in the assembly process. The regions that participate in the trimer multimerization are

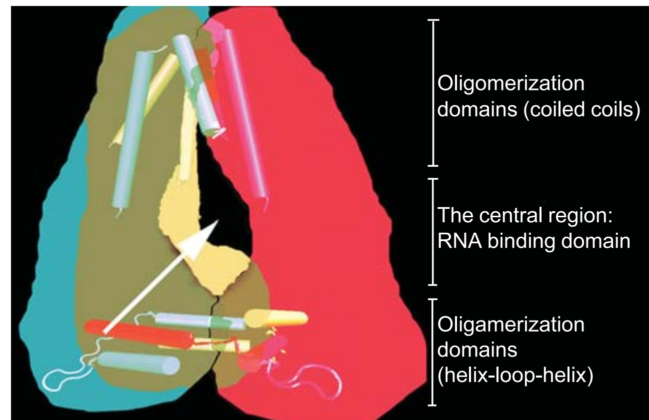

Fig. 3. The model for the hantavirus N protein trimerization. Three monomers (in red, blue and in yellow) interact via oligomerization domains in the N-terminal region (coiled-coils) and in the C-terminal region (helix-loop-helix). The arrow depicts the possible orientation of the viral RNA

probably dispersed along the polypeptide but may also partially overlap with the trimerization regions [49].

Crystallisation of the NP of Borna disease virus (the first crystallization of an NSRV) has revealed some interesting aspects of the molecule's architecture. The bornavirus NP seems to have a novel fold that does not show significant homology to other known protein structures [90]. The crystal analysis showed that the NPs form homotetramers. The tetramer has two potential venues for RNA binding: (1) the central channel inside the tetramer or (2) a groove running diagonally across the tetramer surface. Structural information on hantaviral RNPs and, in particularly, on the N protein monomers is still needed to explain hantaviral N protein oligomerization and RNP assembly in greater detail.

Interactions with other virus proteins

The hantaviral L protein is thought to be associated with the RNPs so that it is ready to initiate viral RNA synthesis immediately after the virus entry into a host cell [94]. One of the lessons learned from the experiments with hantaviral minigenomes was that functional L and N proteins are the only viral proteins needed for replication and transcription [21]. Both proteins were, at least partially, colocalized in the Golgi compartment [56]. Taken together, these data suggested that hantaviral N and L proteins interact with each other resembling, in this respect, N and L proteins of Bunyamwera virus [60].

Hantaviral envelope glycoproteins Gn and Gc form a heterodimer, when they are synthesized on the ER membrane. When both are coexpressed, they localize to the Golgi membrane [91, 104]. Identification of the Gn and Gc protein retention signal was important for clarifying the mechanism and the location of virus budding. The cytoplasmic tail of the Gn protein and the signal sequence of

the Gc protein of Hantaan virus have been suggested to play an important role in the Golgi localization [100]. Similarly, cytoplasmic tail of the Gn protein of Uukuniemi virus (genus *Phlebovirus*) has been shown to contain a Golgi retention signal [5, 6].

The tail of the Gn protein located on the cytoplasmic side of the Golgi membrane could also determine the constituents (i.e., RNPs) that are included in the virion. Therefore, the N protein interaction with Gn or Gc proteins has been thought to be an important step in the virus assembly but direct evidence for such an interaction has been missing. Recently, Puumala hantavirus N protein was shown to interact with the cytoplasmic tail of the Gn protein (V. Koistinen and X. Li, personal communication). The interaction was dependent on the tyrosine residue in the Tyr-Arg-Thr-Leu motif that is highly conserved among hantaviruses and could act as an assembly signal.

The N and the L proteins are peripheral membrane proteins; they associate with membranes electrostatically [56, 86]. The membrane-targeting region was located within 141 C-terminal amino acids of Black Creek Canal virus N protein. That most of the N protein was localized in the Golgi region suggests it was not dependent on vRNA, because N protein expressed alone localized in the same region [56, 88]. Conceivably N and/or L proteins form the hantaviral replication complex that associates with the Golgi membrane. Localization within the Golgi is not surprising since intracellular membranes are the sites of replication of many RNA viruses. Furthermore, the interaction between N protein and Gn protein would secure the incorporation of RNP into virus particles. The current data suggest that N and/or L proteins have an important task in determining the location of RNA synthesis of hantaviruses and in directing RNPs to the site of virus assembly and budding.

