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The nucleocapsid protein of hantaviruses: much more than a genome-wrapping protein

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Abstract
The nucleocapsid (N) protein of hantaviruses represents an impressive example of a viral multifunctional protein. It encompasses properties as diverse as genome packaging, RNA chaperoning, intracellular protein transport, DNA degradation, intervention in host translation, and restricting host immune responses. These functions all rely on the capability of N to interact with RNA and other viral and cellular proteins. We have compiled data on the N protein of different hantavirus species together with information of the recently published three-dimensional structural data of the protein. The array of diverse functional activities accommodated in the hantaviral N protein goes far beyond to be a static structural protein and makes it an interesting target in the development of antiviral therapeutics.

Keywords Multifunctional protein · Nucleocapsid protein · Orthohantavirus

Introduction

Multifunctional proteins are the rule, not the exception. They are widely spread, not particularly in viruses as previously thought, but throughout the evolutionary tree. A subset of multifunctional proteins have been coined moonlighting proteins. They have two or more functions within a single polypeptide chain and they are distinct from enzymes or proteins with broad specificity, multi-enzyme complexes, proteins with different functions as a result of gene fusion, and families of homologous proteins or splice variants [1–6]. Multifunctional viral proteins develop during co-evolution of viruses with their host cells under the pressure to economically use their genetic potential. It applies to both non-structural and structural viral proteins [7].

Capsid proteins are regarded the hallmark of viruses. A characteristic feature of capsid proteins, which can perform multiple functions in the virion and in the intracellular viral growth phase, is the existence of extended disordered regions or arms together with ordered globular domains in the same protein. The arms may be ordered in the condensed macromolecule within the virion, but can be flexible in the isolated protein and therefore available for diverse interactions [6]. They exist as dynamic ensembles of conformations that do not have a stable folded structure and still carry out their respective biological activities. Disordered regions, which apparently have the potential to evolve very rapidly [8], allow viruses to quickly adapt to changes in their environment, survive in both their host and their host's environment, and evade the cellular immune system. Targeting disordered regions in capsid proteins to impair their essential interactions is considered as an attractive antiviral strategy [9, 10]. Moreover, viral capsid proteins assemble into different oligomeric states that, dependent on ligand binding, cellular localization, and post-translational modifications, can generate multiple functional forms. The ability of a viral protein to fulfill multiple functions substantially helps conserve genome space requiring fewer viral proteins for the replication cycle [11].

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Hantavirus infections and medical impact

Hantaviruses are enveloped viruses with a tri-segmented negative-sense RNA genome (Fig. 1). According to the International Committee for the Taxonomy of Viruses (ICTV), they form an own family *Hantaviridae* within the order *Bunyavirales*. Currently, the virus family encompasses only one genus, *Orthohantavirus*, which is subdivided into different species (https://talk.ictvonline.org/taxonomy/).

In contrast to viruses from other families within the *Bunyavirales*, hantaviruses are not transmitted by arthropods. Instead, infection of susceptible organisms occurs by vector-free transmission from infected reservoir hosts [12]. Inhalation of virus-containing, aerosolized excreta and—rarely—animal bites lead to virus uptake into the organisms; however, gastrointestinal infection is also possible [13].

Small mammals are considered as hantavirus hosts; for a long time, rodents were the only known natural carriers of human pathogenic hantaviruses. However, during the last few years many novel hantavirus species have been found in reservoirs such as shrews, moles, and bats. There are first serological indications that those viruses or antigenically related viruses are also able to infect humans [14].

A strong association of different hantaviruses (hantavirus species) exists with particular host species. The factors which determine the host specificity and the species barriers of viruses are mainly unknown but probably include geoecological factors and on the cellular-level components as receptor specificity and modulation of innate host immunity. In contrast to the *Mus musculus*, which does not harbor hantavirus, all the genetics and immunology of the natural hantavirus hosts are poorly investigated.

Hantaviruses are able to "spill over" from their natural hosts to humans. Whereas small mammals are chronically infected by "their" hantaviruses without signs of disease, infected humans can develop hantavirus disease, either hemorrhagic fever with renal syndrome or hantavirus cardiopulmonary syndrome. The disease is characterized by

