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THE STRUCTURE AND FUNCTIONS OF HANTAVIRUS NUCLEOCAPSID PROTEIN

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ACADEMIC DISSERTATION

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Be practical, study, work… but I like long walks & rain ---

- from the film by Jonas Mekas *'He stands in a Desert Counting the Seconds of His Life'* (1985)

Original Publications

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Alminaite, A., Halttunen, V., Kumar, V., Vaheri, A., Holm, L., and Plyusnin, A. 2006. Oligomerization of hantavirus N protein: analysis of the N-terminal coiled-coil domains. Journal of Virology, 80:9073-81.

II. Alminaite, A., Backström, V., Vaheri, A., and Plyusnin, A. 2008. Oligomerization of hantavirus N protein: Charged residues in the N-terminal coiled-coil domain contribute to intermolecular interaction. Journal of General Virology, 89: 2167-74

III. Wang, H.*, Alminaite, A.*, Vaheri, A., and Plyusnin, A. 2010. Interaction between hantaviral nucleocapsid protein and the cytoplasmic tail of surface glycoprotein Gn. Virus Research, 151(2):205-12.

IV. Alminaite, A., Backström, V., Vaheri, A., Holm, L., and Plyusnin, A. 2010. Analysis of putative RNA binding domain of hantaviral nucleocapsid protein. Manuscript

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Abbreviations

aa	amino acid
AD	activation domain
cRNA	complementary RNA
DBD	DNA-binding domain
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
Gc	formerly glycoprotein G1
GFP	green fluorescent protein
Gn	formerly glycoprotein G2
GST	glutathione-S-transferase
GTP	guanosine triphosphate
HFRS	hemorrhagic fever with renal syndrome
HPS	hantavirus pulmonary syndrome
IFA	immunofluorescence assay
IFN	interferon
L	large genome segment of *Bunyaviridae*
M	medium genome segment of *Bunyaviridae*
MAb	monoclonal antibody
NE	nephropathia epidemica
N protein	nucleocapsid protein
ORF	open reading frame
PAb	polyclonal antibody
RdRp	RNA-dependent RNA polymerase
RNP	ribonucleoprotein
S	small genome segment of *Bunyaviridae*
vRNA	viral RNA

Abbreviations for viruses

Genus *Hantavirus*

ANDV	Andes virus
BCCV	Black Creek Canal virus
DOBV	Dobrava virus
HTNV	Hantaan virus
PUUV	Puumala virus
SAAV	Saaremaa virus
SEOV	Seoul virus
SNV	Sin Nombre virus
TULV	Tula virus

Genus *Orthobunyavirus*

BUNV	Bunyamwera virus
LACV	La Crosse virus

Genus *Nairovirus*

CCHFV	Crimean-Congo hemorrhagic fever virus

Genus *Phlebovirus*

RVFV	Rift Valley fever virus
UUKV	Uukuniemi virus

Genus *Tospovirus*

TSWV	Tomato spotted wilt virus

SUMMARY

The structure and functions of hantavirus nucleocapsid protein

Agnė Alminaitė 2010. Doctor's dissertation.

Hantaviruses, members of the genus *Hantavirus* in the *Bunyaviridae* family, are enveloped single-stranded RNA viruses with tri-segmented genome of negative polarity. In humans, hantaviruses cause two diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), which vary in severity depending on the causative agent. Each hantavirus is carried by a specific rodent host and is transmitted to humans through aerosolized excreta of infected rodents. The genome of hantaviruses encodes four structural proteins: the nucleocapsid protein (N), the glycoproteins (Gn and Gc), and the polymerase (L) and also the nonstructural protein (NSs).

This thesis deals with the functional characterization of hantavirus N protein with regard to its structure. Structural studies of the N protein have progressed slowly and the crystal structure of the whole protein is still not available, therefore biochemical assays coupled with bioinformatical modeling proved essential for studying N protein structure and functions. Presumably, during RNA encapsidation, the N protein first forms intermediate trimers and then oligomers. First, we investigated the role of N-terminal domain in the N protein oligomerization. The results suggested that the N-terminal region of the N protein forms a coiled-coil, in which two antiparallel alpha helices interact via their hydrophobic seams. Hydrophobic residues L4, I11, L18, L25 and V32 in the first helix and L44, V51, L58 and L65 in the second helix were crucial for stabilizing the structure. The results were consistent with the "head-to-head, tail-to-tail" model for hantavirus N protein trimerization. We demonstrated that an intact coiled-coil structure of the N terminus is crucial for the oligomerization capacity of the N protein. We also added new details to the 'head-to-head, tail-to-tail' model of trimerization by suggesting that the initial step is based on interaction(s) between intact intra-molecular coiled-coils of the monomers.

We further analyzed the importance of charged aa residues located within the coiled-coil for the N protein oligomerization. To predict the interacting surfaces of the monomers we used an upgraded *in silico* model of the coiled-coil domain that was docked into a trimer. Next the predicted target residues were mutated. The results obtained using the mammalian two-hybrid assay suggested that conserved charged aa residues within the coiled-coil make a substantial contribution to the N protein oligomerization. This contribution probably involves the formation of interacting surfaces of the N monomers and also stabilization of the coiled-coil via intramolecular ionic bridging. We proposed that the tips of the coiled-coils are the first to come into direct contact and thus initiate tight packing of the three monomers into a compact structure. This was in agreement with the previous results showing that an increase in ionic strength abolished the interaction between N protein molecules. We also

showed that residues having the strongest effect on the N protein oligomerization are not scattered randomly throughout the coiled-coil 3D model structure, but form clusters.

We found evidence for the hantaviral N protein interaction with the cytoplasmic tail of the glycoprotein Gn. In order to study this interaction we used the GST pull-down assay in combination with mutagenesis technique. The results demonstrated that intact, properly folded zinc fingers of the Gn protein cytoplasmic tail as well as the middle domain of the N protein (that includes aa residues 80–248 and supposedly carries the RNA-binding domain) are essential for the interaction. Since hantaviruses do not have a matrix protein that mediates the packaging of the viral RNA in other negative stranded viruses (NSRV), hantaviral RNPs should be involved in a direct interaction with the intraviral domains of the envelope-embedded glycoproteins. By showing the N-Gn interaction we provided the evidence for one of the crucial steps in the virus replication at which RNPs are directed to the site of the virus assembly.

Finally we started analysis of the N protein RNA-binding region, which is supposedly located in the middle domain of the N protein molecule. We developed a model for the initial step of RNA-binding by the hantaviral N protein. We hypothesized that the hantaviral N protein possesses two secondary structure elements that initiate the RNA encapsidation. The results suggest that amino acid residues (172-176) presumably act as a ‘hook’ to catch vRNA and that the positively charged interaction surface (aa residues 144-160) enhances the initial N-RNA interacation.

In conclusion, we elucidated new functions of hantavirus N protein. Using *in silico* modeling we predicted the domain structure of the protein and using experimental techniques showed that each domain is responsible for executing certain function(s). We showed that intact N–terminal coiled-coil domain is crucial for oligomerization and charged residues located on its surface form a interaction surface for the N monomers. The middle domain is essential for interaction with the cytoplasmic tail of the Gn protein and RNA binding.

Contents

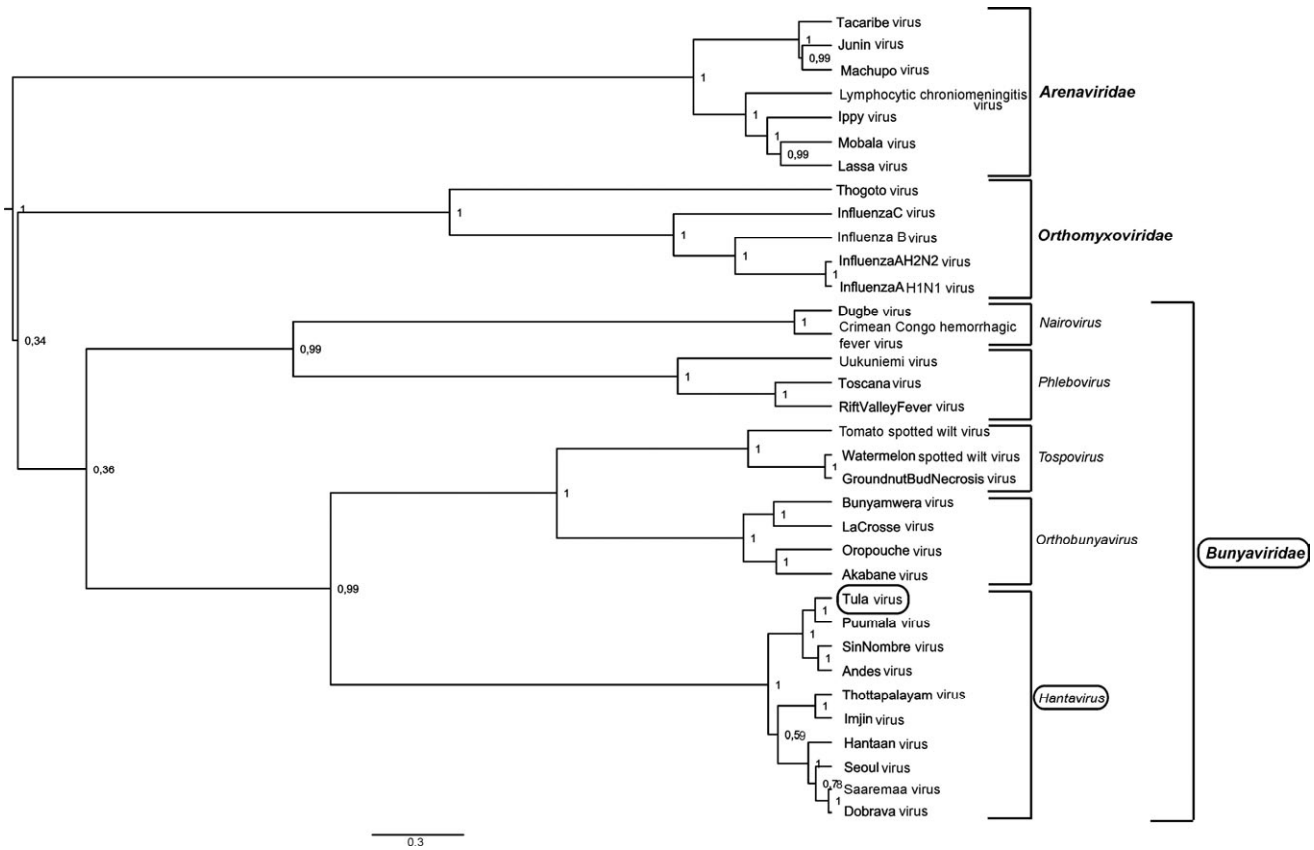
1. INTRODUCTION

1.1 HANTAVIRUS-ASSOCIATED DISEASES

Hantaviruses are enveloped negative-strand RNA viruses with a tripartite genome. Together with four other genera (*Nairovirus*, *Phlebovirus*, *Tospovirus* and *Orthobunyavirus*) the genus *Hantavirus* constitutes the family *Bunyaviridae* (Nichol *et al*., 2005) (**Fig. 1**). Hantaviruses are rodent- and insectivore-borne viruses and each is closely associated with a single rodent or insectivore host species. Hantaviruses have co-evolved with their rodent or insectivore hosts for millions of years and transmission to other species (including humans) is a dead-end for the virus (Plyusnin *et al*., 1996).

Hantavirus infections of the host species are thought to be persistent and non-pathogenic, although some studies have provided evidence that infected rodents grow slower and have a significantly lower overwinter survival probability (Childs *et al*., 1989; Douglass *et al*., 2007; Kallio *et al*., 2007). In general, the mechanisms of persistence of hantaviruses in the rodent hosts are not well understood but thought to depend on both virus-mediated modification of host immune response and alterations of the viral replication (Jonsson & Schmaljohn, 2001).

Fig. 1. *Phylogenetic tree of the segmented negative-strand viruses based on core polymerase domains. The tree was reconstructed using the Bayesian Monte Carlo Markov Chain method. The scale indicates substitutions per site. The family, genus and subject virus of this thesis are framed (By courtesy of Dr. Tarja Sironen).*



Infected rodents may excrete the virus in their saliva, urine and feces for months. When transmitted to humans hantaviruses can cause two diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus (cardio)pulmonary syndrome (HPS) (Vapalahti *et al*., 2003). Humans usually contract the infection through the respiratory tract when the air containing aerosolized viruses is inhaled. Person-to-person transmission has been reported only for Andes virus (Padula *et al*., 1998).

HFRS is a disease most commonly found in the human population in Eurasia, with mortality rates reaching 12%. It typically affects the urinary system and results in vascular hemorrhage and kidney dysfunction. HFRS is caused by the Old World (i.e. Afro-Eurasia) hantavirus species, associated with *Murinae* rodent subfamily (Old World rats and mice) and also by Puumala virus associated with bank vole (*Myodes glareolus*), belonging to *Arvicolinae* rodent subfamily. Hantaan virus (HTNV), the first etiological agent for HFRS was reported in 1978 (Lee *et al*., 1978), this led to recognition of other HFRS-related viruses in Asia and Europe. Now most HFRS cases are diagnosed in China and Korea and are caused by Hantaan and Seoul type viruses. HFRS caused by Puumala, Dobrava and Saaremaa viruses is also highly endemic in Europe. Specifically, several thousand cases, caused by Dobrava hantavirus are reported in the Balkans and Western Europe. Puumala hantavirus causes nephropathia epidemica (NE), which is a milder form of HFRS. Most cases of PUUV infection diagnosed in Europe come from Fennoscandia, notably Finland accounts for most of the hantavirus cases in Europe. In many European countries the number of hantavirus infections and HFRS has been on the increase in recent years (Heyman *et al*., 2008). Some sporadic cases of HFRS are reported worldwide due to the global distribution of the Norwegian rat (*Rattus norvegicus*) carrying SEOV (Lee & van der Groen, 1989).

HPS is characterized by rapid onset of respiratory failure and cardiogenic shock and is caused by the New World (i.e., the Americas) hantaviruses carried by New World rats and mice, belonging to *Neotominae* and *Sigmodontinae* rodent subfamilies. HPS bears some resemblance to HFRS except that the lungs are primarily targeted for capillary leakage instead of the kidneys. The mortality of this rare but severe disease can reach 60%, depending from the type of infecting hantavirus (Zavasky *et al*., 1999; Khan *et al*., 2000; Peters & Khan, 2002; Pini., 2004). The first known causative agent of HPS, the Sin Nombre virus (SNV), was discovered in 1993 within weeks after the outbreak in the Four Corners region in the United States (Nichol *et al*., 1993; Hjelle *et al*., 1994). Since then many different types of hantaviruses carried by rodents belonging to *Sigmodontinae* and *Neotominae* subfamilies have been identified throughout both Americas and more than 2000 cases of HPS have occurred (Jonsson *et al*., 2010).

At present, over 20 hantaviruses that cause illness in humans when transmitted from their rodent reservoirs have been identified, as well as many non-pathogenic hantaviruses. More hantaviruses may remain undiscovered, since in many countries, hantaviral infections are likely undiagnosed and not reported. This is especially evident with the discovery of shrew-borne hantaviruses (Jonsson *et al*., 2010). Furthermore, not all hantaviruses cause illness in humans. E.g. Tula hantavirus (TULV) isolated from the European common vole (*Microtus arvalis*) is widely spread in central and eastern Europe. It has been generally regarded as a non-pathogenic, thus being a perfect model for

experimental studies (Plyusnin *et al*., 1994). Factors, which determine and influence hantavirus pathogenicity, remain mostly unknown but likely include the nature of the rodent host and genetic susceptibility of patients. Hantaviruses carried by *Sigmodontinae* rodents cause, in general, more severe illnesses than those carried by *Murinae* and *Arvicolinae* rodents (Plyusnin *et al*., 1996).

Because of the global distribution of hantaviruses efforts are continuing to develop effective hantavirus vaccines. However, there is no WHO-approved vaccine available against hantavirus infections so far (Schmaljohn, 2009). Both rodent brain- and cell culture-derived inactivated vaccines for HFRS have been developed and tested in humans in Asia. Currently formalin-inactivated rodent brain-derived HTNV is used as a vaccine in South Korea. The vaccine is considered safe, however, the protective response to it is limited and requires continuous vaccination to gain sufficient protection (Sohn *et al*., 2001; Cho *et al*., 2002).