Interactions of hantaviral N protein with cellular proteins

As discussed in previous sections, the hantaviral N protein has important functions in RNA replication, encapsidation and the virus assembly. Moreover, the N protein seems to interact with cellular proteins and hence could modulate the metabolism of infected cells. These “ambassador” activities are discussed below.

N protein interactions with actin microfilaments

Many viruses interact with actin at different stages during the infection cycle, both disrupting and rearranging the actin cytoskeleton to their own advantages [15]. Actin polymerization is required for endocytosis of some viruses and actin filaments are used at the budding step through cellular membranes [102]. Like cellular membrane structures, actin filaments may form anchoring sites for viral RNA replication complexes, thus providing a suitable microenvironment for replication. In nonmuscle cells, actin is present in both globular monomeric and filamentous forms. Several viral proteins have been reported to bind to filamentous actin. The host cell actin participates in viral RNA synthesis during Human parainfluenza virus 3 and Respiratory syncytial virus infection [32, 36]

and in transporting measles virus RNPs to the cell surface [74]. It is also possible that cellular proteins mediate the RNP interaction with actin filaments, as in the case of Rabies virus NP that was shown to interact with a neuron-specific, actin-binding phosphoprotein, synapsin I [14]. In the case of Influenza A virus, the NP-actin interaction has been suggested to cause the cytoplasmic retention of viral RNPs that are destined to be packaged into progeny virions [9, 17].

Recently, interaction with actin has been reported for Crimean Congo hemorrhagic fever virus (genus *Nairovirus*) N protein [8]. Actin filaments were involved in the perinuclear localization of the N protein and in the virus assembly. As for hantaviruses, it seems that at least some of them use the actin network of infected cells for their benefit. The N protein of Black Creek Canal hantavirus (which is known to bud not from the Golgi but from the plasma membrane) has been found to bind monomeric actin and also to colocalize with the actin filaments [88]. Filamentous actin seems to play an important role in the infection cycle of this hantavirus since the disruption of actin filaments led to inhibition of the virus release. It has been shown that actin filaments are involved at the stage of Black Creek Canal virus assembly and it has been suggested that they transport viral RNPs to the plasma membrane where the assembly and release of this virus occur. The release of Punta Toro virus (genus *Phlebovirus*) was not affected by the disruption of actin filaments suggesting that viruses budding to the Golgi do not need actin filaments for maturation. Our data on Tula hantavirus N protein support the same conclusion, since the disruption of actin filaments did not affect the N protein distribution in Tula virus-infected cells (P. Kaukinen and A. Plyusnin, unpublished observations). Perhaps, utilization of the actin network is not uniform for hantaviruses and other bunyaviruses.

N protein interactions with the regulatory cellular protein Daxx and its potential role in apoptosis signaling

PUUV N protein was shown to interact with the Daxx protein [62]. Daxx is involved in competitive interactions with many cellular proteins, including transcription factors, Fas-death receptor, promyelocytic leukemia (PML) protein and ubiquitin conjugating enzyme-9 [40]. Interaction(s) with the hantaviral N protein might interfere with these functions. It is thought that Daxx mainly acts as a transcription repressor. Most notably, some studies have shown its anti-apoptotic activity [72, 108]. There is growing evidence for hantavirus-induced apoptosis [2, 45, 61, 66]. We hypothesize that hantaviral N proteins might bind Daxx in the cytoplasm and thus prevent Daxx-mediated transcriptional repression; consequently, this could allow the Fas-apoptosis pathway in hantavirus-infected cells. To substantiate this hypothesis further studies are needed. Interestingly, the Daxx-interacting domain rests within the terminal 100 aa residues of the hantaviral N protein molecule [62], i.e. in the same region that carries one of the N-N-oligomerization domains. Whether it is a monomer or a trimer/oligomer form of the N protein that reacts with Daxx remains to be determined.