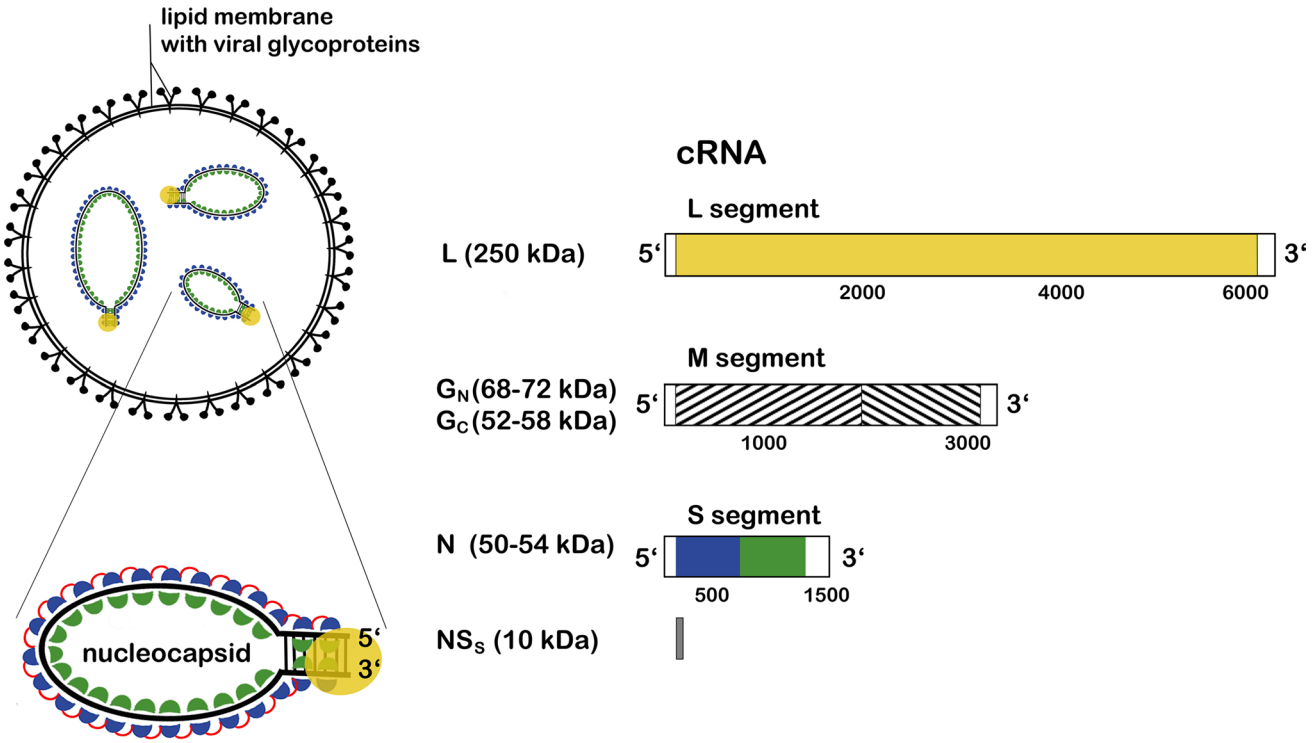

Fig. 1 Schematic overview of hantavirus virion composition and genome structure. Left: enveloped hantavirus particle. Lipid membrane presents viral glycoproteins Gn and Gc (black) and surrounds the three ribonucleocapsids S, M, and L. The S nucleocapsid is shown enlarged: single-stranded negative-sense RNA of the S gene segment forms a "panhandle" structure due to complementary bases at both RNA ends (black); RNA molecule is covered by numerous copies of the capsid protein N composed of an N- and a C-terminal lobe (blue and green, respectively) embracing the RNA strand. N proteins make contacts to the neighboring N molecules (red thin line). The ribonucleoprotein is the template for RNA replication and transcription by the viral RNA-dependent RNA polymerase (yellow). Right: complementary RNA of the L gene with ORF for the RNA polymerase (yellow), of the M gene coding for glycoproteins Gn and Gc (striped), and of the S gene coding the capsid protein (and in some hantaviruses for a nonstructural protein NSs). Untranslated regions at the ends of the RNA molecules are white. Some details integrated in the figure were taken from [27, 28, 97] (Color figure online)

high initial fever, myalgia, hypotonia (shock), renal and/or pulmonary edema, and development of renal and/or pulmonary failure. The severity of the clinical course is influenced by the particular virus type which had caused the infection; there are hantaviruses which are considered not to be pathogenic for humans and, at the other end of the spectrum, viruses which cause case fatality rates of up to 50% [15].

The small hantaviral genome which encompasses only three segments coding for not many more proteins enforces a high efficiency and economy in the function of these few proteins: nucleocapsid protein (referred to in the following text as N protein or N), glycoprotein precursor, which is cleaved into two final envelope proteins, RNA polymerase, and sometimes a putative nonstructural protein. This review will focus on the multiple functions of the N protein, which contains only 429–433 amino acid (aas) residues and has a mass of 50–54 kDa, as a new example of a viral moonlighting protein. In the experimental studies to be described here, members of various hantavirus species have been utilized by the different authors. Table 1 gives an overview of these hantaviruses, their natural hosts, geographical distribution, and clinical importance for humans.