Several vaccines have been developed for hantaviruses using molecular approach, but only two have been tested in humans. The first was a recombinant vaccinia virus-derived vaccine containing the proteins encoded by the M and the S segments of HTNV. This vaccine was tested but the trials were not pursued further due to preexisting vector immunity problems (Schmaljohn *et al*., 1992). The other molecular hantavirus vaccine approach that had advanced to clinical studies is plasmid DNA delivered by a gene gun. The vaccines based on plasmids with HTNV and PUUV genes have been developed and tested in animals. A phase 1 clinical study of these vaccines in humans is in progress (Schmaljohn, 2009).

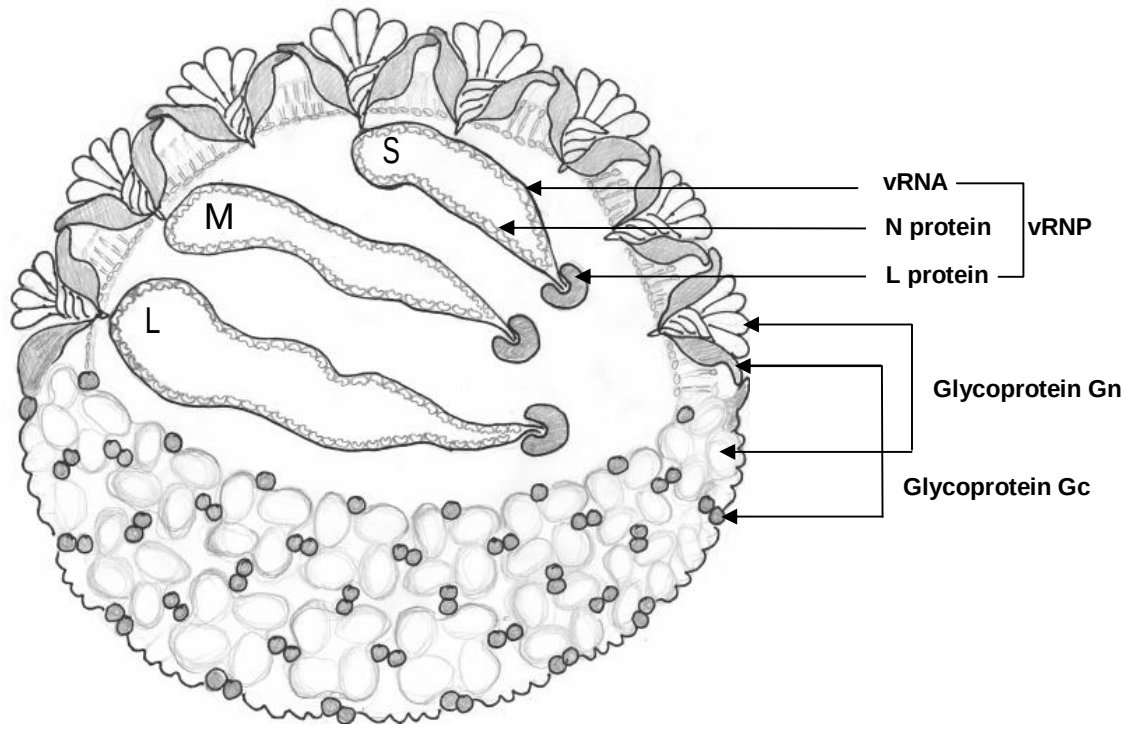
Currently, no approved specific antiviral drug is available for treatment of the hantavirus caused diseases either. Ribavirin has been shown to have *in vitro* activity and to some extent also *in viv*o activity against some hantaviruses (Severson *et al*., 2003). Vero E6 cells, pretreated with human interferons IFN-α, IFN-β and IFN-γ resulted in dose-dependent inhibition of HTNV replication. Of the three interferons, IFN-β inhibited the virus replication most effectively (Tamura *et al*., 1987). Human MxA protein might as well be a promising antihantaviral agent. It is a type I interferon-inducible protein that mediates antiviral actions against several members of the *Bunyaviridae* family after interferon stimulation (Frese *et al*., 1996). The human MxA protein has the capacity to inhibit PUUV and TULV replication, and RNA accumulation in virus-infected Vero E6 cells (Kanerva *et al*., 1996).

1.2 VIRION

Virions of bunyaviruses are enveloped, spherical and have a diameter of approximately 100 nm (Obijeski *et al*., 1976; Lee & Cho, 1981; Martin *et al*., 1985). Electron microscopy studies show that compared to other members of *Bunyaviridae*, hantavirus particles are remarkably different in shape. They are pleomorphic, tubular, variable in size, ranging from 70 to 210 nm (Hung *et al*., 1985; Goldsmith *et al*., 1995; Huiskonen *et al*., 2010; Battisti *et al*., 2010). The two glycoproteins Gn and Gc are embedded in the viral membrane and form tetrameric spikes on the surface of the virion. In

contrast to icosahedrally symmetric organization of the glycoproteins of Uukuniemi and Rift Valley fever viruses, Tula hantavirus spike complexes form ordered patches on the viral membrane via specific lateral interactions (Huiskonen *et al*., 2010) (**Fig. 2**). The viral membrane with the embeded glycoprotein complexes encloses viral RNA and proteins. Inside the virion S, M, and L vRNAs are not naked but encapsidated by the N protein forming ribonucleoprotein (RNP) complexes. Small amounts of the L protein are also associated with the encapsidated vRNA (Elliott *et al*., 1984; Gott *et al*., 1993; Kukkonen *et al*., 2004). S, M and L viral ribonucleoprotein complexes are found in equimolar amounts (Hutchinson *et al*., 1996). Threadlike RNPs fill the particles evenly, despite their pleomorfic shapes. This suggests that different amounts of RNPs can be incorporated in the virions (Huiskonen *et al*., 2010).

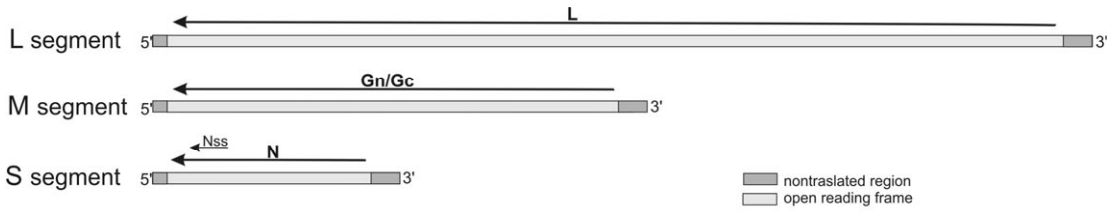
Fig. 2. *Schematic structure of hantavirus virion. The virion is enveloped by a phospholipid bilayer and contains three vRNPs: L (large), M (medium), and S (small), with one L protein bound to each. On the top of the virion picture the tetrameric glycoprotein spikes are shown half-sectioned.*



1.3 GENOME

Membrane of hantaviruses encloses a ribonucleoprotein core, which consists of three types of encapsidated RNA segments that encode four structural proteins (**Fig. 3**). The S mRNA encodes the N and NSs proteins from overlapping reading frames (Plyusnin, 2002); the M mRNA encodes a polyprotein that is cleaved to yield the Gn and Gc envelope proteins and the L mRNA encodes the L protein that is the RNA-dependent RNA polymerase (Elliott, 1996). Puumala virus has the longest open reading frames for all four structural proteins: L 2156 aa, Gc 658 aa, Gn 490 aa, N 433 aa; Seoul virus has the shortest open reading frames for all of the four viral proteins: L 2151 aa, Gc 647 aa, Gn 486 aa, and N 429 aa (Plyusnin, 2002). Hantavirus particles contain equimolar amounts of L, M, and S genomic RNAs (Hutchinson *et al*., 1996).

Fig. 3. *Hantavirus genome organization. The three negative stranded RNA segments encode fours structural proteins (L, Gn, Gc, S) and one nonstructural protein (NSs). Flanking these open reading frames are non coding regions containing cis-acting signals important for replication, transcription, encapsidation and packaging.*



Virus genomic RNA segments contain noncoding sequences on both the 3' and 5' termini. The NCRs are unique for each of the three vRNAs, but the very terminal NCR nucleotide sequences of all three segments are identical, conserved through genus and complementary:

```
3' AUCAUCAUCUG...
   |||||||| :|
5' UAGUAGUAUGC...
```

Due to sequence complementarity these terminal sequences presumably form base pairs and genome segments adopt a ring-like shape with a 'panhandle' (Plyusnin *et al*., 1996). 'Panhandle' structures of tomato spotted wilt virus (genus *Tospovirus*) and Uukuniemi virus (genus *Phlebovirus*) have been studied by microscopy and ring-like structures of three different lengths were visualized (Kellmann *et al*., 2001; Jonsson & Schmaljohn, 2001).

The terminal sequences of hantaviral RNA segments supposedly have several functions. They are important for recognition by the L protein and encapsidation by the N protein. It was proposed that N

protein trimer recognizes these structures specifically and after that RNP elongation is achieved by addition of further N molecules in a sequence independent manner (Mir at al., 2004). Located within these non-coding sequences are promoter elements that control replication of the segments and transcription of the encoded reading frames (Severson *et al*., 1999a; Flick *et al*., 2003a; Kohl *et al*., 2004; Barr *et al*., 2005). The BUNV transcription termination signal lies within the genomic 5' NTR (Barr *et al*., 2006). In general, complementarity, as well as defined sequences within very terminal NCR nucleotides are crucial for virus life cycle.

1.4 REPLICATION CYCLE

1.4.1 Attachement and entry

Hantavirus life cycle can be divided into viral attachment, entry, transcription, translation and replication, virion assembly and viral progeny release (**Fig. 4**). Enveloped viruses enter cells via recognition of the cell surface receptors. Subsequent fusion of the virus and cell membranes follows. In both their rodent hosts and humans, hantaviruses predominantly infect endothelial cells that constitute the lining of capillaries. Endothelial cells were found susceptible to hantavirus infection exclusively through the apical surface (Mou *et al*., 2006; Krautkramer & Zeier, 2008). Glycoprotein Gn mediates the entry presumably by interacting with β1 and β3 integrins (Gavrilovskaya *et al*., 1998; Gavrilovskaya *et al*., 1999). Pathogenic viruses causing HFRS and HPS use β3-integrins while apathogenic viruses use β1-integrins (Gavrilovskaya *et al*., 2002). This implies a role for receptors in determining the virulence. β3-integrins are known to regulate vascular permeability and platelet activation therefore hantavirus interactions with β3-integrins may contribute to viral pathogenesis (Mackow & Gavrilovskaya, 2001).

In addition to β3-integrins two other proteins, denoted '70K' and '30K' according to their mass, have been found to serve as receptors for HTNV (Kim *et al*., 2002; Mou *et al*., 2006). A decay-accelerating factor (DAF), glycosylphosphatidylinositol-anchored protein that serves as a receptor for a number of viruses, was proved to be crucial cofactor for entry of pathogenic PUUV and HTNV, too (Mou *et al*., 2006; Krautkramer & Zeier, 2008).

Attachment of enveloped viruses to cells is a critical early event in infection. More precisely, productive infection is achieved when the receptor-binding domain (RBD) of glycoprotein binds to receptors on cell surfaces (Godet *et al*., 1994). Using a phage-displayed peptide library crucial motifs of the RBD of HTNV were identified. The Gn region located between aa residues 96 and 105 (YPWHTAKCHY) may be a key motif of HTNV RBD which recognizes the receptor on target cell surface (Lu *et al*., 2009).

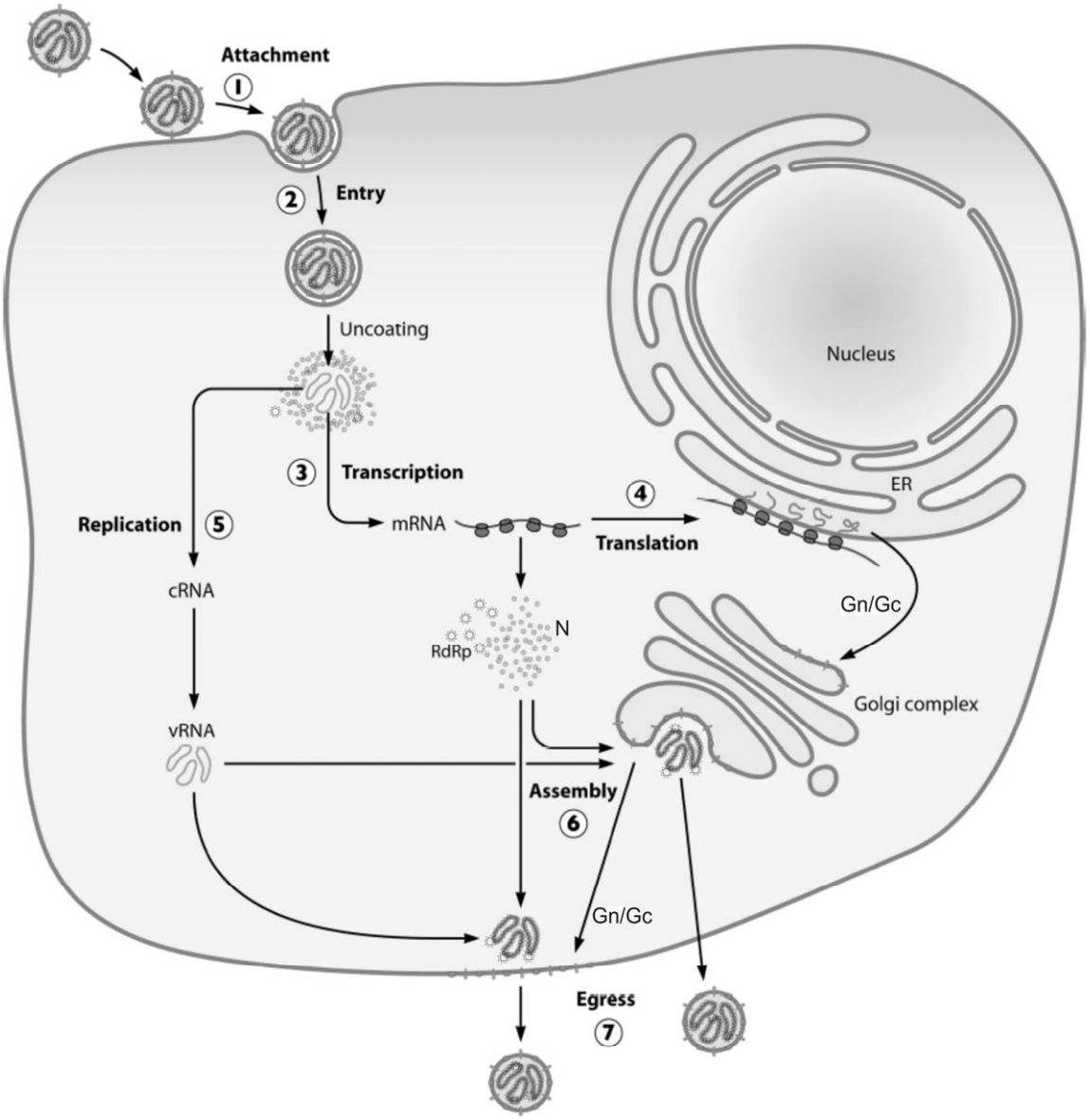


Fig. 4. *The hantavirus life cycle. Basic steps include the attachment of the virion particle through interactions between the cell receptors and the viral glycoprotein (1); entry through the use of receptor-mediated endocytosis and the uncoating and release of the viral genomes (2); transcription of complementary RNA (cRNA) from the viral RNA (vRNA) genome using host-derived primers (3); translation of S, M, and L mRNAs into viral proteins (4); replication and amplification of vRNA, assembly with the N protein, and transport to the Golgi apparatus (5); assembly of all components at the Golgi apparatus or, possibly for New World viruses, at the plasma membrane (6); and viral budding via the fusion of the Golgi vesicle harboring the mature virion particles with the plasma membrane (7). Modified from Jonsson et al., 2010.*

Following attachment, hantaviruses must penetrate the plasma membrane and target the genome to the proper cellular compartments. Glycoproteins are responsible for cell recognition, followed by virus internalization and subsequent fusion of the virus and cell membranes (Ogino *et al*., 2004). The entry of hantaviruses into cells was first studied with HTNV. The mode of entry appeared to be consistent with dynamin-regulated clathrin-mediated endocytosis (Jin *et al*., 2002). In support of these studies it was later shown that entry of HTNV, BCCV and SEOV also occurs via clathrin-mediated endocytosis. Only ANDV appeared to have different mode of entry that may include yet uncharacterized pathways that are clathrin- and caveolae-independent (Ramanathan & Jonsson, 2008). Later in the infection HTNV was shown to move to early endosomes, and subsequently to late endosomes or lysosomes (Jin *et al*., 2002).