N protein interactions with the components of sumoylation pathway(s)

Small ubiquitin-like modifiers (SUMOs), molecules of approx. 100 amino acids in length, are members of the growing family of ubiquitin-related proteins [71]. Despite its name, ubiquitin conjugating enzyme-9 conjugates not a ubiquitin but SUMOs to target proteins; the conjugation occurs covalently, via the lysine residue in the consensus motif, ΨKXE (Ψ represents a hydrophobic residue and X represents any amino acid). SUMOs, especially SUMO-1, are conjugated to a large number of cellular and viral proteins: some 60 target proteins have been already reported [96]. Sumoylated proteins are mainly nuclear proteins, such as transcription factors, DNA repair proteins and components of nuclear pore complex and PML nuclear bodies (PML NBs) that are thought to be involved in transcription and replication of DNA viruses as well as in antiviral responses [69].

Several viruses have been known to interfere with the host cell sumoylation reactions [115]. Viruses that replicate in the nucleus (e.g., Influenza A virus) have been documented to alter the structure of PML NBs [1, 31, 75, 79]. The viral proteins have been shown to decrease sumoylation of PML protein that builds the framework of PML NBs. SUMO-1 removal has led to disruption of PML NBs to their components followed by degradation through ubiquitin/proteosome pathway [20]. In contrast to these viruses, hantaviral infection has not been found to affect the distribution of SUMO-1 although the N protein and SUMO-1 were found to colocalize near the nuclear membrane in virus-infected cells [50]. Since Ran-GTPase activating protein (RanGAP) at the cytoplasmic fibers of the nuclear pore complex has been shown to be sumoylated [68], we speculate that the cytoplasmic side of the nuclear membrane could be the actual site for the N protein-SUMO-1 interaction. However, only a small portion of the N protein molecules were found in that region suggesting that the interaction might be transient, e.g. it might occur during a certain phase of the cell cycle and/or virus infection [50]. Moreover, neither in this nor in other studies has a sumoylated form of hantaviral N protein been detected.

Hantaviral N protein has been found to interact with Ubc-9 and SUMO-1 in the yeast two-hybrid assay [50, 65]. It was shown that amino acid residues 188 to 191 (Met-Lys-Ala-Glu) in Hantaan virus N protein are crucial for its interaction with both Ubc-9 and SUMO-1 proteins [65]. As the removal of residues MKAE relocalized the N protein from the Golgi region to the cytoplasm, it was speculated that the N protein – SUMO-1 interaction might be responsible for retaining the N protein in the cytoplasm [65]. The MKAE/D sequence is conserved among hantavirus species suggesting that it is associated with important function(s) of the N protein. Further yeast two-hybrid screenings revealed interactions of the N protein with several other components of sumoylation pathway: PIAS1 (protein inhibitor of activated STAT 1), PIASxβ, TTRAP (TRAF- and TNF receptor-associated protein), CHD3 (chromodomain helicase DNA-binding protein) and HIPK2 (human homeodomain-interacting protein kinase 2) [59]. All these proteins interacted also with Ubc-9 and SUMO-1. These data suggest that the hantaviral

N protein has a complex network of interactions involving both several proteins in sumoylation pathway (Ubc-9, PIAS proteins and SUMO-1) and sumoylated proteins, e.g. Daxx. Further studies are needed to understand the consequences of these interactions.

N protein and the MxA-mediated antiviral response?

Human MxA protein, an interferon-inducible gene product with antiviral activity, provides resistance to a wide range of RNA viruses [97]. Two interesting observations have been made about MxA protein and hantaviruses. First, MxA was found to inhibit hantaviral infection in MxA-expressing Vero cells [23, 44]. Second, the data from DNA microarray analysis of cellular mRNAs showed that MxA-specific transcript was induced ∼160-fold 1 day after infection by non-pathogenic Prospect Hill virus compared with ∼4-fold and <2-fold by the pathogenic New York virus and Hantaan virus, respectively [28]. Although it was observed that 4 days p.i. all three hantaviruses induced MxA about 200-fold, the authors speculated that rapid MxA activation by the non-pathogenic hantavirus could slow the infection and thereby assist the defense system to clear it. Taken together, these data suggest that the MxA-mediated antiviral response is an important part of the innate immunity against infection with certain hantaviruses. In addition, it appears that stimulation of the MxA response might be a viral characteristic, rather than a host characteristic.