Functions of hantaviral N protein

Interaction with RNA

The canonical function of hantaviral N protein is to recognize and to protect the viral tripartite negative-sense RNA genome. The three genes exist as "panhandle" structures formed by imperfect base-pairing of the sequences at the 5′- and 3′-end of the viral segments. Specific recognition of viral RNA by N occurs at this panhandle structures with dissociation constants in the nanomolar range [16–18], and discrimination between viral and nonviral RNA was reported to be achieved by the formation of highly stable N trimers [19]. Besides a nonspecific RNA-binding region that was found in the C-terminal 93 aas of HTNV-N and PUUV-N [20], the major specific RNA-binding determinant was assigned to a central, conserved region between aas 175 and 217 of N protein [21, 22]. More detailed analyses using synthetic peptides and site-specific mutagenesis revealed aas E192, Y206, and S217 as essential for RNA binding, but also stated that several lysine residues distributed between aas 175 and 429 are also indispensable for RNA binding [22].

Based on competition experiments and mutational analyses, Mir et al. reported that N has independent binding domains for viral RNA and for mRNA-cap structures. An N variant lacking the RNA-binding domain aas 175–217

Table 1 Members of the family *Hantaviridae* referred to in this review

Orthohantavirus species	Species abbreviations	Reservoir host	Geographical distribution	Usual clinical severity in humans
Andes	ANDV	*Oligoryzomys longicaudatus* *Oligoryzomys* sp.	South America	Severe
Black Creek Canal	BCCV	*Sigmodon hispidus*	North America	Severe?[b]
Dobrava–Belgrade virus	DOBV	*Apodemus agrarius* *Apodemus flavicollis* *Apodemus ponticus*	Central and Eastern Europe	Mild/moderate and severe[c]
Hantaan	HTNV	*Apodemus agrarius*	Asia	Moderate/severe
New York[a]	NYV	*Peromyscus leucopus*	North America	Severe?[b]
Prospect Hill	PHV	*Microtus pennsylvanicus*	North America	Probably not pathogenic
Puumala	PUUV	*Myodes glareolus*	Europe	Mild/moderate
Seoul	SEOV	*Rattus norvegicus* *Rattus* sp.	Asia and probably worldwide	Moderate
Sin Nombre	SNV	*Peromyscus maniculatus*	North America	Severe
Uluguru	ULUV	*Myosorex geata* (shrew, not rodent!)	Africa	Moderate?[b]
Tula	TULV	*Microtus arvalis* *Microtus* sp.	Europe	Mild?[b]

[a]Belongs to the SNV species according to the latest ICTV vote

[b]Only few clinical cases verified yet

[c]The genotypes of DOBV (associated with different *Apodemus* hosts) show different virulence in humans

was found to still bind a capped RNA decamer [23]. In virus-infected cells, N protein accumulates in cytoplasmic processing bodies (P bodies), where it binds with high affinity to the 5′ cap of cellular mRNAs protecting them from degradation by the cellular de-capping machinery. N can protect at least 180 5′-terminal nucleotides of capped RNAs in P bodies [24, 25]. Viral RNA-dependent RNA polymerase (RdRp) preferentially snatches caps from nonsense mRNAs and cleaves the sequence primarily at a G located 8–18 nucleotides downstream of the 5′ cap [25, 26]. Finally, N facilitates annealing of a capped RNA primer that has a single terminal G residue complementary to one C residue at the 3′ terminus of vRNA template and this complex can be used by the hantaviral RdRp for transcription initiation [23].

Recently, the three-dimensional structure of the core region of SNV-N and ANDV-N (aas 111–398) and of HTNV-N (aas 113–429) was resolved [27, 28]. All three proteins were considerably truncated to obtain stable proteins for crystallization. Hantavirus N proteins were found to consist of an N-terminal and a C-terminal lobe forming together the compact core. The three structures exhibit a positively charged RNA-binding pocket between the two lobes. In ANDV-N and SNV-N, aas R146, Q185, R197, R199, R313, R338, N339, R366, and R367 predominantly contribute to the formation of the RNA-binding crevice. Within the RNA-binding crevice, a deep hydrophobic pocket was reported including aas M220, F307, S310, F331, and Y364 that is assumed to bind discrete nucleotides [27]. For HTNV-N, aas S180, N183, S186, S187, and T194 as well as the positively charged K189, R197, and R199 line the RNA-binding groove, positions that concur with earlier data from biochemical studies [21, 22, 28]. However, in the crystal structure of HTNV-N (at least in the truncated form) no evidence for a conserved RNA cap-binding site was found as predicted earlier by Mir et al. [23].

Recently, inhibitors have been identified which interrupt RNA–N protein interaction and inhibit hantavirus replication with the same potency as ribavirin, a commercially available suppressor of RNA virus replication. The authors suggest that this discovery could lead to the development of first anti-hantaviral therapeutics [29].