Following the cell entry, virus-cell fusion occurs in early endosomes, where low pH was shown to play an important role (Arikawa *et al*., 1985). In addition to Hantaan virus, low pH-dependent cell fusion has been shown for several other hantaviruses including, PUUV, SEOV and ANDV (Okuno *et al*., 1986; Ray *et al*., 2010; Hepojoki *et al*., 2010a). Hantaan, Seoul and Puumala virus-infected cells have been shown to form syncytia with both infected and uninfected cells upon brief incubation at low pH (McCaughey et al., 1999; Tischler *et al*., 2005; Zheng *et al*., 2007). The ANDV pseudovirions also demonstrated pH-dependent entry where endosomal pH played a critical role in inducing fusion (Arikawa *et al*., 1985; Okuno *et al*., 1986; Ray *et al*., 2010). To summarize, acidic environment in endosomes induces the fusion of the virus membrane with the endosome membrane leading to the viral RNP release into the cytoplasm where synthesis of virus-specific mRNA begins.

1.4.2 Transcription and replication of the genome

The general features of hantavirus genome transcription and replication are similar to those of other negative-stranded RNA viruses. During replication, the negative stranded genome is copied to yield a complementary positive-stranded anti-genome, which acts as an intermediate for the generation of further genomic strands. Hantaviruses replicate exclusively in the host cell cytoplasm; more precisely perinuclear region. This was demonstrated when the N protein of Black Creek Canal, Puumala, and Seoul hantaviruses was found to localize in the cytoplasm in the perinuclear region (Ravkov & Compans, 2001; Kariwa *et al*., 2003a). Furthermore, Tula hantavirus nucleocapsid protein and polymerase have also been shown to colocalize in the Golgi region, indicating that Golgi region might be the site of replication (Kukkonen *et al*., 2004). Interestingly, N protein of the Crimean-Congo hemorrhagic fever nairovirus localizes in the perinuclear region even when Golgi is disrupted (Andersson *et al*., 2004a).

Virus genome replication often takes place in specific, virus-initiated compartments where cell organelles are recruited, viral components concentrated and different steps of the virus life cycle are sequentially connected. Replication complexes are frequently actin-associated. Besides cytoskeletal

components, also mitochondria and cytoplasmic membranes participate in the formation of these complexes, named viral factories (Novoa *et al*., 2005). The N protein of Black Creek Canal virus was shown to interact with actin (Ravkov *et al*., 1998). Localiztion of the N protein of Crimean-Congo hemorrhagic fever virus (genus *Nairovirus*) in Golgi region was abolished when actin filaments were disrupted (Andersson *et al*., 2004a). Therefore, it is possible that the RNA synthesis machinery of bunyaviruses would be associated with actin filaments in the perinuclear region.

It has been recently observed that a large structure is formed in the perinuclear area of cells infected with BUNV (Salanueva *et al*., 2003; Novoa *et al*., 2005). It was proposed that the virus modifies Golgi complex to build factories, which are used from early to late steps of the virus life cycle. The most stable component of Golgi stacks, the actin containing matrix scaffold, connected viral replication and morphogenesis inside viral factories. Actin and myosin-I were identified in isolated tubes while actin and the viral non-structural protein (NSm) were detected in the tubes' lumen. Tubes anchored cell organelles, such as mitochondria and rough endoplasmic reticulum to Golgi stacks and make contacts with intracellular viruses. Cellular translation elongation factor 2 and ribosomal proteins were indentified within the tubes as well (Novoa *et al*., 2005; Fontana *et al*., 2008), indicating that orthobunyavirus replication might occur in the newly built viral factories.

Naked genomic RNA from negative strand viruses cannot act as a template for replication. Each of the hantavirus genome segments, S, M and L, serves as template for two distinct RNA-synthesis activities: (i) replication, to generate antigenomes that are in turn replicated to yield more genomes; and (ii) transcription, to generate a single species of mRNA from which protein can be translated. After uncoating these viruses introduce RNPs to the cell, a vRNA segments that are bound by the N protein and the RNA-dependent RNA polymerase. Shortly after the RNP release primary transcription, i.e., synthesis of mRNA begins (Ortin & Parra, 2006).

Viruses from *Orthomyxoviridae*, *Bunyaviridae*, and *Arenaviridae* families all initiate viral transcription through the process of ‘‘cap-snatching,’’ which involves the acquisition of capped 5' oligonucleotides from cellular mRNA (Jin & Elliott, 1993; Mir *et al*., 2008). During transcription, each genomic strand is copied to yield a single mRNA that possesses a capped 5' extension, and is truncated at its 3' end relative to the negative stranded template. The scheme of hantavirus transcription is the following: first the L protein cleaves a primer from the 5´ termini of cellular mRNA molecules. Then initiation of transcription continues according to the “prime-and-realign” mechanism (Garcin *et al*., 1995). In this mechanism capped cellular oligonucleotides with the 3' terminal G residue are generated. These Gs form the base pairs with one of three possible C residues within the triplet repeat at the 3' terminus of the RNA template. After synthesis of a few nucleotides complementary to the triplet repeat the nascent mRNA realigns to a position upstream on the vRNA template. Without delay, the nascent virus-specific mRNAs are translated into viral proteins and, after reaching a threshold level of these proteins in the cytoplasm, the replication of viral genome starts. First, complementary RNA (cRNA) is synthesized; this RNA is used to produce new vRNA, which, in turn, activates secondary transcription (Jonsson & Schmaljohn, 2001). The newly synthesized cRNA and vRNA are immediately encapsidated by the N protein.

1.4.3 Translation

After transcription of vRNA, nascent virus specific mRNAs are translated into five viral proteins. Recently, several roles were ascribed to the hantaviral N protein during translation initiation. It was observed that N specifically binds the viral RNA panhandle and, using its RNA chaperone activity, unwinds the panhandle. N remains associated with the 5' end of the viral RNA, which facilitates access of the 3' end of the template for translation initiation (Mir & Panganiban, 2004; Mir & Panganiban, 2006a; Mir & Panganiban, 2006b). N also binds the 5' caps of cellular mRNA and sequesters these caps in cytoplasmic processing bodies for use (presumably by the viral polymerase) in cap-snatching and translation initiation (Mir *et al*., 2008). Additionally, it was observed that hantavirus N protein binds directly to the cellular 43S pre-initiation complex facilitating loading of ribosomes onto capped mRNA and functionally replacing cellular cap-binding complex eIF4F (Mir & Panganiban, 2008). N protein's colocalization with P-body (processing body) proteins, led to the hypothesis that both translation and replication of hantavirus genome might take place within the P-body. This hypothesis is under investigation (Melanson & Panganiban, 2010).

1.4.4 Assembly and budding

During the hantavirus assembly the four structural proteins and genomic RNA have to be packaged to form a virus particle. For enveloped viruses, the site of viral assembly and maturation is largely determined by the glycoproteins. Gn and Gc proteins are translated from the same M mRNA, translocated to ER, cotranslationally cleaved by signal peptidases, glycosylated, and transported to the Golgi complex or to the plasma membrane, the places of hantavirus maturation and budding (Kariwa *et al*., 2003a). Studies with Uukuniemi virus (genus *Phlebovirus*), Bunyamwera virus (genus *Orthobunyavirus*) and hantaviruses SNV and ANDV showed that not a single glycoprotein but Gn/Gc heterodimers constitute the Golgi retention signal that retains them in the Golgi compartment for virus budding (Shi & Elliott, 2002; Deyde *et al*., 2005; Overby *et al*., 2007b).

As soon as new vRNA is synthesized it is encapsidated by the N protein. This process involves specific recognition of the viral genome. Conserved central region of HTNV N protein binds RNA (Xu *et al*., 2002), lysine residues dispersed between positions 175 and 429 and also three residues, E192, Y206, and S217, located in the RNA-binding domain were shown to be important for the RNA binding (Severson *et al*., 2005). It was also observed that bunyavirus N protein binds the viral RNA with higher affinity than it does the nonviral RNA (Mir & Panganiban, 2004). Additionally, N specifically

binds the viral RNA panhandle. This also likely aids encapsidation and packaging of the viral genome. The encapsidation process can start as early as 2 hours post-infection since at that time the first newly synthesized N protein appears in the cytoplasm of infected Vero E6 cells (Kariwa *et al*., 2003a).

It is also possible that the initiation of encapsidation requires that the L protein binds to vRNA first and then the N protein would bind the L protein/vRNA complex. With influenza virus it has been shown that it is the polymerase subunits and not the nucleoprotein that binds the termini of vRNAs (Klumpp *et al*., 1997) and the influenza virus nucleoprotein has been shown to bind the RNA polymerase (Biswas *et al*., 1998; Medcalf *et al*., 1999; Coloma *et al*., 2009). There are, however, no published studies on interaction between L and N proteins of bunyaviruses, except that they have been found to colocalize in the perinuclear region (Kukkonen *et al*., 2004).

Viruses in the family *Bunyaviridae* lack the matrix protein, which in other negative-strand viruses such as orthomyxo-, paramyxo- and rhabdoviruses plays a key role in virus assembly (Suryanarayana *et al*., 1994; Ye *et al*., 1999; Schmitt & Lamb, 2004). Therefore it was suggested that bunyavirus vRNPs could interact directly with the membrane-associated viral glycoprotein(s) thus helping the virus to assemble (von Bonsdorff & Pettersson, 1975). Recently, interactions between the RNP and the glycoprotein cytoplasmic tails have indeed been observed for bunyaviruses (Overby *et al*., 2007a; Shi *et al*., 2007). Interaction was also demonstrated between N and both glycoproteins of a plant infecting bunyavirus – TSWV (genus *Tospovirus*). The analysis demonstrated that TSWV N co-localizes and interacts with both glycoproteins, with a preference for Gn (Snippe *et al*., 2007; Ribeiro *et al*., 2009).

So far for hantaviruses there is no direct evidence on interaction between the N protein and the glycoproteins, but in Tula virion tomogram slices, connections of the RNP to the membrane, where the cytoplasmic tail of the Gn protein is located, were occasionally observed (Huiskonen et al., 2010).

The members of family *Bunyaviridae* are known to mature at the Golgi apparatus (Andersson et al., 1997; Jantti et al., 1997). Lateral growth of the glycoprotein spike lattice on the Golgi membrane appears to be the force that drives the budding. Spike complexes (made of Gn and Gc) form ordered patches on the viral membrane by means of specific lateral interactions. These interactions may be sufficient for creating membrane curvature during virus budding (Huiskonen *et al*., 2010).

After newly formed hantavirus virions have acquired glycoproteins and vRNPs have been packed, the vesicles bud into the lumen of the Golgi and are transported to the plasma membrane. Cellular recycling endosomes and the Rab proteins associated with vesicular transport are needed for hantavirus release from the cells (Pensiero *et al*., 1988; Ravkov *et al*., 1997; Novoa *et al*., 2005; Fontana *et al*., 2008; Schmaljohn, 2001). First hantavirus particles can be detected within 24 h post infection (Kariwa *et al*., 2003a). However, the electron microscopy of New World hantavirus Sin Nombre have suggested that its maturation did not occur in the Golgi compartment but on the cell surface (Goldsmith *et al*., 1995). New World and Old World hantaviruses differentially utilize host cytoskeleton components during their life cycles, suggesting that cell surface maturation is possible for some hantaviruses (Ramanathan & Jonsson, 2008).

1.5 PROTEINS

1.5.1 Glycoproteins, Gn and Gc

Hantavirus glycoproteins are expressed from the medium (M) genome segment as a polypeptide, which is cotraslationally cleaved by a cellular protease after a conserved amino acid sequence WAASA. The cleavage yields Gn and Gc glycoproteins, that are translocated to the endoplasmic reticulum. The members of *Bunyaviridae* family require the presence of both newly synthesized Gn and Gc glycoproteins for their transport from endoplasmic reticulum to the Golgi apparatus, but only Gn was reported to posses the Golgi localization signal. Thus the process is assumed to be driven by a signal formed by the Gn-Gc heterocomplex (Pettersson, 1991; Spiropoulou, 2001; Andersson *et al.,* 1997). Hantaan virus studies have showed that Gn and Gc proteins localize in Golgi when the two are coexpressed. However, the electron microscopy of Sin Nombre hantavirus suggested that its maturation did not occur in the Golgi compartment but on the cell surface (Goldsmith *et al*., 1995; Ravkov *et al*., 1997).

Glycoproteins are responsible for cell recognition via surface receptors, followed by virus internalization and subsequent fusion of the virus and cell membranes. Evidence is accumulating that the hantaviral Gc protein is a class II fusion protein. Computer modeling of Andes virus Gc protein structure supports the hypothesis that this glycoprotein of hantaviruses and also other members of the family *Bunyaviridae* directs the viral fusion activity (Tischler *et al*., 2005).

Hantaviral glycoproteins are type-I integral membrane proteins: their N-terminal domains are oriented towards the ER or Golgi lumen while the C-terminal domains are facing the cytoplasm. The Gn protein presumably consists of an external domain, a transmembrane domain and a C-terminal cytoplasmic domain (also called cytoplasmic tail, Gn-CT). The Gc protein consists of a long external domain, a transmembrane domain and a short C-terminal cytoplasmic domain (Pettersson *et al*., 1995; Spiropoulou, 2001). The C-terminal part of hantaviral Gn-CT contains one or two conserved YxxL motifs. These motifs are known to direct receptor signaling within immune and endothelial cells (Geimonen *et al*., 2003). Recently other conserved cysteine-histidine rich region in the Gn-CT was reported to form a dual-fold, CCHC-type zinc fingers that presumably interact with each other and fold into a unique compact structure (Estrada *et al*., 2009). The glycoproteins are glycosylated with the sugar moieties being mainly of the high-mannose type (Schmaljohn *et al*., 1986).

Glycoproteins form heteromeric complexes that give the virions their typical surface symmetry. Tula virus appears to have unique grid-like surface pattern, described earlier by thin-section electron microscopy (Hung *et al*., 1985; Martin *et al*., 1985; Goldsmith *et al*., 1995) and recently by electron cryotomography (Huiskonen *et al*., 2010). This pattern is made of spikes likely containing four subunits of Gn glycoprotein and two subunits of Gc. The protruding volume of the spike consists of

Gn subunits while homodimeric Gc lies below (Hepojoki *et al*., 2010a). This feature may be shared by other hantaviruses, since very similar patten was shown for Hantaan virus (Battisti *et al*., 2010).

1.5.2 RNA Dependent RNA Polymerase, the L protein

RNA-dependent RNA polymerases (RdRp) are key enzymes in the replication of RNA viruses. The largest hantavirus genome segment L encodes the RdRp participating in both transcription and replication of the hantavirus genome. To execute these activities it must have several enzymatic functions such as endonuclease, transcriptase, replicase and, possibly, also RNA helicase activity (Jonsson & Schmaljohn, 2001). The RNA polymerase of influenza A virus has been shown to act as the endonuclease in the cap-snatching process. Bunyamwera virus replication studies also suggest that the L protein has the endonuclease activity which generates the primers (Jin *et al*., 1993). Indeed, La Crosse (LACV, genus *Orthobunyavirus*) virus L protein analysis confirmed that influenza-like endonuclease domain, essential for viral cap-dependent transcription is located in the N terminus of LACV L protein (Reguera *et al*., 2010). Recently, the N protein of SNV was shown to bind 5' caps of cellular mRNAs in cytoplasmic processing bodies, where presumably the polymerase cleaves and generates capped oligonucleotides that are then transported by the N protein and anneal at the 3' of vRNA. The annealed primers are elongated by RdRp to initiate the viral transcription (Panganiban & Mir, 2009).