Other bunyaviruses, La Crosse virus (LACV) (genus *Orthobunyavirus*), Rift Valley fever virus (genus *Phlebovirus*), Dugbe virus and Crimean Congo Hemorrhagic Fever virus (genus *Nairovirus*), have been shown to be susceptible to MxA as well [7, 13, 23]. For these viruses, direct involvement of the N protein has been documented. It appeared that MxA binds to the N protein in La Crosse-, Bunyamwera- and Rift Valley Fever virus-infected cells and targets the N protein to filamentous structures in the perinuclear region [53]. It seems that MxA binds the N protein and reduces the amount of free N protein crucial for vRNA replication. The current model for MxA action suggests that MxA adopts two different conformations; homo-oligomers as a storage form in the absence of infection and co-polymers with N proteins in infected cells [33]. Whether a similar mechanism is operating during the hantaviral infection and whether the hantaviral N protein reacts directly with MxA remains to be determined.

Concluding remarks

This review was, to a large extent, inspired by excellent summary of Portela and Digard on the influenza virus nucleoprotein [84]. Analysis of the data accumulated so far on the hantaviral N protein clearly shows that, very much like the influenza virus, it is a multifunctional molecule. Besides protecting the virus genome RNA, the hantaviral N protein seems to be actively involved in transcription, replication, and also in packaging of RNPs into virus particles. On the top of these "housekeeping" duties, it appears to perform interactive "ambassadorial" functions by interfering with important cellular regulatory pathways. All the tasks the N protein

executes are based on its exquisite capability to interact with different viral and cellular macromolecules. It thus presents an excellent model for basic research on structure-functional organization of a multifunctional protein. But not only that. The hantaviral N protein is the major antigen in early serological response in infected humans [112], most probably, due to large amounts of the protein are produced, and several diagnostic tests are based on its use. The N protein has been shown to contain several B- and T-cell epitopes. B-cell epitopes are localized mainly in the N-terminal part of the molecule [30, 63, 112] while T-cell epitopes are distributed more randomly [19, 111]. The N protein has been shown to elicit protection from hantavirus infection in experimental animals and thus could be considered a component of a vaccine (see, e.g. [16, 63]). More detailed knowledge of the N protein would, naturally, help in the development of better diagnostics and vaccines and even, perhaps, new antivirals that would target this component of the virus and hence target crucial steps in the replication cycle in which it is involved.

Recently, crystallization of the first nucleocapsid protein of NSRV, Borna disease virus, has been reported [90] raising the hope that other advances on this front will follow. Structural studies of the hantaviral N protein have so far been progressing slowly, since it has a strong tendency to form aggregates. This particular feature has been hampering all efforts to crystallize the protein. Based on the current knowledge on the structural organization of the oligomerization domains (which could be responsible for the protein's "stickiness") it might become possible to bypass this obstacle and finally resolve the 3D-structure of the N protein. If *bona fide* crystals would remain elusive, "crystallization *in silico*" could, perhaps, offer a reasonable alternative. Promising examples here are provided by Influenza A virus and Rabies virus: nucleocapsid proteins of these NSRV incorporated in mini-RNPs were visualized using electron microscopy, followed by the image processing and 3-D reconstruction [67, 95].

The hantaviral N protein also presents a good model for evolutionary studies. Current collection of natural variants of the N protein is among the largest of any NSRV and its gene, the S segment, has been successfully used to estimate the speed of hantaviral evolution, which appeares to be relatively slow [101]. Notably, the primary structure of the protein is highly conserved within a given hantavirus, with only a few of non-homologous substitutions permitted per molecule among more than 400 aa residues. The overall outfit of the N protein molecule also seems to be well preserved among dozens of known hantaviruses. Within the *Bunyaviridae* family, however, even the size of the N protein varies greatly: in orthobunya-, phlebo- and tospoviruses, the N protein is approximately half of that in hanta- or nairoviruses [18]. At the same time, the S segment of orthobunya-, phlebo- and tospoviruses encodes an additional protein, NSs, using either ambisense or overlapping coding strategy. Most, but not all, hantaviruses carry an NSs ORF as well [83] but whether it is functional or not remains to be determined. This suggests the intriguing likelihood that the larger N proteins of nairoviruses and of at least some hantaviruses might effectively combine in one molecule the functions of both the smaller N protein and the NSs protein. Taking into consideration that

bunyaviral NSs proteins are powerful antagonists of interferon responses and host cell transcription in general [12, 58, 106, 114], it would be safe to assume that studies of the hantaviral N protein might reveal some additional interesting twist in the nearest future.

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