N shows ATP-independent RNA chaperone activity

Proteins with RNA chaperone activity have important roles in cellular mechanisms. They resolve misfolded RNA structures without ATP consumption [30]. Hantavirus N protein has nonspecific chaperone activity resulting in the dissociation of misfolded RNA structures. The activity depends on Mg^{2+} ions, but not on ATP. To unwind double-stranded RNA helices, N needs an adjacent single-stranded RNA region. It has been suggested that N dissociates the panhandle to present an accessible 3′ RNA end that can be used for transcription initiation [31, 32]. Investigation of N protein variants point to the N-terminal 100 aas as being responsible for RNA chaperone activity [33]. The authors propose that this region of N exists in two potential states—(i) in a highly disordered state with RNA chaperone activity or (ii) in a trimeric and ordered state mediating high-affinity vRNA panhandle recognition. In contrast to specific RNA recognition, RNA chaperone activity does not require N trimerization [33]. Although the exact chaperoning mechanism is not yet understood, it is probable that in general disordered regions within the proteins play an important role in chaperone activity [30]. Disordered regions were described in all three N structures guaranteeing a highly flexible architecture of the N protein. However, the amino-terminal 100 aas with chaperone activity are missed in the published three-dimensional structures [27, 28].

N protomer as the structural building block of viral core formation

During the process of encapsidation of viral nucleic acid, the N protein molecules must interact not only with each other, but also with other viral and host proteins as well as with the viral genome to form the structural cage. N protein has been found in different quaternary structures—as mono-, di-, tri-, and in multimeric form [34–37]—and there have been a number of studies over the years that reported crucial regions in the N protein for N–N interaction [34–36, 38–43]. A consensus region for N–N oligomerization appears to include the N- and C-termini of the N polypeptide chain.

Earlier work exploiting biochemical and genetic methods had reported that the C-terminal half and the N-terminal 40 aas of N are essential for trimerization of the protein [34]. By investigating the features of the N terminus using synthetic peptides it was described that residues 43–75 efficiently trimerize at low concentrations and therefore probably carry a coiled-coil trigger sequence [35]. Alminaite et al. also reported aas D35, D38, L44, V51, and L58 as important for N–N interaction of TULV-N [41, 42]. In 2007, Boudko et al. published the atomic resolution structure of the N-terminal 75 and 93 aas of SNV-N, respectively, which showed two α-helices (residues 1–34 and 38–75) that form an antiparallel coiled coil with linker residues (35-DPD-37) [43]. However, in the crystal structure of truncated SNV-N proteins (aas 1–73 and 1–93) they did not find evidence for a trimeric association and, thus, deduced that the amino-terminal part of the N protein is insufficient to initiate trimerization of the full-length molecule [43].

Recently, when the crystal structure of three N-terminally truncated hantaviral N proteins was solved, the authors reported that they purified monomeric SNV-N and ANDV-N proteins for crystallization (comprising aas 111–398) [27]. Olal and Daumke [28] confirmed this fact for HNTV-N

amino-terminally fused to the maltose-binding protein (comprising N-derived aas 113–429). However, when these authors investigated the full-length HNTV-N protein by size-exclusion chromatography, purified after denaturation/renaturation, they mostly found hexameric structures. This underlines a role for the N-terminal arm of N in oligomerization. Visualizing N and N variant structures by negative-stain electron microscopy, they pointed to a coupling of RNA binding and N oligomerization [28].

Oligomerization models have been constructed by comparing three-dimensional structure data of hantaviral N protein core with other bunyavirus N multimer structures. Both models conclude that N- and C-terminal structures of the hantavirus N protein contact the N and C lobe structures, respectively, of adjacent N protomers [27, 28, 44]. Whereas in HNTV-N the core is flanked by an N- and a C-terminal arm that are connected with the N core by flexible hinges [28], in the structures of ANDV-N and SNV-N horn-like extensions from the core called N- and C-bulge structures were found [27]. Molecular dynamic simulations supported a model that ANDV-N and SNV-N oligomerize by contacts of the C-terminal arm of an N protomer with the C lobe of a preceding N protomer on the left, and between the linker region connecting the N-terminal coiled-coil structure with the N core and the N lobe of the adjacent protomer on the right [27]. For the HTNV-N, it was reported that oligomerization between N protomers occurs by binding of the C-terminal arm into a hydrophobic pocket in the C lobe of the following molecule and by binding of the N-terminal arm to the N lobe of the preceding N protomer. Highest structural similarity was found between ANDV-N, SNV-N, and HNTV-N and orthobunyavirus N proteins. It was concluded that N proteins of orthohanta-, orthobunya-, and also phleboviruses have a common progenitor, but have diverged considerably during evolution [27, 28].

Very recently, the N structure of Tomato spotted wilt virus (TSWV), which belongs to the only plant-infecting genus *Tospovirus* in the *Bunyaviridae* family, was solved. The three-dimensional structural analysis of TSWV N protein showed an asymmetric trimeric ring structure [45, 46]. Co-crystal structures proved that the positively charged central cleft within the N protein can not only accommodate viral RNA sequences, but also DNA [45].