The polymerases of negative-stranded viruses are either large in size or constitute large complexes (Ortin et al., 2006). Hantavirus L protein is a 250 kDa perinuclear membrane-associated protein. It shares conserved sequence regions with RdRp's of both positive-strand and negative-strand RNA virus polymerases as well as core structural polymerase subdomains (the thumb, the finger, and the palm) that adopt typical right-hand fold (Kukkonen *et al*., 1998; Kukkonen *et al*., 2005). Of the subdomains, the palm is the most conserved between the polymerases (Butcher *et al*., 2001). Crystal structures of influenza virus polymerase subdomains covering roughly half of the enzyme have been determined (Ruigrok *et al*., 2010) but for the hantaviral L protein the structure has not been solved yet. The influenza virus polymerase is a heterotrimer composed of the PB1, PB2 and PA subunits. The PB1 subunit contains the conserved polymerase domain which is responsible for the RNA elongation activity of the polymerase, PB2 is the subunit that binds the 5' cap of cellular mRNA and the PA subunit carries the cap-snatching endonuclease activity (Ruigrok *et al*., 2010).

Genomic RNA segments are encapsidated by the N protein and associate with the L protein, both in infected cells and in virions, and only these ribonucleoprotein particles are the functional templates for both mRNA synthesis and RNA replication by the viral polymerase (Elliott, 1996.). Recently, the high-resolution cryo-EM structure of a recombinant RNP of influenza virus has been described. The RNP contained RNA molecule of 248 nucleotides, 9 nucleoprotein protomers and a polymerase complex. The main nucleoprotein-polymerase interactions there were mediated by the PB1 and PB2

polymerase subunits (Coloma *et al*., 2009). Hantavirus L protein is also 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 (Schmaljohn & Dalrymple., 1983). So far there is no direct evidence for this model except that it was observed that hantavirus L protein, at least partially, is colocalized with the N protein in the Golgi region (Kukkonen *et al*., 2004).

1.5.3 Nonstructural protein, NSs

In viruses from genera *Orthobunyavirus*, *Tospovirus*, *Phlebovirus* and *Nairovirus* (**Fig. 1**) the M segment encodes not only two surface glycoproteins but also a non-structural protein (NSm), and the small S segment of first three aforementioned genera encodes nonstructural protein (NSs) in addition to the N protein (Schmaljohn *et al*., 2001).

The S RNA genome segment of hantaviruses carried by Arvicolinae and Sigmodontinae rodents encodes the nucleocapsid protein and has an overlapping open reading frame for a putative nonstructural protein (NSs) (Plyusnin, 2002). Recently the ORFs Tula and Puumala hantaviruses were shown to be functional. The produced NSs protein accumulated in the perinuclear area in infected and transfected cells and inhibited activation of the IFN-beta promoter. A portion of the NSs protein was colocalized with the N protein. There is also some evidence that the NSs protein might be expressed earlier in infection than the N protein (Jääskeläinen *et al*., 2007; Virtanen *et al*., 2010).

Innate immune response-regulating activity of NSs has been proposed for other viruses from the family *Bunyaviridae,* too. The NSs proteins of Bunyamwera (BUNV, genus *Orthobunyavirus*), and Rift Valley Fever Virus (RVFV, genus *Phlebovirus*) have been shown to act as antagonists of IFN induction (Weber *et al*., 2002; Bridgen *et al*., 2004; Billecocq *et al*., 2004; Ikegami *et al*., 2009). The NSs protein of RVFV is a general inhibitor of transcription; it prevents the formation of mature transcription factor TFIIH, suppresses the transcription of host mRNAs, including interferon-beta mRNAs (Le May *et al*., 2004), and induces post-transcriptional downregulation of dsRNA-dependent protein kinase (PKR), to promote viral translation in infected cells (Ikegami *et al*., 2009). The silencing of the NSs ORF converts BUNV from an interferon noninducer to an inducer (Bridgen *et al*., 2001). In adittion BUNV NSs suppresses activation of a nuclear factor kappa B (NF-kB)-dependent promoter and inhibits signaling downstream of IFN regulatory factor 3 (IRF-3) (Bridgen *et al*., 2004; Weber *et al*., 2002; Kohl *et al*., 2003). The NSs protein from the plant virus TSWV (genus *Tospovirus*) suppresses post-transcriptional gene silencing (Takeda *et al*., 2002). Tula and Puumala hantavirus N and NSs proteins did not suppress post-transcriptional gene silencing when *Nicotiana benthamiana* plants were transformed with plasmids expressing these proteins (Alminaite, unpublished data).

The role of NSm, another nonstrucural protein encoded by BUNV virus M segment and synthesized early in the infection is related to BUNV morphogenesis. NSm was shown to accumulate in the Golgi region of infected cells (Lappin *et al*., 1994; Shi *et al*., 2006). Recently it was shown that NSm and

actin are key factors in virus factory tube formation and the virus assembly in Golgi (Novoa *et al*., 2005; Fontana *et al*., 2008).

1.5.4 Nucleocapsid protein, N

All viruses with negative-sense RNA genomes encode a single-strand RNA-binding protein. Hantaviral N protein plays central role in the life cycle of the virus. It is the most abundant viral component in both virions and infected cells (Kaukinen *et al*., 2005). It has a well-established role in the formation of intracellular and virion-associated nucleocapsids, encapsidating both minus-strand viral RNA and plus-strand cRNA. For negative stranded viruses genomic RNA encapsidated by the nucleocapsid (N) protein is the template for transcription and replication by the viral polymerase. The N protein is synthesized in the cytoplasm and is later transported to the perinuclear region, where new virions assemble (Ravkov & Compans, 2001; Kukkonen *et al*., 2004; Kaukinen *et al*., 2005).

The N protein of hantaviruses contains 429 to 433 aa residues. Analysis of the sequence conservation reveals three conserved regions separated by two more variable regions spanning aa 50–80 and 230–310 (Kaukinen *et al*., 2005). The central conserved region contains a cluster of positively charged residues that overlaps with a putative highly conserved RNA-binding domain (Xu *et al*., 2002). The N protein is not mererely a structural RNA-binding protein. It functions also as a key adapter between virus and host cell processes (**Table 1**).

Table 1. *Macromolecules interacting with hantaviral N protein*

Macromolecule	Interacting region	Reference
Gn	Central region	Hepojoki *et al*., 2010b
Gc	Central region	Hepojoki *et al*., 2010b
RNA	Central region (HTNV 175-217)	Xu *et al*., 2002
Caps	Central region	Mir *et al*., 2008; Mir *et al*., 2010
SUMO	Central region (HTNV 188-191)	Kaukinen *et al*., 2003b; Maeda *et al*., 2003
Daxx	C- terminus (PUUV 339-433)	Li *et al*., 2002
Actin		Ravkov *et al*., 1998
DCP1(P-body)		Mir *et al*., 2008
Ubc9	Central region	Maeda *et al*., 2003
PIAS1		Lee *et al*., 2003a
TTRAP		Lee *et al*., 2003a
CHD3		Lee *et al*., 2003a
HIPK2		Lee *et al*., 2003a
Importin alpha		Taylor *et al*., 2009b

In the course of transcription and replication, N protein interacts with the L protein (Kukkonen *et al*., 2004); it also reacts with the cytoplasmic tail of Gn protein during assembly of new virions (Hepojoki *et al*., 2010a). Recently, the N protein was shown to act as an RNA chaperone (Mir & Panganiban,

2006b). In addition, N protein performs some ambassadorial duties by interacting with actin filaments (Ravkov *et al*., 1998) and also Daxx and SUMO-1 pathway components in infected cells (Li *et al*., 2002; Kaukinen *et al*., 2003b; Lee *et al*., 2003a; Maeda et al., 2003). The N protein also inhibits the translocation of the transcription factor NF-kB to the nucleus, which is consistent with a role in blocking induction of innate and acquired immune response by the host (Taylor *et al*., 2009a).

Recent observations indicate that the hantaviral N protein has additional unexpected biological activities that intersect with both the cellular mRNA translation and mRNA degradation apparatus. It has an activity that mimics the cellular cap-binding complex, eIF4F, in the initial stages of translation initiation (Mir & Panganiban, 2008). N is likely to be the major switching factor that determines whether genomic vRNA is transcribed into mRNAs that encode viral proteins or used as a template to synthesize cRNA for genome replication (Skorko *et al*., 1991). If, during cRNA and vRNA synthesis, the N protein is required to prevent the base pairing within the nascent RNA or between nascent RNA and the template, then the genome replication can proceed only when N protein has been synthesized in sufficient amounts to encapsidate the nascent RNA.

The N protein localization has been studied using several hantaviruses as models. In cells infected with BCCV, the N protein co-localizes with the medial Golgi marker (α-mannosidase II) in the perinuclear region. The N protein is located in perinuclear region also in most of cells infected with PUUV and SEOV, as well as in TULV-infected cells (Ravkov et al., 2001; Kaukinen *et al*., 2003b). In another study the N protein of SEOV did not co-localize with the Golgi protein giantin and formed granules in the cytoplasm (Kariwa *et al*., 2003a). In addition, in studies with BCCV and TULV, N protein was found to be a peripheral membrane-associated protein. Microsomal membrane and cytosol fractions were separated from cells transfected with a plasmid expressing BCCV N protein and the N protein was found in the microsomal membrane fraction. Apparently the C-terminus of the protein mediates the membrane association (Ravkov & Compans, 2001; Kukkonen *et al*., 2004).

Several experiments indicated that the N protein of SNV is located in cellular processing bodies (P bodies), discreet cytoplasmic foci that host proteins involved in RNA degradation and RNA regulation. The N protein fused with GFP co-localized strongly with DCP1, a signature protein of the P bodies. The N protein was shown to store and protect cellular 5' mRNA caps in P bodies for viral cap-snatching (Mir *et al*., 2008). In conclusion, the hantaviral N protein carries out many different functions at different time points during virus life cycle and probably localizes to several cellular compartments.

N protein oligomerization

Oligomerization of the nucleocapsid protein is crucial for providing a major structural framework for the assembly of viral ribonucleoproteins. The N protein multimerization requires contacts with both viral RNA and other N protein molecules. It was noticed that nucleoprotein (NP) molecules of negative-strand RNA viruses could self-assemble into helical nucleocapsid-like particles in the absence of viral RNA and of any other viral proteins. Marburg virus NPs (*Filoviridae*) assembled into large aggregates, which were seen in close association with membranes of the rough endoplasmic

reticulum in cells infected with the virus or transiently expressing viral nucleoprotein (Kolesnikova *et al*., 2000; Mavrakis *et al*., 2002a). Evidence for self-assembly of NP was shown for both Sendai and measles viruses (*Paramyxoviridae*) (Bankamp *et al*., 1996; Myers *et al*., 1997). The first RNP-like structures for hantavirus have been observed in insect cells that were expressing HTNV N protein in a baculovirus driven system (Betenbaugh *et al*., 1995). Later, TULV N protein expressed in insect cells has been shown to form 'spaghetti'-like structures, probably by assembling on nonspecificaly bound cellular RNAs (Plyusnin *et al*., 2008). Evidence that hantavirus N protein molecules interact with each other came also from the observation that the N protein monomers are difficult to purify because they tend to aggregate (Vapalahti *et al*., 1996a; Severson *et al*., 1999a).

The modes of the N-N interaction vary. The most studied nucleocapsid protein among segmented negative-strand RNA viruses is the influenza virus NP. In the influenza virus particle, the RNP complexes are organized into a distinct pattern, as visualized by transmission electron microscopy (Noda *et al*., 2006). To maintain the RNP structure influenza NP forms oligomers (Prokudina-Kantorovich & Semenova, 1996). Influenza virus NP is composed of head and body domains and a tail-loop linker region. The tail-loop region (aa 402 to 428) is important for oligomerization, which is mediated by the insertion of the structurally conserved tail-loop of one NP molecule to a groove of a neighboring NP (Ng *et al*., 2008; Chan et al., 2010).

The interaction regions of the influenza virus NP are organized differently than those in the NPs of *Paramyxoviridae* and *Rhabdoviridae*. In these latter virus families, the N-terminal part of viral NP is responsible for the self-assembly and RNA binding while the C-terminal part interacts with other viral proteins. At least two regions involved in the N-N interactions have been identified. The first is located within the first 255 aa residues (aa 189-239 in measles virus NP). The second is the central, mainly hydrophobic, conserved region (aa 258-357 in measles virus NP) (Bankamp *et al*., 1996; Myers *et al*., 1997; Kouznetzoff *et al*., 1998; Nishio *et al*., 1999; Karlin *et al*., 2002).

The assembly of RNP-like structures of negative-strand RNA viruses usually occurs without defined intermediate structures, but for bunyaviruses this may not be the case. For the hantaviral N protein, a trimer seems to be a stable and functional intermediate. From experiments with various hantaviruses it became apparent that the N protein forms trimers (Alfadhli *et al*., 2001; Kaukinen *et al*., 2001; Mir & Panganiban, 2004). Notably, the N protein trimers have been documented for BUNV (genus *Orthobunyavirus*) (Osborne & Elliott, 2000) and UUKV (genus *Phlebovirus*) (Katz *et al*., 2010). Analysis of other phlebovirus, the RVFV, showed that after chemical crosslinking treatment, the nucleoprotein migrated mainly as dimers. The N terminus of RVFV N was required for dimer formation (Le May *et al*., 2005). However, the conserved residues tested in the latter study (Tyr4, Phe11) were not identified as part of a dimer interface in crystalyzed RVFV N. The observed loss of dimer formation was likely due to destabilization of helices and, indirectly, the dimer interface (Raymond *et al*., 2010).

Which domains of the hantaviral N protein associate with each other to facilitate oligomerization? Initially, TULV N protein was studied by pepscan and linear interaction sites were indentified in both: the N-terminus (aa 13-30; 41-57) and the C-terminus (aa 340-379; 391-407; and 410-419). These regions were further studied with the alanine scan and the data pointed out that basic amino acid

residues (K and R) were crucial. This was in agreement with the earlier findings showing that the N protein molecules associate via electrostatic interactions and positively charged aa residues may facilitate the process. Notably, non-basic amino acid residues were found to be important for the interaction as well (Kaukinen *et al*., 2003a).

Later on N-N interacting domains were mapped more precisely by means of the mammalian two-hybrid (M2H) assay using the N protein mutants. The results pointed out that the contributions of the N and C-terminal regions to the N-N interaction are not equal. Deletion of 43 N-terminal amino acid residues from N protein constructs, used in M2H assay, reduced their interaction capacity to approx. 80%. This relatively weak reduction suggested that while the N-terminal region is involved in the homotypic interaction, other regions participate in the interaction too and their contribution might be even more substantial. To evaluate this hypothesis a set of C-terminal deletion constructs was prepared and the region VNHFHL, (aa residues 393-398) in TULV N was found to be essential for N-N interaction. This region probably scaffolds the structure formed by two alpha-helices (aa residues 374-394 and 404-421), since deletions shorter than 1-392 abolish the N-N interaction in M2H assay. In general, the C terminal regions were found to be crucial for N-N interaction.

The involvement of N- and C- terminal regions in the oligomerization of the N protein could be characteristic feature for other hantaviruses and also bunyaviruses. It was also shown for SNV that the C-terminal (aa 357-428) and the N-terminal (aa 1-40 regions are involved in the homotypic interaction (Alfadhli *et al*., 2001). For HTNV and SEOV N proteins, the N-terminal interaction region was mapped between aa 50 and 155 (HTNV), and 100 and 125 (SEOV). The C-terminal interaction region was mapped within aa 404 and 429, for HTNV, and aa 413 and 420, for SEOV (Yoshimatsu *et al*., 2003). For other bunyavirus, TSWV (genus Tospovirus), the N protein interaction regions were defined to the first 39 aa and the last 16 aa (Uhrig *et al*., 1999). For RVFV-N protein (genus *Phlebovirus*), dimer formation was suggested, and the N-N interacting domain was mapped to the first 71 N-terminal residues (Le May *et al*., 2005). UUKV (genus *Phlebovirus*) N proteins ability to oligomerize depended on the presence of intact alpha-helices on both termini of the N protein molecule. Notably, a helical structure in the N-terminal region, rich in aa residues with aromatic (W7, F10, W19, F27, F31) or long aliphatic (I14, I24) side chains, played a crucial role in the N-N interaction(s) (Katz *et al*., 2010). Chemical cross-linking studies of deletion mutants of orthobunyavirus BUNV indicated that both N- and C-terminal aa residues are involved in self-interaction, suggesting the head-to-head and tail-to-tail mode of interaction (Leonard *et al*., 2005).

Comparison of N structures

The capacity of forming RNPs is a unique feature of negative-strand RNA viruses. It is the viral RNP that is involved in the process of replication, since the viral polymerase complex can only copy the RNA in the RNP form. In addition, the virion assembly also requires the RNPs to be packaged in the virion. These unique functions that are common among negative-strand RNA viruses may lead to conservation of certain structural motifs in the N protein. Such conservation has been observed in the

capsid proteins of spherical viruses which share a β-barrel motif (Rossmann & Johnson, 1989). Structural similarities have also been noticed in the coat proteins of three other icosahedral virus groups: dsDNA virus (e.g. adenovirus), tailed dsDNA viruses (e.g. bacteriophages), and dsRNA viruses (e.g. reovirus) (Bamford *et al*., 2005).

Structures of the nucleoprotein of four negative-strand RNA virus families *Bornaviridae* (BDV), *Rhabdoviridae* (VSV and RABV), *Orthomyxoviridae* (FLUAV) and *Bunyaviridae* (RVFV) are now available. When the VSV N structure was compared to BDV and FLUAV N structures, it appeared that the C-terminal domain is more conserved than the N-terminal domain, with secondary structure elements arranged by a similar topology. Interestingly, nucleoproteins of the three first virus families share common structural motifs despite the fact that there is no detectable homology at the amino acid sequence level. There are five helices in the N terminal domain and three helices in the C-terminal domain that are common among the N structures of the three virus families. This '5H+3H' motif may be a common fold of negative-strand RNA viruses and can be compared to the β-barrel fold in the capsid proteins of spherical viruses (Luo *et al*., 2007).

On the other hand the crystal structures of RVFV N and RNP reveal a substantially different organization of the encapsidated genome than in other negative-sense RNA virus families. The RNP has an extended structure without helical symmetry. Free RVFV N protein has a novel fold that is mostly alpha-helical, compact and well ordered at both the N and C termini. Homology of the N protein within the *Bunyaviridae* family members is not apparent from the sequence data and the N proteins from different genera appear unrelated. Nevertheless it is possible that bunyaviruses from other genera have similar N protein folds (Raymond *et al*., 2010).

The N-terminal regions of hantavirus N protein have been shown to form the coiled-coil structure (Alfadhli *et al*., 2002). Coiled-coils consist of two or more alpha-helices that wrap around each other in a highly organized manner. A single α-helix is formed by heptad repeat of amino acid residues of which the first and the fourth are generally non-polar or hydrophobic. When helices coil around each other, the first and the fourth amino acid are internalized in a "knobs-into-holes" manner thus stabilizing the structure, while other aa residues are exposed on the surface of the protein (Crick, 1953). The N terminal coiled-coil domains of TULV and ANDV were crystallized and their structures solved (Boudko *et al*., 2007; Wang *et al*., 2008).

Secondary structure predictions suggested that the C-terminal region of the hantaviral N protein forms a helix-loop-helix structure. For TULV helix I formed by aa 373-387 is separated by a loop from helix II formed by aa 404-421. Removal of latter helix from one of the interacting N protein partners is not enough to disrupt the interaction, but when helix II is removed from both partners in the M2H assay, the interaction is abolished, suggesting that helix II is crucial for the interaction. It was experimentally proved that hydrophobic aa residues I380, I381 (helix I) and L413 and I414 (helix 2) are crucial for the interaction suggesting that they could form a shared hydrophobic space that provides stability to the N-N interaction (Kaukinen *et al*., 2004).

It is known that hydrophobic contacts do not usually initiate the protein-protein interactions. The aromatic (Phe, Tyr, Trp) and positively charged (Arg or Lys) side chains of amino acids are best known to form a special type of noncovalent molecular interaction with an adjacent cation (e.g. Li+,

and Na+) (Gallivan & Dougherty, 1999). For TULV, the N protein residue W388 could play an important role in the interaction initiation. The mutation of W388 to alanine abolished the M2H interaction totally and when transiently expressed in mammalian cells mutant proteins were localized diffusely. However, none of the R residues in the C-terminal region appeared to be a partner for W388 (Kaukinen *et al*., 2004). Interestingly, the W119A mutation in SEOV N protein and mutations of F242A and F246A of TSWV (genus *Tospovirus*) reduced the interaction of the viral proteins studied as well (Uhrig *et al*., 1999; Yoshimatsu *et al*., 2003). Thus, these data suggest that the aromatic aa residues might be crucial for maintaining the overall structure of the N protein.

So far five crystal structures of negative-strand RNA virus nucleocapsid proteins are available. Rhabdovirus RABV and VSV N were crystallized in complex with RNA, their structures are basically identical (Green *et al*., 2006; Albertini *et al*., 2006). The remaining three, the BDV N protein, FLUAV N protein and RVFV N protein structures were determined as a tetramer, trimer, and dimer, respectively, in the absence of RNA. The presence of multimers can indicate that multimeric N protein is functional in certain processes of virus life cycle, such as RNP formation. Experiments with RVFV N that carried a single W125 mutation in a dimer interface suggested that W125, and perhaps an N dimer, is essential for transcription. This could also be due to RNP-mediated molecular interaction(s), most probably with the RdRp (Raymond *et al*., 2010). For the hantaviral N protein, a trimer seems to be a stable and functional intermediate (Osborne & Elliott, 2000; Alfadhli *et al*., 2001; Kaukinen *et al*., 2001). Both C- and N-terminal domains of the N protein contribute to the trimerization. The current data are consistent with the “head-to-head, tail-to-tail” model for hantavirus N protein trimerization (Kaukinen *et al*., 2004). This model suggests that trimerization is a two-step process that involves an initial interaction between the N-terminal domains followed by a consolidating interaction between the C-terminal domains. Hantavirus N-trimers are able to discriminate between viral and nonviral RNA molecules and bind with high affinity the viral RNA panhandle structure (Mir & Panganiban, 2004). N protein trimers have also been proposed to be the intermediates that further assemble on vRNA (Alfadhli *et al*., 2001; Kaukinen *et al*., 2004). So far the crystal structure of the whole molecule of the hantavirus N is not available, only the N-terminal coiled-coil domain of SNV was crystallized. Notably, dimeric form of it was dominant in the preparations. At acidic pH, which mimics the N protein environment, these dimers formed an infinite band of head-to-head packed proteins. This might be a biologically relevant structure (Boudko *et al*., 2007).

RNP assembly

Successful formation of virus progeny depends on incorporation of viral RNA into the newly assembling viral particles. For all RNA viruses this process involves specific recognition of the viral genome by a nucleocapsid protein. In turn, oligomerization of the nucleocapsid protein is crucial for providing a major structural framework for the assembly of viral ribonucleoprotein particles.

The length of RNA molecule determines the stoichiometry of the N protein binding to RNA. There are estimations of how many N molecules are needed to form the N-RNA complexes. In all negative-

strand RNA viruses this nucleotide:N ratio is a multiple of three (Mavrakis *et al*., 2002a). The ratio determined for influenza virus was 24 nt/N (Ortega *et al*., 2000; Coloma *et al*., 2009), for VSV and rabies virus 9 nt/N (Iseni *et al*., 1998; Iseni *et al*., 2000) and for Marburg virus (family *Filoviridae*) 12 or 15 nt/N (Mavrakis *et al*., 2002a). The rule of six in the *Paramyxoviridae* family states that the virus genome is copied efficiently only if its length in nucleotides is a multiple of six (Vulliemoz & Roux, 2001). It was determined that for the prototypic virus of *Bunyaviridae* family, the BUNV, the stoichiometry of N-RNA association is 12 nucleotides per N monomer (Mohl & Barr, 2009).

Influenza is one of the best-studied viruses when it comes to the RNP formation. In the influenza virus particle, the RNP complexes are organized into a distinct pattern, as visualized by transmission electron microscopy. NP not only encapsidates the viral RNA but also forms oligomers to maintain the RNP structure. Oligomerization of the NP protein forms a major part of the RNP complex, as observed for purified intact viral RNPs and most recently for mini-RNPs (Coloma *et al*., 2009; Chan *et al*., 2010). The RNA-binding property of influenza N protein is known to involve the protruding element and the flexible basic loop between the head and body domains, both having high degree of primary sequence conservation. To bind RNA, NP protein must first capture the RNA by the flexible basic loop and then the RNA is clamped by the protruding element (Ng *et al*., 2009). In the influenza mini-RNPs observed by cryo-electron microscopy an estimated interaction among the NP monomers indicated that additional side-by-side interactions are possible due to the tighter packing of the monomers. The structure of the RNP was compatible with the RNA-binding site located in the groove between the head and body domains of the NP, a connecting mass was even apparent in the appropriate position (Coloma *et al*., 2009).

Other crystallized nucleocapsid proteins of negative-stranded viruses have similar topology of the RNA binding region. Borna disease virus and rhabdovirus N proteins also fit the RNA into a cavity formed by the two distinct domains of the nucleoprotein. RNA binds nonspecifically in a positively charged cleft between the lobes of the N subunits. It binds either the outside or inside of a ring of 9–11 N subunits. In all cases, protrusions from the N subunits make specific contacts with adjacent subunits to maintain the ring structure (Luo *et al*., 2007).

Contrary to the aforementioned viruses, RNP of RVFV, a member of *Bunyaviridae* family, lacks helical symmetry (Raju & Kolakofsky, 1989; Raymond *et al*., 2010). Instead the symmetry is similar to that observed in electron micrographs of another phlebovirus UUKV RNP, which forms large macrocircles (Pettersson & von Bonsdorff, 1975). Circular structures of bunyavirus RNP are formed due to pairing of complementary bases at the 3' and 5'ends of each genomic segment (Raju *et al*., 1989). The crystal structure of RVFV N is also consistent with the lack of helical symmetry. It is highly compact and has no protruding loops or termini that could link it to other N protein molecules in a superstructure like the rings of the *Mononegavirales* and orthomyxoviruses (Rudolph *et al*., 2003; Green *et al*., 2006; Coloma *et al*., 2009; Tawar *et al*., 2009), although major conformational changes of the N are possible upon RNA binding. In addition, there was no dominating RVFV N multimer found using the image analysis or gel filtration experiments, but cross-linking data showed a preponderance of weakly associated hexamer species. Therefore the model for the phlebovirus RNP structure is that the N protein binds cooperatively to RNA, in groups of 4–7 subunits. The RNP lacks a strong helical

structure, and the N-N contacts are weak. The exact RNA-binding manner of the RVFV N protein remains unclear but it is expected to engage the phosphate backbone and take place in a positively charged, highly conserved N lobe of the RVFV N protein (Raymond *et al*., 2010).

Formation of unusual N-RNA multimers and nonhelical RNPs may be a general feature of bunyaviruses. The observed multimer species of RVFV N-RNA are similar to those reported 109-kDa recombinant RNP from BUNV (Mohl & Barr, 2009). Details of the hantaviral RNP assembly remain largely unknown. Current data suggest that the N protein binds to vRNA as a trimer (Alfadhli *et al*., 2001; Kaukinen *et al*., 2004). Hantavirus RNA-binding domain has been located to the central conserved region of the N protein (Xu *et al*., 2002).

There are several models of the initiation of hantavirus RNA encapsidation. In one of the proposed models, the encapsidation starts when the N protein binds the specific region within the vRNA molecule that posseses a unique encapsidation signal (Severson *et al*., 1999a). The N protein starts the encapsidation process at the 5' end of the vRNA that forms a single-stranded stem-loop structure. The N protein binds specifically to the loop; this is followed by a subsequent N–N interaction that encapsidates the remaining part of vRNA in a non-specific fashion (Severson *et al*., 2001).

Terminal panhandle-forming regions of the vRNA molecules seem to possess a unique binding region for the hantavirus N protein (Mir & Panganiban, 2004; Mir & Panganiban, 2005; Mir & Panganiban, 2006b). The corresponding sequences of the cRNAs are recognized with lower affinity. A conformational change of the nucleocapsid protein molecule upon binding the RNA is probably essential for the RNA encapsidation. This conformational change results from an interaction not only between the N protein and viral RNA but also between two (or more) molecules of the N protein. This hypothesis is in agreement with experimental data on *in vitro* binding of recombinant N-trimer to the viral RNA panhandle. The trimer works as an RNA chaperone causing dissociation of the panhandle and thus making the 3´ terminus of RNA available for the viral polymerase (Mir & Panganiban, 2006b). It is worth mentioning that single monomers cannot perform this task and it has to be a trimer, which acts as a chaperone. The chaperone activity was proposed to reside in the amino terminal sequence (aa residues 1-90) of the N protein (Mir & Panganiban, 2006b).

Hantaviral N protein binds the viral genomic RNA with high affinity. The RNA-binding domain of the HTNV N protein has been mapped to the central conserved region (aa 175–217) (Xu *et al*., 2002). Experiments with SNV suggested that the hantaviral N protein binds not only RNA, but also has distinct cellular mRNA cap-binding site that enables N to interact with both the capped primer and vRNA template simultaneously. The binding capacity of the N protein for capped RNA is weaker than that for the viral RNA. Moreover, the N protein adopted different conformational states when its cap- and RNA-binding sites were occupied with their substrates either separately or simultaneously (Mir *et al*., 2010).

Secondary structures located near 5' terminus have been proposed to be important for BUNV vRNA encapsidation (Osborne & Elliott, 2000). Therefore, BUNV virus was studied in order to find out whether the encapsidation of the bound RNAs by the N protein requires the presence of a defined secondary structure. Experimental results suggested that the RNAs bound to the N protein tetramers were not encapsidated because of any obligatory signals, neither a linear sequence, nor a secondary

structure. The authors, however, did not exclude the possibility that BUNV replication products contain a signal that allows preferential binding over other RNAs. Furthermore, they pointed that if an encapsidation signal that drives preferential binding does exist, it will be interesting to understand how it is recognized by the RNA-binding groove within the BUNV N protein (Mohl & Barr, 2009).

Association of the N protein with other viral proteins

All negative-strand RNA viruses have their own RNA-dependent RNA polymerase, which transcribes and replicates the virus genome. For example, influenza virus genomic RNA segments are found in association with a tripartite RNA polymerase (PB1, PB2 and PA subunits) and stoichiometric quantities of nucleoprotein. Three-dimensional reconstruction of a recombinant influenza virus RNP particle showed that the NP interacts with PB1 and PB2 subunits of the polymerase (Coloma *et al*., 2009). Similarly, the hantaviral L protein is also thought to be associated with the RNPs so that it is ready to initiate viral RNA synthesis immediately after the virus entry to a host cell (Schmaljohn & Dalrymple, 1983).

Little sequence specificity has been found with N protein of hantavirus binding to RNA alone. With influenza virus it has been shown that it is the RNA polymerase, not nucleoprotein, that binds the very termini of the vRNAs (Klumpp *et al*., 1997). Thus it is possible that the initiation of encapsidation first requires the L protein to be bound to the vRNA and then the N protein would bind to the L protein/vRNA complex and encapsidation would proceed. Hantaviral N and L proteins have been found to colocalize in the perinuclear region near the cis-Golgi, suggesting that this is likely to be the site of RNA synthesis (Kukkonen *et al*., 2004).

Hantaviral glycoproteins Gn and Gc are co-translationally cleaved from a single precursor and targeted to the Golgi compartment where they are processed and transported to the virus assembly sites. Identification of the Gn and Gc protein retention signal has been important for clarifying the mechanism and the location for the virus budding. The cytoplasmic tail of the Gn protein and the complete signal sequence of the Gc protein have been suggested to play an important role in the Golgi localization (Shi & Elliott, 2002; Spiropoulou *et al*., 2003). The Gn protein presumably consists of an external domain, a transmembrane domain and a C-terminal cytoplasmic domain, also called cytoplasmic tail, Gn-CT. The Gc protein consists of a long external domain, a transmembrane domain and a short C-terminal cytoplasmic domain (Melin *et al*., 1995; Spiropoulou, 2001). Recently crystallized hantaviral Gn-CT zinc finger domain showed no RNA-binding activity thus pointing in the direction of a protein–protein interaction (Estrada *et al*., 2009).