N directly interacts with viral RdRp

Nucleocapsids composed of vRNA and N protein serve as a template for transcription and replication of the viral genome by the viral RdRp. It was concluded from numerous reverse genetic systems that RdRp from negative-strand RNA viruses requires the assistance of N protein in order to perform its function in virus-infected cells. Cheng et al. [47] localized a direct interaction between N protein and RdRp in the amino-terminal 50 aas of N and the N-binding pocket in the C-terminal part of RdRp of yet unknown function (residues 1291–2153). They propose that the N–RdRp interaction might play a role in cap-snatching in that N helps RdRp to bind the mRNA.

N guides intracellular movement of viral glycoproteins

Glycoproteins Gn and Gc from hantaviruses are transmembrane proteins and form the envelope with a lipid membrane derived from the host cell. Gn and Gc derive from proteolytic cleavage of a glycoprotein precursor and are involved in receptor binding, membrane fusion, and induction of protective immunity. Gn and Gc assemble into spike complexes with apparent fourfold symmetry on the viral membrane [48–50]. The N-terminal domains of Gn and Gc are ectodomains projected into the outer environment, and the C-terminal domains contain the cytoplasmic tails, which face to the cytosolic environment. While hantaviral Gc glycoprotein was reported to be the membrane fusion effector, the role of Gn is not quite clear [49, 50]. It is assumed that the cytoplasmic tail of Gn glycoprotein executes the function of a matrix protein that normally acts as an anchor between the viral membrane and the nucleocapsid, but is absent in hantaviruses [51]. Indeed, it was shown earlier by co-immunoprecipitation that the N protein has multiple binding sites in the cytoplasmic tails of Gn (and also in Gc). Moreover, it was demonstrated that the carboxy-terminal part of Gn-CT encompasses a sequence-unspecific nucleic acid-binding capability [52, 53].

Specific recognition of the ribonucleoprotein complex by the glycoprotein(s) is crucial in the assembly and egress of enveloped viruses. Using NMR, Estrada et al. [54] found that Gn-CT harbors two classical ββα fold zinc fingers that are joined together and thus would be expected to bind nucleic acid. However, they could not show RNA binding by the core zinc fingers [54]. Formation of progeny hantaviruses is thought to take place in the Golgi compartment. By co-expression and immunofluorescence studies, Shimizu et al. presented evidence that aas 1–30 and 116–155 of HTNV-N promote Golgi localization of Gc by interacting with Gc cytoplasmic tail. This effect was observed also with N proteins of other hantaviruses, e.g., PUUV, SEOV, or SNV [55]. Moreover, N protein probably also helps protect Gn from autophagic degradation by the host later in the infection cycle [56], whereas shortly after synthesis the Gn glycoprotein was described to be degraded by the host autophagy machinery [57, 58].

The majority of the N protein is localized in the perinuclear region, at least for the Black Creek Canal virus (BCCV) [59]. Using amino- and carboxy-terminally truncated N variants, the authors suggested that the sequence

responsible for this Golgi-like localization of N is situated in the last 141 carboxy-terminal amino acids.

N absorbs the cellular translation machinery

Modulation of the translational processes in the host cell is a crucial point of viral infection strategies. A well-known example how viruses manipulate translation is the use of internal ribosomal entry sites that allows the virus to maintain or enhance the virus-specific cap-independent translation while stopping the cap-dependent host translation. Hantaviruses appear to apply a novel strategy that relies on the N protein replacing parts of the cap-binding complex [60]. In 2008, Mir et al. illustrated that the N protein of Sin Nombre virus is capable of functionally replacing the entire cellular eIF4F complex in translation initiation [61]. They described that N facilitates the translation of capped mRNA, preferentially of viral origin, but also of heterologous indicator mRNA. From co-precipitation experiments in rabbit reticulocyte lysates and sedimentation analyses, the authors determined that N interacts directly with the 43S pre-initiation complex, more precisely with the small ribosomal 40S subunit, without the need for eIF4F cap-binding complex [61]. Haque and Mir [62] demonstrated that N specifically interacts with the ribosomal protein S19 (RPS19), located at the head region of the 40S subunit. They further suggested that N bound to RPS19 can load the 40S subunit onto the 5′-UTR of capped hantaviral mRNA during N-mediated translation initiation [63]. By mutational analysis, the RPS19-binding region was assigned to aas 151–175 of N [64]. A similar scenario was very recently published for the nairovirus CCHFV. However, unlike hantavirus N protein CCHFV N-mediated translation enhancement depends on the presence of factor eIF4G [65].