Bunyaviruses lack a matrix protein, which in other negative-strand viruses, such as orthomyxo-, paramyxo- and rhabdoviruses plays a key role in the virus assembly (Suryanarayana *et al*., 1994; Ye *et al*., 1999; Schmitt & Lamb, 2004). Therefore it was suggested that bunyaviruses vRNPs can interact directly with the membrane-associated viral glycoprotein(s) thus helping the virus assembly (von Bonsdorff & Pettersson, 1975). The cytoplasmic tail of the Gn protein is presumably located on

the cytoplasmic side of the Golgi membrane and it probably determines which RNPs would be included in the forming virus particle. For UUKV (genus *Phlebovirus*), the interaction between vRNPs and Gn/Gc proteins was first demonstrated by immunoprecipitation (Kuismanen *et al*., 1984). Later, it was shown that it is the cytoplasmic tail of Gn protein of Uukuniemi virus that interacts with the vRNP (Overby *et al*., 2007a). For BUNV (genus *Orthobunyavirus*) the interaction between RNP and Gn-CT glycoprotein has been shown in the infectious virus-like particles (Shi et al., 2006). Similarly, in TSWV (genus *Tospovirus*) direct interaction of the N protein, a key component of the vRNPs, and Gn glycoprotein was demonstrated by co-localization assay (Snippe *et al*., 2007; Ribeiro *et al*., 2009).

In fact, recently PUUV Gn and Gc proteins were shown to interact with the ribonucleoprotein. Native PUUV proteins were used in co-immunoprecipitation experiments where N protein was detected together with the glycoprotein complex. Mapping of the interaction sites revealed that Gn-CT has multiple N protein binding sites and even the predicted short CT peptide of Gc was able to bind to the N protein. In addition, the N protein of PUUV was demonstrated to interact with peptides from Gn and Gc of a variety of hantavirus species suggesting that RNP recognition is a conserved feature (Hepojoki *et al*., 2010b). Hantavirus RNP interaction with cytoplasmic tails of glycoproteins is an important point in virus morphogenesis that probably directs RNPs to the site of virus budding.

Interactions of the N protein with cellular factors

The primary function of hantavirus N protein is to encapsidate the virus genome for the purposes of RNA transcription, replication and packaging. However the molecule does not work merely like a structural RNA-binding protein, but also functions as a key adapter molecule between virus and host cell. Hantavirus infection has certain impact on cellular metabolism and the N protein has an important role therein. Notably, it is the most abundant viral component in both virions and infected cells (Kaukinen *et al*., 2005).

Viruses entering a host cell often make use of cellular structural scaffold molecules such as actin or myosin. In nonmuscle cells, actin is present in both globular monomeric and filamentous forms. Viral proteins usually bind to the filamentous actin. The involvement of cytoskeletal proteins in the morphogenesis of hantaviruses was studied with BCCV. In BCCV-infected Vero cells N was partially colocalized with actin microfilaments; this association was confirmed by coimmunoprecipitation with beta-actin-specific antibody. The authors found that filamentous pattern of the N protein was disrupted by an actin microfilament depolymerizing drug, cytochalasin D. Moreover, disruption of actin filaments led to the inhibition of virus release. It was proposed that actin filaments transport viral RNPs to the plasma membrane where the virus assembly and release occurs. Generally, the results indicated that actin filaments may play an important role in hantavirus morphogenesis (Ravkov *et al*., 1998). Immunofluorescence studies of ANDV also showed an association of the N protein with actin in the perinuclear region (Ramanathan *et al*., 2007).

It was demonstrated that the N protein of many hantaviruses traffics on microtubules to the ER-Golgi intermediate compartment (ERGIC) prior to its movement to the Golgi for virus assembly. The N

protein was also shown to require an intact ERGIC for viral replication. However, early entry events were distinct for the Old World (HTNV) and the New World (ANDV) hantaviruses. ANDV appeared to have a different mode of entry that may include yet uncharacterized pathways that are clathrin- and caveolae-independent. Besides, ANDV replication depended on intact actin while the microtubule cytoskeleton was important for HTNV (Ramanathan & Jonsson, 2008). Release of Punta Toro virus, a bunyavirus from genus *Phlebovirus*, was not affected by the disruption of actin filaments, but the N protein of the Crimean-Congo hemorrhagic fever virus, a member of genus *Nairovirus* was reported to interact with actin that was essential for the assembly of infectious CCHFV particles (Andersson *et al*., 2004a).

Like cellular membrane structures, actin filaments may form anchoring sites for viral RNA replication complexes thus providing a suitable microenvironment for replication. Recently, BUNV was reported to build a new structure among enveloped viruses, a viral factory. During the BUNV replication factory construction, organelles such as Golgi and mitochondria, were recruited. Actin and myosin-I were identified in the the factory and numerous ribonucleoproteins were released from purified tubes disrupted *in vitro*. Notably, no direct interaction was reported for the N protein and actin in this new tubular structure. On the other hand, NSm protein was detected in the tubes' internal proteinaceous scaffold by immunogold labeling (Fontana *et al*., 2008).

One type of widespread virus–host interaction is functional modulation of the viral proteins by post-translational modifications, such as phosphorylation, glycosylation, ubiquitinylation, and sumoylation. Sumoylation involves the conjugation of a small ubiquitin-like modifier (SUMO-1) to the lysine side chain of a target protein (Saitoh *et al*., 1997). The effect of sumoylation on viral proteins appears to be substrate-specific, but has functional consequences that are important for the viral replication cycle (Rangasamy *et al*., 2000). Interaction with SUMO-1 was discovered with the N protein of TULV (Kaukinen *et al*., 2003b), SEOV (Lee *et al*., 2003a) and HTNV (Maeda *et al*., 2003). The region spanning aa 100-125 of SEOV N, which represents a critical region for the N-N oligomerization, was found responsible for the interaction with SUMO-1 (Lee *et al*., 2003a). The N protein of Hantaan virus also interacts with SUMO-1-conjugating enzyme 9 (Ubc9) which conjugates SUMO-1 to target proteins and modulates signal transduction, transcription regulation, and regulation of cell growth (Maeda *et al*., 2003). Using co-expression, yeast two-hybrid and mammalian two-hybrid screenings with SEOV and HTNV N proteins it was detected that the N protein also associated with SUMO-1-interacting proteins PIAS1, PIASxβ, HIPK2, CHD3, and TTRAP (Lee *et al*., 2003a).

It is worth mentioning that the N protein is also involved in different aspects of the antiviral response. Proteins of the Mx family are universal interferon-inducible gene products with antiviral activity, that provide resistance to a wide range of RNA viruses, including members of *Orthomyxoviridae*, *Paramyxoviridae*, *Rhabdoviridae*, *Togaviridae* and *Bunyaviridae* families (Haller & Weber, 2007). Hantaviruses are susceptible to MxA protein. MxA is responsible for inhibiting hantavirus infection and colocalizes with the hantaviral N protein in infected cells (Kanerva *et al*., 1996; Khaiboullina *et al*., 2005). The mechanism of this inhibition has not been studied yet but similar studies with other bunyaviruses (LACV, RVFV) showed that MxA sequesters N protein into fibrillary

structures in the perinuclear region thus making it unavailable for the virus assembly (Kochs *et al*., 2002).

Recently it was demonstrated that the N protein participates in yet another process, namely in the blocking of the innate immune response. The N protein of HTNV inhibits the translocation of the transcription factor NF-κB to the nucleus (Taylor *et al*., 2009b). NF-κB is a protein complex that controls the transcription of DNA and plays a key role in the regulation of the immune response to virus infection. HTNV N protein can sequester NF-κB in the cytoplasm, thus inhibiting NF-κB activity. It probably does so by binding the importin-alpha, a nuclear import molecule responsible for shuttling NF-κB to the nucleus. This is consistent with the idea that the N protein can block the induction of both innate and acquired immune responses by the host (Taylor *et al*., 2009b).

The N protein of PUUV was found to interact with Daxx, an enhancer of apoptosis. Domains of this interaction were mapped to the carboxyl-terminal region of 142 aa residues in Daxx and the carboxyl-terminal 57 residues in PUUV-N, respectively. The binding sites of Daxx to PUUV-N were further narrowed to two lysine-rich regions, of which one overlaps with the sequence of the predicted nuclear localization signal of Daxx (Li *et al*., 2002). These data indicate that the N protein has a complex network of interactions occurring in hantavirus-infected cells. Further studies are needed to find out what effect these interactions have on the host cell.

In conclusion, N protein is a multifunctional molecule involved in various interactions during the life cycle of the virus. It probably interacts with all the viral proteins and the list of its cellular interaction partners is increasing. It has essential functions in viral RNA replication, encapsidation, and also in virus assembly.

2. AIMS OF THE STUDY

It is known that protein's function is greatly determined by its structure. Hantavirus N protein is most abundant viral component executing wide variety of functions. This study aimed to decipher the functions of the N protein in the hantavirus life cycle with the relation to the structural organization of the molecule. TULV N protein was studied in four sub-projects, which together constitute this doctoral thesis. The specific aims of the study were:

- to evaluate the role of N-terminal coiled-coil structure in the oligomerization of TULV N protein and to identify amino acids critical for the stability of the coiled-coil domain

- to identify the amino acid residues forming the interaction surfaces of the coiled-coil domain and assess their contribution to the N protein oligomerization

- to study the interaction of the hantavirus N protein and the cytoplasmic tail of hantavirus Gn protein and to identify the interacting domains of the two partners

- to analyse the hantavirus N protein RNA-binding domain and to evaluate whether conserved motifs in the domain participate in the RNA-binding initiation

3. METHODS

STRUCTURAL ANALYSIS (I; II; III; IV)

The secondary structure of TULV N protein was predicted using the following programs: (i) PsiPred, which uses feed forward neural networks and PSI-BLAST (Jones, 1999); (ii) Sam-T99, based on position-specific scoring matrices (Karplus, 1998; Park, 1998); (iii) Jufo (http://www.jens-meiler.de/jufo.html), which uses primary structure only; and (iv) PROFsec, an improved method of PHDsec (Rost, 1996). The program Coils was used to predict coiled-coils (Lupas *et al*., 1991).

Analysis of hantaviral Gn protein sequences was performed using the following transmembrane-region prediction programs: HMMTOP based on a hidden Markov model with special amino acid architecture (Tusnady & Simon, 2001); TMHMM based on a hidden Markov model (Krogh *et al*., 2001); SOSUI based on the predication of hydrophobic helix and TMPRED based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins (Hirokawa *et al*., 1998).

The three-dimensional (3D) structure prediction for TULV N coiled-coil domain was performed using a meta server (http://www.bioinfo.pl/meta/) (Ginalski *et al*., 2003) that combines results of various threading methods that are used to scan a representative set of structures in the Protein Data Bank (Berman *et al*., 2000) taking into account both sequential and structural information of the query and the target proteins. Phyre (http://www.sbg.bio.ic.ac.uk/_phyre/) was used in order to generate a model containing both main-chain and side-chain coordinates.

SITE-DIRECTED MUTAGENESIS (I; II; III; IV)

Initial plasmids encoding the N protein of TULV, strain Moravia (Vapalahti *et al*., 1996b), were created by PCR from the cDNA clone that contained the complete coding region of the S segment sequence. Point mutations were created in the initial pM1-TULVN, pVP16-TULVN and pcDNA3-TULVN plasmids using site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene). All the mutations were confirmed by sequencing.

CELL CULTURES (I; II; III)

COS-7 (African green monkey kidney) and 293FT (Human Embryonic Kidney) cells were cultivated in Dulbecco Minimal Essential Medium (DMEM) and HeLa (Cervical cancer) cells in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin and streptomycin at +37°C in a humified 95% air - 5% CO_2 atmosphere.

DNA TRANSFECTION USING FUGENE REAGENT, LIPOFECTAMINE AND CALCIUM PHOSFATE (I, II, III)

Cells were grown in MEM or DMEM supplemented with 10% fetal bovine serum, 1% of L-glutamine, penicillin and streptomycin mix in appropriate plates 24 h to approximately 70% confluence. Transfections with FuGene™6 reagent (Roche Diagnostics) or Lipofectamine™2000 (Invitrogen) were performed according to the manufacturer's instructions. For transfection by calcium phosphate method, 10 µg DNA was mixed with 1 ml sterile water and 155µl 2M $CaCl_2$. After addition of 1,25ml of 2xHBS buffer (42 mM HEPES, 10 mM KCl, 1.8 mM sodium phosphate dibasic, 0.27 M NaCl, 11 mM glucose, pH 7.05) the mixture was added to the cells and incubated for 7 hours before changing the medium. The cells were harvested 36 hours after transfection.

MAMMALIAN TWO-HYBRID ASSAY (I; II; IV)

The mammalian two-hybrid assay (M2H) was used to evaluate the interaction capacity of modified N protein. HeLa cells were co-transfected with DNA constructs expressing N protein mutants fused to the DNA-binding domain (DBD), N protein mutants fused to the activation domain (AD), firefly luciferase (FF-luc) reporter and Renilla luciferase (RLluc). The assay was performed 24 h after transfection. The luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega). Due to inherent variations the RL-luc values were used to normalize FF-luc values by the following way: Normalized value of the experiment = [(RL-luc value from N-N / RL-luc value of the experiment) x FF-luc value of the experiment. The formula for interaction is: (%) = (normalized value of the experiment / normalized value of N-N) x100.

IMMUNOFLUORESCENCE ASSAY (I)

Immunofluorescenc assay was performed to study the intracellular localization and immunofluorescence pattern of transiently expressed truncated N-protein molecules. COS-7 cells trasfected with pcDNA3-N constructs were grown on coverslips. After 24 h, cells were fixed with 3.2% paraformaldehyde and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS). Cells were stained either with polyclonal N protein antibody (1/300 in PBS, 1 h at room temperature) (Vapalahti *et al*., 1995) or monoclonal N-protein antibodies (3D3,1C12) (Lundkvist *et al*., 1996) (1/50 in PBS, 1 h at room temperature) and then with fluorescein isothiocyanate-conjugated rabbit anti-mouse secondary antibodies (1/30 in PBS, 1 h at room temperature). The samples were examined using a Zeiss Axioplan microscope with a x63 oil immersion lens.

GST PULL-DOWN ASSAY (III)

GST pull-down assay was carried out to analyse the interaction between the TULV N protein and Gn-CT. GnCT and its modifications were expressed as GST fusions from the plasmid pGEX-4T-3-CT in *E.coli*. TULV-N protein and its modifications were expressed from pcDNA3-TULVN in 293FT cells. Diluted 293FT-cell lysates were incubated with the slurry of GST-bound Sepharose glutathione beads for 4 hours at 4°C. Simultaneously GST control or GST-fused protein Sepharose glutathione beads were incubated with 100 µM ZnSO4 at 4°C using end-over-end mixing. Beads were washed three times for 10 min with cold ST buffer (10 mM Tris pH 8.0, 150 mM NaCl). The precleaned 293FT-cell lysates were centrifuged at maximum speed and were then mixed with an equimolar amount of washed GST-CT or its mutant-bound beads and incubated overnight at 4°C. Samples were centrifugated, supernatants stored at 4°C and the beads washed five times with 1ml of ice-cold ST buffer. Supernatants and beads were then analyzed by SDS-PAGE followed by immunoblotting with anti-TULV N monoclonal antibody 1C12 (1:500) (Lundkvist *et al*., 1996) or anti-FLAG antibody (1:1000) (Sigma). Secondary antibodies were either an anti-mouse HRP conjugate (1:1000) or an anti-mouse DyLight680 conjugate (1:10,000) (Pierce Biotechnology).

IMMUNOBLOTTING (I, II, III)

Proteins were separated on SDS-acrylamide gel and transferred onto a nitrocellulose membrane. Membrane was blocked with 4% milk powder, 0.05% Tween 20, TEN buffer (20mM Tris-HCl pH 8, 1mM EDTA, 50mM NaCl)–by incubating overnight at +4°C. Membrane was then incubated with primary antibody (1-2 h, RT) and after washing step (3 x 10 min) with secondary antibody (30-60min, RT), both diluted in 1% milk powder, 0.05% Tween 20, TEN-buffer. Proteins were detected using enhanced chemiluminescence (ECL) or fluorescence.

4. RESULTS AND DISCUSSION

Coiled-coil structure stabilization (**I**)	N-N interacting surface formation (**II**)	N-Gn CT interaction (**III**)	RNA-binding initiation (**IV**)
NΔ44-429* NΔ51-429 NΔ58-429 NΔ65-429 NΔ78-429 L4Q I11Q I18Q I18L L25Q V32Q V32P L44Q V51Q L58Q L65Q I18Q-L25Q L25Q-V32Q L44Q-V51Q V51Q-L58Q	R22A K24A K26A E29A K30A E33A D35A D37A D38A D37A-D38A K41A R47A R48A R63A D67A V69Q K73A E76A	NΔC25 NΔC31 NΔC100 N1-248 (D1D2) N80-248 (D2) N1-79 (D1) I380A-I381A* W388A*	G154Δ G154I RR172,173AA G154I, R172A,R173A G154Δ,R172A,R173A K160A

Table 2. *Mutated variants of TULV N protein used in this study.* ***** *these constructs were available from earlier studies.*

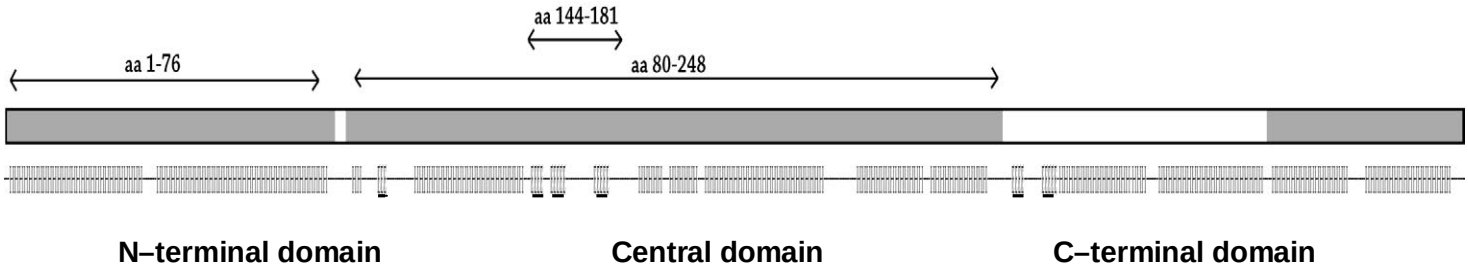


Fig. 5. *TULV N protein regions analysed in this study. Conserved domains indicated by grey shading, from left to right: N-terminal domain, central domain, C-terminal domain. Arrows are showing the regions analysed in this study: aa1-76 N–terminal oligomerization region that folds into a coiled-coil domain (**I, II**); aa 80-248 central conserved domain, that is crucial for interaction with the cytoplsmic tail of the glycoprotein Gn (**III**); aa 144-181 beta sheet forming region that supposedly initiates the RNA binding (**IV**). Secondary structure prediction for TULV N protein is shown in the bottom. 'H'- α helix, 'E' – β sheet.*

4.1 ANALYSIS OF THE N-TERMINAL COILED-COIL DOMAIN OF TULV N PROTEIN (I)

Before the start of this project, it was apparent that the hantaviral N protein can oligomerize and the trimers were found to be most stable intermediates in the processes of oligomerization and RNP formation. The association of the monomers was shown to invove both the amino- and carboxyl-terminal assembly domains. In fact, data were consistent with the "head-to-head, tail-to-tail" model for the hantaviral N protein trimerization which suggested that the trimerization is a two-step process that involves initial interaction between the N-terminal domains followed by a consolidating interaction between the C-terminal domains (Alfadhli *et al*., 2001; Alfadhli *et al*., 2002; Kaukinen *et al*., 2001; Kaukinen *et al*., 2003a; Kaukinen *et al*., 2004).

Using structure prediction algorithms it has been proposed that the N-terminal oligomerization domain of the hantaviral N protein most likely folds into a coiled-coil structure. In addition, synthetic peptides representing stretches of SNV N protein (aa 3 to 35, 43 to 75, and 3 to 75) were shown to oligomerize (Alfadhli *et al*., 2001; Alfadhli *et al*., 2002). We further characterized the N-terminal oligomerization domain of the TULV N protein and evaluated its role in the oligomerization. The study was performed using secondary and tertiary structure prediction algorithms, *in silico* modeling and point-mutagenesis to find structural requirements for the interaction in the N-terminal part of TULV N protein molecule.

Initial sequence analysis and structure predictions. The TULV N-protein N-terminal domain was analysed using bioinformatics approach. An alignment of the 77 N-terminal aa residues of the N protein of 20 distinct hantavirus species revealed that the sequence is highly conserved between all hantaviruses (identity of 43 to 99%). A typical coiled-coils pattern was also highly conserved in the 20 virus species: a heptad repeat with a hydrophobic residue in the first position and another hydrophobic residue in the fourth position (**I**: Fig.1A).

Various algorithms were used for secondary structure predictions for the TULV N-protein N-terminal sequence. The prediction was also performed for other five virus species (PUUV, SNV, ANDV, HTNV, and SEOV) representing three evolutionary distinct hantavirus groups. All algorithms that were used predicted the coiled-coils structure in the N-terminal domain of the N protein. Different prediction methods revealed that the N-terminal part of the protein folds into two alpha-helices; the first helix includes residues 4 to 31, and the second one includes residues 38 to 72. Almost identical results regarding the occurrence of coiled-coils were obtained for other hantaviral species (**I**: Fig.1B).

Next, the tertiary structure prediction for the putative N-terminal coiled-coil-forming domain (residues 1 to 79) was performed. Due to the lack of significant sequence identity between the N-terminal domain of the TULV-N protein and proteins with a resolved structure, the three-dimensional (3D) structure prediction for this domain was performed using a meta server (Ginalski *et al*., 2003) that

combines results of both sequential and structural information. This led to the detection of hits that might not have sufficient sequence identity but nevertheless share important structural features with the query. Most of the models provided by the servers were based on coiled-coil domains in different proteins. After manual inspection of the highest scoring models, one was chosen as the working model. It was based on the coiled-coil forming a dispensable insert domain of the human DNA topoisomerase I. The alignment showed the sequence identity of 25.7%. Further analysis of the hantaviral N protein relied on the model based on the DNA topoisomerase I structure with TULV N protein sequence adapted to it. The final model showed that the N-terminal region of TULV-N protein forms a coiled-coil in which two antiparallel alpha-helices interact via their hydrophobic seams. Based on the model the crucial aa residues that interact and stabilize the structure were the hydrophobic residues L/I/V at the “a” position of the heptad repeats. In the following experiments these residues were selected for point mutagenesis: L4, I11, L18, L25, and V32 in the first helix and L44, V51, L58, and L65 in the second helix of the TULV N protein. However before proceeding to more detailed, point mutation based, analysis of the N-terminal coiled-coil domain, the deletions of different lengths were tested. This model, based on a modest sequence identity, nevertheless proved useful for prediction of amino acid residues that stabilize the coiled-coil (**I**: Fig. 2).

Analysis of the N-terminal oligomerization domain of TULV-N protein by M2H. The mammalian two-hybrid assay was used to compare the interaction capacity of modified N protein in HeLa cells. Earlier, it was shown that the deletion mutant N44-429 retained approximately 80% of the interacting capacity of the full-length N protein (Kaukinen *et al*., 2003), therefore in our experiments only deletions longer than first 44 residues were introduced to the N protein. Altogether, four mutants (fused with either GAL4 DNA-binding domain [DBD] or the VP16 activation domain [AD]) lacking the first 50, 57, 64, and 77 aa residues were tested. The summary of interaction of N- and C-terminal truncation mutants in the M2H assay can be found in (**I**: Fig. 3). In brief: the molecule with the longest deletion, N78-429, that included the whole coiled-coil-forming region plus several extra residues, retained approximately 40% of the initial interacting capacity. This result was repeatedly observed when the deletion was introduced into the N protein fused with the DBD, AD, or both. Two other mutants, N51-429 and N58-429, showed a reduced interacting capacity as well. These results were in agreement with the earlier observations that the N-terminal domain is important for the homotypic interaction seen in the M2H assay (Kaukinen *et al*., 2003). Surprisingly, in the mutant N65-429 the interacting capacity measured in the M2H assay remained unaffected. There are several possible reasons for that: the truncations shorter than 65 aa residues might destroy the structure of the coiled-coil and hence induce a misfolding of the remaining part of the N-terminal region. In addition, the part of the molecule between positions 65 and 77 might contain aa residues crucial for the oligomerization. This hypothesis was investigated, but point mutants, D67A, K73A, and V69Q, in which conserved charge residues or a conserved hydrophobic “d” residue was replaced, did not affect the oligomerization of the N protein (**I**: Fig. 5).

All five truncated N-protein molecules (lacking the first 44, 50, 57, 64, and 77 aa and fused with AD) were next tested together with the mutant N1-398 (fused with DBD). This mutant lacks the last 31 aa

residues that form one of the two interacting alpha-helices in the C-terminal part of the molecule and therefore can react only with the partners carrying an intact C-terminal sequence. When used with wildtype N protein, the mutant N1-398 showed complete interacting capacity; when used with another N1-398 molecule, it showed no interaction at all. This mutant was used to ensure that two N-protein mutants will react exclusively "head-to-head" and "tail-to-tail." Experiments indicated that mutants which carried N-terminal truncations reacted with the mutant N1-398 as efficiently as with the full-length N protein. Truncated N-protein molecules with gradually removed coiled-coil forming heptads showed a reduced interacting capacity. The mutant with the longest truncation, N78-429, showed the lowest interacting capacity. Notably, an interacting capacity of the mutant N65-429 again remained unaffected, and the mutant N58-429 showed a reduced interacting capacity (72%) in only one of three experiments performed. These results confirmed that both N-terminal coiled-coils and C-terminal alpha-helices contribute to the oligomerization.

In a control experiment, when the N-terminal oligomerization domain was removed from one partner, AD–N78-429, and the C-terminal oligomerization domain was removed from the other partner, DBD–N1-392, an interacting capacity of the pair was almost completely abolished, confirming the invovment of both N protein termini in the oligomerization (**I**: Fig. 3).

Intracellular localization of transiently expressed truncated N-protein molecules. Previous studies of TULV N protein showed good correlation between an interacting capacity of the mutated N protein in the M2H assay and a perinuclear localization, as well as a granular fluorescence pattern of the transiently expressed polypeptide (Kaukinen *et al*., 2004). While non-mutated N protein localized in the perinuclear region and showed a bright granular pattern, the N-protein mutants (both point mutants and truncations) showed a more diffuse localization. To study the intracellular localization of the N-protein mutants with the N-terminal truncations, they were transiently expressed in COS-7 cells and then visualized with TULV-specific monoclonal antibody 3D3, which recognizes the central part of the protein spanning aa residues 226 to 293 (Lundkvist *et al*., 1996). There was a steady increase in the number of transfected cells, showing a diffuse immunofluorescence pattern with the length of aa stretch removed from the N terminus of the molecule. An increase as high as six fold (from 2% to 12%) was observed (**I**: Fig. 4). However, these IFA results alone cannot be taken as direct evidence for a reduced oligomerization capacity of the N-protein mutants. An intracellular transport of the N protein is supposedly a complex process, which may include an interaction with a cellular component(s). Although it is quite possible that this interaction would occur only if the N protein exists in a proper form (e.g. trimer/oligomer), the results also suggest that mutations affect the ability of the N protein to interact with its yet unknown cellular counterparts.

Point mutagenesis of aa residues located at positions "a" in the coiled-coil heptads. Since previous experiments suggested that gradual removal of the coiled-coil-forming region affected the oligomerization capacity of the N-protein molecule, the L/I/V residues located at positions 4, 11, 18, 25, 32, 44, 51, 58, and 65 were the next targets of the mutagenesis. These aa residues are supposedly keeping together the 3D structure of the N-terminal coiled-coil and therefore should be

crucial for the homotypic interaction. A set of the N-protein mutants in which these residues were replaced with glutamine (Q), a polar amino acid with an aliphatic chain, was created. In theory, this mutation (L/I/V →Q) should have a negative effect on the coiled-coil formation. In addition to single point mutants, four double mutants, I18Q-L25Q, L25QV32Q, L44Q-V51Q, and V51Q-L58Q, were produced.

It was observed that three out of ten single mutants, I11Q, V32Q, and L65Q, retained the interacting capacity of the wild-type N protein; in 7 others this capacity was reduced. Notably, for the mutations residing in the first alpha-helix, L4Q, I18Q, and L25Q, the reduction was minimal. The mutant V32P was included to test whether this aa residue is located within the helix or in the loop/turn which connects two alpha-helices of the coiled-coil. If V32 is located within the helix, the change to proline (known as a "disruptor" of alpha-helices) should have a greater effect on the N-protein-interacting capacity. The results suggested that V32 is located within the helix. Of the four double mutants, two (I18Q-L25Q and L25QV32Q) showed unaffected interacting capacity, while this capacity in the other two mutants, L44Q-V51Q and V51Q-L58Q, was reduced to approximately half. Moreover, a cumulative effect of the L/V and V/L replacements was observed. Taken together, these results suggested that an intact coiled-coil structure of the N terminus is crucial for the oligomerization capacity of the N protein and that the hydrophobic "a" residues from the second a-helix are especially important (**I**: Fig. 4).

IFA was also performed with some point mutants. However, the double mutant L44Q-V51Q that demonstrated reduced interacting capacity in the M2H system showed an immunofluorescent pattern indistinguishable from that of the wild-type N protein. It seems that the remaining oligomerization capacity of the N-terminal domain is still sufficient to bring together interacting C-terminal alpha-helices and hence to secure both perinuclear localization of the mutant protein and the granular pattern of immunofluorescence.

4.2 ANALYSIS OF THE INTERACTING SURFACE OF THE COILED-COIL DOMAIN (II)

Improving the working TULV N model based on the crystal structure of SNV N-terminal coiled-coil domain and prediction of interacting surfaces. Following the release of the SNV N protein coiled-coil domain crystal structure (PDB 2IC6) (Boudko *et al*., 2007), a new molecular model for TULV N protein coiled-coil domain was constructed. It replaced the previously used topoisomerase dispensable insert domain-based model of TULV N. However, a superimposition of the new model and the old model showed almost identical topography. The only visible difference was that the new model had a slightly stronger curvature than the old one. The new model thus confirmed that our selection of the first set of aa residues, which supposedly stabilize the coiled-coil structure, was well justified (**II**: Fig.1).

The new model of TULV N protein coiled-coil domain was used to predict interacting surfaces of the monomers. Three monomers were docked together and trimerization modes were examined both manually and automatically. Two trimer models were chosen for prediction of interacting surfaces. Previous results showed that an increase in ionic strength abolished the interaction between N protein molecules (Kaukinen *et al*., 2003a). This suggested that at least some charged aa residues belonged to the interacting surface. Conservation of charged residues among different hantaviruses was examined and, considering their spatial position, the most probable candidates to form interacting surfaces were selected. The following charged residues were considered to be part of interacting surface and therefore subjected to mutagenesis: R22, K24, K26, E29, K30, E33, D35, D37, D38, K41, R47, R48, R63, D67 and K73. Residue E76, which is located just outside the crystallized part of the coiled-coil but, according to the docking results, also belonged to the interacting surface, was also selected for mutagenesis. According to the crystal structure of the SNV N protein N-terminal domain, three of these residues, R22, E29 and R48, are involved in the formation of intramolecular ionic bridges: R22–E55 and E29–R48 (Boudko *et al*., 2007). Based on these data, E55 was added to the mutagenesis list.

Point mutagenesis of charged amino acid residues. 16 selected charged aa residues out of 29, that are found in the TULV N-terminal coiled-coil region, were mutated to alanine (**II**: Fig. 2). The interacting capacity of the resultant mutants was evaluated using a M2H assay. The results showed that the majority of charged aa residues did contribute to the interaction indeed. There were 9 residues that when mutated moderately affected oligomerization capacity (remaining interacting activity ¢80%): R22A, K26A, E29A, K30A, E33A, D37A, R48A, D67A and E76A. Intrestingly four mutants showed substantially strongly affected oligomerization (remaining interacting activity 44–80%): D35A, D38A, E55A and R63. None of the mutations totally abolished the ability of the N protein to interact, suggesting that even impaired N termini were still able to bring together C-terminal alpha helices, the key players in the oligomerization process. Moreover, the aa residues that affected the oligomerization capacity of the N protein were not scattered randomly throughout the coiled-coil 3D structure (**II**: Fig. 4). Some formed clusters (e.g. R22, E29 and E33 in the first helix and D35, D37 and D38 in the tip), whilst the side chains of others were similarly oriented and also came into close proximity to each other (e.g. R48 and E55 in the helix). Notably, two of the most crucial residues, D35 and D38, are located at the tip, and the double mutation D37A/D38A had a statistically significant cumulative effect, suggesting that the tips of the coiled-coils are highly reactive and are especially important for the oligomerization. Results are graphically presented in (**II**: Fig. 3). Probably the tips are the first to come into direct contact and thus to initiate tight packing of the three coiled-coils.