MA3-like domains of tumor suppressor PDCD4 in the N protein might be involved in the inhibition of cap-dependent translation

Olal and Daumke [28] reported for the HTNV-N structure that the C-terminal lobe shares structural similarity with two MA3 domains of the eukaryotic tumor suppressor protein programmed cell death 4 (PDCD4). In eukaryotic cells, tumor suppressor PDCD4 inhibits cap-dependent translation initiation by binding of its two MA3 domains to eIF4A [66–68]. Therefore, the interesting question arose whether HTNV-N structurally mimics PDCD4 in order to promote translation of viral mRNA and/or to actively repress translation of host mRNA [28]. A complex intervention of hantavirus N protein in cellular translation was described earlier by Mir and Panganiban [61].

N exhibits DNA-degrading activity

Recently, a new enzymatic activity of the N protein was discovered in different hantaviruses; it was demonstrated that N possesses a DNA-specific metal-dependent endonuclease activity [69]. Upon incubation of SNV-N protein with DNA in the presence of magnesium or manganese in vitro, DNA digestion in a sequence-unspecific manner was observed. In contrast, RNA was not affected under the same conditions. Structure-based protein fold prediction using known structures from the PDB database revealed that Asp residues in positions 88 and 103 of SNV-N show sequence similarity with the active site residues Asp93 and Asp108 of the restriction endonuclease HindIII. Crystal structure of HindIII predicted that residues Asp93 and Asp108 are essential for coordination of the metal ions required for HindIII DNA cleavage. Replacing the homologous residues in SNV-N, Asp88, and Asp103 by alanine led to an SNV-N protein almost completely abrogated for endonuclease activity [69]. Currently, one can only speculate that this cytoplasmic nuclease activity might (i) prevent premature binding of cellular nucleic acid molecules to the monomeric N protein before genome encapsidation (assuming that there is an autoinhibited form of N that precludes premature nucleic acid binding as reported for rhabdo-, phlebo-, and orthobunyavirus), (ii) counteracts the sensing of cytoplasmic DNA which induces innate IFN responses, (iii) prevents the formation of DNA/RNA hybrids, which interfere with virus replication, or (iv) counteracts the accumulation of ssDNA in the cytosol which triggers IFN production [69]. The fact that Asp88 and Asp103 in the N protein are highly conserved in all hantaviruses known so far implies that the DNA-specific metal-dependent endonuclease activity is a novel common feature of the multifunctional hantaviral nucleoprotein.

N impairs the innate immune system

Cytoplasmic entry of nucleocapsids of negative-strand RNA viruses with a 5′-ppp dsRNA panhandle acts as a RIG-I activator and triggers antiviral signaling mediated through the adaptor protein MAVS and the transcription factor IRF-3 [70, 71]. Interestingly, some negative-strand RNA viruses (including hantaviruses HTNV and PHV) present only monophosphates at their 5′-RNA ends and thereby should not trigger RIG-I [26, 70, 72]. However, Hantavirus-induced RIG-I signaling has been reported [73] and for ANDV-N it was reported that it inhibits IFN signaling responses directed by cytoplasmic dsRNA sensors RIG-I and MDA5 [74]. However, N from other hantaviruses (SNV-N, NYV-N, and PHV-N) had no effect on RIG-I/MDA5-directed transcriptional responses. ANDV-N inhibits IFN signaling responses by interfering with TBK1 activation, upstream of IRF3 phosphorylation, and NF-kB activation [74]. Pan

et al. communicated a dose-dependent effect of HTNV-N on the regulation of the IFNβ pathway—in low doses HTNV-N stimulates IFN response, whereas high doses significantly inhibit IFNβ production. N protein constitutively inhibits NF-kB activation [75]. This has been reported earlier by Taylor et al. [76] who found that HTNV-N and ANDV-N bind to importin α, a nuclear import molecule responsible for shuttling NF-kB to the nucleus, and thereby inhibit TNFα-induced activation of NF-kB by sequestering NF-kB in the cytoplasm. Very recently, attempts were made to identify mechanisms that mediate this inhibition. However, no interactions of N with TBK1, TANK, or TRAF3 could be detected [77]. The authors suggested that ANDV-N impacts ancillary regulators of TBK1 activation. By LC/MS/MS proteomics approach, they found that ANDV-N in primary human endothelial cells only co-precipitated with the E3 ubiquitin ligase TRIM21 and concluded that TRIM21 and ANDV-N synergistically inhibit IFNβ.

Interaction of hantaviral N with components of the ubiquitin proteasome system and modulating effects on apoptosis has been published earlier by different groups [78–90]. Park et al. [90] presented data indicating that N ubiquitinates and degrades tumor suppressor p53 in an MDM2-dependent manner. MDM2 is the primary negative regulator of p53. The authors concluded that this is one of the antiapoptotic mechanisms employed by HTNV [90]. Furthermore, Wang and Mir [91] described that in ANDV-infected cells protein kinase R (PKR) is overexpressed. However, the enzyme is inactive due to missing autophosphorylation of the protein. The authors found out that ANDV-N inhibited PKR dimerization that is an important step for PKR autophosphorylation to attain activity. Normally, PKR as a product of an IFN-stimulated gene, phosphorylates eIF2α and thereby causes translational shutdown of the host cell to hamper viral protein synthesis. N acts as a PKR inhibitor and can be regarded as a hantavirus virulence factor [91].

Moreover, ANDV-N also contributes to endothelial cell permeability by interaction with TSC2 and Rac1/RhoA regulatory proteins. It was found that ANDV-N expressed in endothelial cells activates mTOR, which regulates hypoxia-directed cell permeability, and increases cell size by forming a complex with TSC2 [77, 92].

In some hantaviruses, the mRNA of the S gene carries a second ORF (TULV, PUUV, ANDV, SNV, PHV) that was shown to be translated during virus infection into a small nonstructural protein designated NSs in PUUV and TULV [93–97]. The gene product moderately interferes with IFNβ gene expression by inhibiting the activity of IFNβ-, NF-kB-, and IRF-3-promoters in infected cells [93, 94]. No other functions of hantaviral NSs have been proven so far. It was suggested that NSs expression results from a leaky scanning mechanism when ribosomal subunits bypass the upstream N protein initiation codon in Andes virus [95].

Immunogenicity

N is the immune dominant protein inducing a strong immunity in patients and infected animals. Multiple efforts were undertaken to map linear and conformation-dependent epitopes in the protein. It seems that some epitopes are conserved between the hantavirus species of a major phylogenetic group and others are rather serotype (species) specific [98, 99].

The immunodominant region contains mainly linear B-cell epitopes within the first 100 aas of the N terminus, and this region is serologically cross-reactive between viruses of the same phylogenetic group but not between those from different groups ([99] and references therein). However, epitopes can be distributed over the whole protein [100]. It is thought that the C-terminal half of N contains rather conformation-dependent (and multimerization-dependent) epitopes which should be more serotype specific ([99] and references therein). Still, it is difficult to pinpoint locations of distinct epitopes in Fig. 2 since the various studies used different viruses and described different epitopes.

Because of the strong immunity induction by N during hantavirus infection of the organism, perhaps caused by infection of dendritic cells [101, 102], the anti-N antibody response can be used for serodiagnostics of infection, i.e., N is used as the main diagnostic antigen. After the development of first ELISA assays for antibody detection based on recombinant N ([103, 104] and references therein), the current ELISA, immunoblot, rapid immunochromatographic, and other assays use this antigen [99, 105, 106]. Since the N terminus of N contains cross-reactive epitopes but epitopes in the C-terminal half of the protein are serotype (species) specific, assays with truncated N lacking the first 50 or 100 aas were developed. These assays show enhanced serotype specificity but lower sensitivity [99]. Moreover, attempts were made to use peptides for serodiagnostic approaches [107].

Despite cross-reactivity between hantaviral N proteins, diagnostic use of homologous virus antigen in the assays is preferred to search for antibodies after infection by a particular hantavirus. For instance, low-titer anti-DOBV sera cannot be detected in ELISAs based on HTNV-N protein (HTNV is highly related to DOBV) but can on DOBV-N protein [106]. New serological assays using recombinantly expressed N protein of Uluguru virus allowed the conclusion that not only rodent-borne but also shrew-borne hantaviruses such as ULUV are able to infect humans [14].

The immunoreactivity of N can also be used for vaccination purposes. The N-terminal 120 aas of PUUV-N, recombinantly expressed or inserted into virus-like particles, are able to protect bank voles against subsequent PUUV challenge. It seems that the main protective effect is associated with aas 1–45 of this region [108–111].

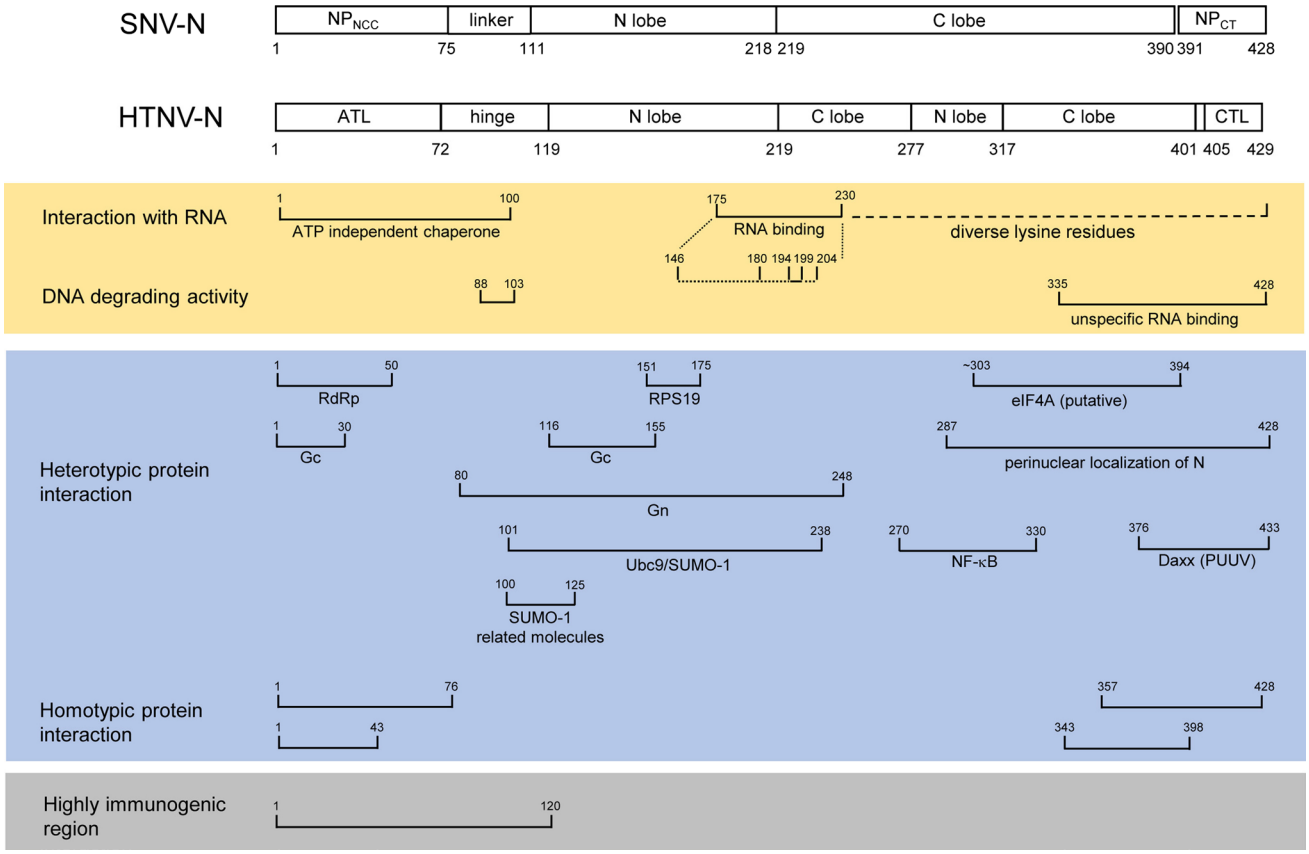

Fig. 2 Hantavirus N protein and multiple functional regions assigned from different studies cited in this review. Structural details for the schemes of SNV-N and HTNV-N above were taken from Guo et al. [27] and Olal and Daumke [28], respectively. NP_{NCC} N-terminal coiled-coil domain of the N protein, NP_{CT} C-terminal tail of the N protein, *ATL* amino-terminal arm, *CTL* carboxy-terminal arm. In the three panels below, experimentally confirmed or predicted interacting regions within the N primary sequence with nucleic acids (yellow), proteins (light blue), and a highly immunogenic region (gray) are compiled (Color figure online)

Following the idea that neutralizing antibodies have to be directed against surface proteins (glycoproteins) of the virion, the protection by N, an internal protein, can be best explained by induction of cellular immunity.

Some hantaviruses (mainly HTNV, PUUV, SNV) have been investigated for the presence of CD8 and CD4 CTL epitopes in their N. It seems that these epitopes are distributed over the whole protein and, again, that some are virus specific but others are cross-reactive [112–115]. On the basis of predicted human proteasome cleavage, transporter-associated antigen processing efficiency, and antigenicity, there are bioinformatic approaches to define HLA-restricted T-cell epitopes in the N protein. The authors speculate that the identified epitopes could be considered for the development of subunit peptide vaccines [116].

Conclusions

Viruses co-evolve with their hosts under the pressure of genome minimization [6]. On the genome level, the principle of overlapping genes has resulted in a highly efficient viral gene expression by use of mechanisms such as suppression of termination, leaky scanning, usage of different reading frames, alternative splicing, ambisense RNA, and RNA editing. On the protein level, polyproteins and multifunctional proteins are synthesized for reasons of genetic economy. The very limited genome length of hantaviruses makes multitasking of the few encoded proteins necessary. N is a typical viral moonlighting protein which can be found both in the cytoplasm of the infected cells and in the matured virion. It encompasses properties as diverse

as genome packaging, RNA chaperoning, intracellular protein transport, DNA degradation, intervention in host translation, and restricting host immune responses. These functions all rely on the capability of N to interact with RNA and other viral and cellular proteins (Fig. 2). The awesome diversity of N-derived functions in the hantaviral replication cycle makes it a promising target in antiviral drug development [10].

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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