Analysis of amino acid residues forming intra- and intermolecular ionic bridges (**II**: Fig. 5). Residues R22–E55 and E29–R48 are located at the ‘e’ position of the coiled-coil and stabilize the structure by forming intramolecular ionic bridges (Boudko *et al*., 2007). It cannot be excluded that, due to their flexibility, they could also be involved in intermolecular interactions between the N protein monomers. The mutants E29A and R48A showed a reduced interacting capacity and, most

importantly, the cumulative effect of the two mutations was apparent. Unexpectedly, the double mutant R22A/E55A did not show a substantially reduced oligomerization capacity, although mutation of one of the residues, E55, had a strong effect. The reason(s) for this remains unknown.

Residues E33 and R48, that, according to *in silico* docking, form an intermolecular ionic bridge between the side chains of the N protein monomers were also tested in M2H assay. The double mutation E33A/R48A showed a cumulative effect when compared with the single mutations E33A and R48A. Next, R48 was mutated to glutamic acid to prevent a putative interaction between opposite charges at positions 33 and 48. Finally, we tried to reconstitute the ionic bridge between these two residues by mutating them to arginine and glutamic acid, respectively. The single mutation R48E had a statistically significant effect on oligomerization (64%), suggesting an important role for the R48 residue in the interacting surface. Interestingly, the oligomerization capacity was not restored in the mutant E33R/R48E, suggesting that not only charges but also the nature of the two aa residues is important.

Altogether these results imply that the contribution of charges, which are involved in the formation of ionic bridges is important in the oligomerization process. The capacity of the 'e' residues R22, E29, E55 and especially R48 to both stabilize the coiled-coil and form an interacting surface raises the intriguing possibility of conformational change(s) of the N protein molecule, which might follow trimerization or oligomerization. For instance, 'switching' of R48 from intra- to intermolecular ionic bridging when the other N protein molecule comes into close proximity could cause a conformational change and imply a certain degree of asymmetry to the N-trimer structure, which is probably needed for oligomerization to continue along the viral RNA molecule. Naturally, the binding of the N protein to the viral RNA is another likely candidate for the driving force behind the conformation changes.

Importance of the N-terminal coiled-coil domain in the N-protein oligomerization, final remarks (I; II). Formation of RNPs depends on interactions of the N protein with viral RNA and most importantly with other N protein molecules. Hantaviral N protein can form stable trimers in infected cells. These trimers represent intermediates of the N protein oligomerization and RNP formation. The N protein trimers then interact with each other gradually encapsidating the RNA (Alfadhli *et al*., 2001; Alfadhli *et al*., 2002; Kaukinen *et al*., 2001; Yoshimatsu *et al*., 2003). Mapping of the homotypic interactions sites for TULV N protein revealed that the C-terminal region is crucial for the interaction. Computer modeling predicted that the interaction between the C termini occurs via helix protrusions, and the shared hydrophobic space formed by aa residues 380-IILLF-384 in the first helix and 413-LI-414 in the second helix is responsible for stabilizing the interaction. It was experimentally proved that aa 1-43 in the N terminus are also involved (Kaukinen *et al*., 2003a).

Our study aimed to analyse the role of N-terminal coiled-coil domain in the oligomerization of the N protein in more detail. The results confirmed that the N-terminal coiled-coils are involved in the oligomerization of hantaviral N protein indeed. Taken together with the previously published results (Alfadhli *et al*., 2001; Alfadhli *et al*., 2002; Kaukinen *et al*., 2001; Yoshimatsu *et al*., 2003; Kaukinen *et al*., 2004), they support the model of trimerization based on the "head-to-head, tail-to-tail" mode of interaction. The intact coiled-coil structure that is kept by alpha-helices interacting via their

hydrophobic seams is crucial for the N-N protein interaction. The hydrophobic aa residues at the position “a” of the heptad repeats are responsible for stabilizing the structure.

There are at least three different ways in which the N-terminal coiled-coils can form a trimer (**I**: Fig.6). In the first model, antiparallel coiled-coils of the monomers remain intact and the interacting forces are generated mainly by charged or polar residues, which are located on the opposite side of the helix relative to the hydrophobic “a” residues. The other scenarios include conformational changes that involve opening of the intramolecular coiled-coils. We favor the first possibility for the following reasons. First, it is the simplest model that does not require an opening of the coiled-coils of three monomers (**Fig. 6;** right). Second, this conclusion is in good agreement with the results of *in silico* docking. Three N-protein monomers using the 3D model predicted by the Rosetta *ab initio* protocol (Bonneau *et al*., 2001) were docked (as rigid bodies) into a trimer. One of the obtained solutions was a trimeric structure similar to model that we favour (**Fig. 6;** below). Also cryo-EM analysis of recombinant N protein confirmed the existence of the N-trimer made of curve-shaped N protein monomers, which resemble influenza and rabies virus N protein monomers in the reconstructed 3D models (Albertini *et al*., 2006; Ye *et al*., 2006). Importantly, contacts between the monomers via their N- and C-termini of the monomers were clearly seen (Plyusnin *et al*., 2008).

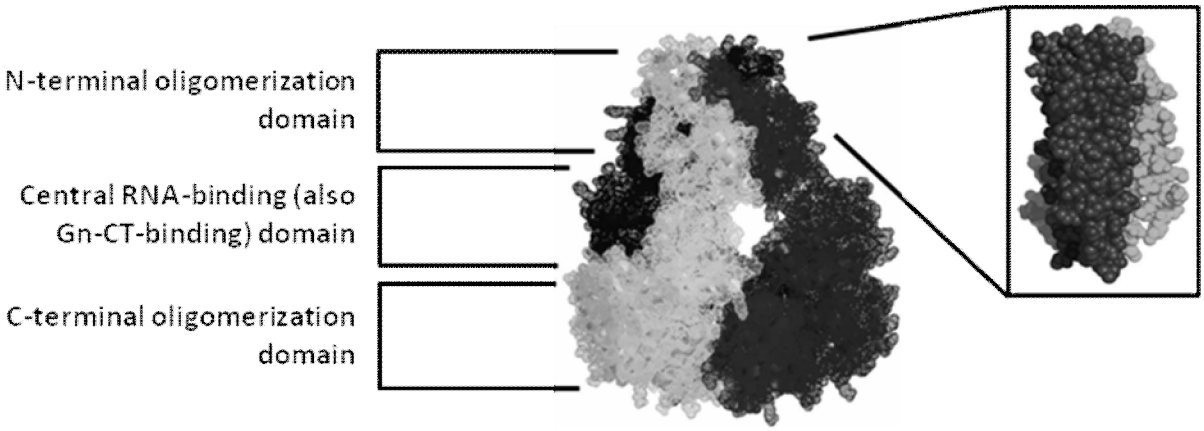


Fig. 6. *Model of hantavirus N-protein monomer (space-fill view) docked into a trimer. Framed on the right, independent docking of three N-terminal coiled-coils. Notably, the structure of the N protein monomer is similar to that obtained by negative staining of baculovirus-expressed hantavirus N-protein (Plyusnin et al., 2008) and the mode of trimerization is in agreement with the ‘head-to head, tail-to-tail’ mode of interaction. Domains of the monomer and their specific function are indicated on the left.*

Analysis of the conserved charged residues involved in the formation of an interacting surface of the monomers indicated that most crucial residues form a cluster at the tip of the coiled-coil domain. This confirms that the structure remains intact and suggests that the tips of the coiled-coils are probably the first to come into direct contact followed by a consolidating interaction between the C-

terminal domains. Moreover the aa residues supposedly contributing to interactions between the coiled-coils appeared clustered within the 3D structure of the coiled-coil.

These studies demonstrated an important contribution of N-terminal charged residues, which are involved in the formation of ionic bridges in the oligomerization process. This brings us to the following step of the RNP formation, where the N protein multimerizes to encapsidate the entire RNA molecule. Intra- and intermolecular ionic bridge formation opens the possibility of conformational change(s) of the N protein molecule, which might follow trimerization or even be a prerequisite for the formation of longer oligomers. Flexibility of the interaction between charged residues in the N-terminus also implies a certain degree of asymmetry to the N-trimer structure, which is probably needed for the oligomerization to continue along the viral RNA molecule. The conformational change can also result from the N protein interaction with the viral RNA, in a way similar to other RNA viruses. Recently solved 3D structures of the N proteins of rhabdoviruses implied a conformational change of the nucleocapsid protein molecule, which is essential for RNA encapsidation (Albertini *et al*., 2006; Green *et al*., 2006). In fact, it was also shown that SNV N protein undergoes distinct conformational changes after binding with either mRNA cap or vRNA or both mRNA cap and vRNA simultaneously (Mir & Panganiban, 2005; Mir *et al*., 2010).

In summary, the role of N-terminal coiled-coil domain in the oligomerization seems to be essential in several ways: (i) intact coiled-coil domain is needed for the N-N interaction; (ii) highly conserved, charged aa residues on the tip of the coiled-coil are the first to come into direct contact and thus to initiate tight packing of the coiled-coils; (iii) flexibility of the of the charged residues in the N-terminus probably allows the conformational changes needed for oligomerization to continue along the viral RNA molecule.

4.3 INTERACTION BETWEEN HANTAVIRAL NUCLEOCAPSID PROTEIN AND THE CYTOPLASMIC TAIL OF THE SURFACE GLYCOPROTEIN Gn (III)

The tail of the hantaviral Gn protein is located on the cytoplasmic side of the Golgi membrane. Since hantaviruses do not code for a matrix protein that usually mediates the packaging of viral RNA, RNPs probably require a direct interaction between the intraviral domains of the envelope-embedded glycoproteins. Recently crystallized hantaviral Gn-CT zinc finger domain showed no RNA-binding activity thus suggesting that it is involved in a protein–protein interaction (Estrada *et al*., 2009). In addition, N proteins of several members from the family *Bunyaviridae* have been reported to interact with the glycoproteins directly (Overby *et al*., 2007a; Snippe *et al*., 2007; Ribeiro *et al*., 2009). This can be a determining step in virus assembly. When this study was initiated an interaction between hantavirus N protein and the glycoproteins was not reported. Our aim was to show that N and Gn proteins of TULV interact and to identify regions of both partners involved in the interaction. Most

recently glycoprotein complex and RNP interaction was described for PUUV (genus *Hantavirus*) (Hepojoki *et al*., 2010b).

Sequence analysis and selection of the protein regions for the study. The region of TULV Gn protein that included aa residues 521–653 was considered the wt-Gn-CT. The Gn-CT sequence was analyzed using transmembrane-region prediction and multiple sequence alignment programs. It was apparent that both zinc finger motifs and one ITAM motif were strictly conserved in all hantavirus species while the second ITAM was conserved only in Tula and Sin Nombre- and Andes-like viruses. The overall level of sequence conservation in this region was relatively high (>40%). Furthermore, aa residues supposedly involved in maintaining the 3D structure were identical in all hantavirus sequences, suggesting a similar mode of folding, and hence similar function of the Gn-CT in different hantavirus species.

Using various methods of secondary structure prediction the central domain of TULV N protein was mapped approximately between aa residues 80 and 250. It is rich in positively charged aa residues and is thought to carry the RNA-binding domain (Xu *et al*., 2002). The N-terminal domain and the C-terminal domain are involved in the oligomerization (Kaukinen *et al*., 2005). All three domains were analysed in this study.

Analysis of the Gn-CT/N interaction. The interaction of TULV N protein with the cytoplasmic tail of glycoprotein Gn was studied using mutagenesis and GST pull-down assays. The pull-down was first established with the wild type Gn-CT fused with GST and the transiently expressed full length TULV N protein. N protein could be detected by immunoblotting with the hantavirus genus-specific MAb 1C12 (**III:** Fig. 2 and 3). Notably, the treatment of the N protein-containing lysates with RNase did not influence the efficiency of pull-down assay (**III**: Fig. 4A and B), suggesting that, to interact with the Gn-CT in these experimental settings, the N protein does not need to be a part of long RNP-structures formed via nonspecific encapsidation of cellular RNA.

Next, the role of two structure-functional elements within the Gn-CT: zinc finger motifs and ITAM motifs, was analyzed. Point mutations were introduced into zink finger CCHC-motifs and the resulting constructs were subjected to pull-down assay. The results clearly demonstrated that, for the successful pull-down, both zinc fingers should stay intact. Introducing single H→Q and C→S mutations in two HxxxC motifs totally abolished the Gn-CT/N interaction. As expected, the double hit abolished the interaction as well. Notably, the pull-down experiments confirmed that Zn^{2+} cations are required for proper folding of the entire zinc fingers containing domain (Estrada *et al*., 2009), and for the Gn-CT/N interaction as well. The amount of pulled-down N protein in $ZnSO_4$ -treated samples was substantially higher than in those where Zn^{2+} was not included.

To test if the conserved ITAM motifs are involved in the Gn-CT/N protein interaction, two deletion mutants of the Gn-CT were constructed. In one of the mutants, 21 C-terminal aa residues, which included the second YxxL-sequence, were deleted. In another mutant, GST-CTΔ34, both ITAM motifs were deleted. Both mutants with deletions showed the capability to pull-down the N protein (**III**: Fig.5).

The result indicates that the complete ITAM-carrying region is not crucial for the interaction with the N protein.

To find out which domains in the N protein are involved in interactions with the Gn-CT, several N protein mutants were tested in the pull-down assay (**III**: Fig.1B). First, three earlier described C-terminal deletion mutants lacking 25, 31 and 100 aa residues respectively (Kaukinen *et al*., 2004), were analyzed. The mutant lacking 100 C-terminal residues was not able to oligomerize as seen in the M2H assay, while first two mutants were oligomerization-competent (Kaukinen *et al*., 2003a). In the current study none of the deletions affected the ability of the N protein to be pulled-down. Similarly, two point mutants, II380381AA and W388A with impaired oligomerization function (Kaukinen *et al*., 2004), behaved in the pull-down assay exactly like the wild type N protein. These data suggested that the C-terminus of the N protein does not influence the Gn-CT/N interaction.

To evaluate the possible contribution of the N-terminal and central N protein domains, three more constructs were tested. The first construct carried the coiled-coil-forming aa residues 1–79; the second construct carried aa residues 80–248; the third construct included both domains (aa 1-248). Of these three polypeptides, only the first domain (aa 1-79) could not be pulled-down. These results showed that the central domain of the N protein is both critical and sufficient for the interaction with Gn-CT. It is of interest that the treatment of the construct (that supposedly carries the RNA-binding domain, aa 80-248) with RNase did not affect its performance in the pull-down assay either.

Role of Gn-CT/N interaction in the assembly of hantavirus progeny. In hantaviruses direct interaction between Gn-CT and the N protein is supposedly a necessary step for virion assembly. Obtained data support the hypothesis on the interaction between the hantaviral Gn protein tail and the viral RNPs that is realized via its key component, the N protein. These data are in agreement with most recent results of co-immunoprecipitation of the N protein of Puumala virus with the glycoprotein complex (Hepojoki *et al*., 2010b).

Taken together our results demonstrated that the 34 C-terminal aa residues of the Gn-CT carrying a conserved ITAM motif are dispensable for the Gn-CT/N interaction. In sharp contrast, zinc fingers appeared crucial for the interaction. For the other interacting partner, the N protein, the pull-down assay allowed to map the interacting region to the middle domain of the molecule that includes aa residues 80–248. It seems that this region alone is able to maintain the proper configuration required for its interaction with the Gn-CT. Interestingly the treatment of lysates containing the N protein (or its central domain) with RNase did not abolish or even influenced the pull-down. It is expected that short RNA fragments would “survive” the RNAse degradation, probably being protected by the N protein. Thus it is probable that the N protein interacting with Gn-CT is a part of RNPs but not necessarily long RNPs such as vRNPs in infected cells (Goldsmith *et al*., 1995) or those formed by nonspecific encapsidation of cellular RNA in transfected cells (Plyusnin *et al*., 2008).

One of critical steps in the infectious cycle of a virus is the packaging of the progeny genome to new viral particles. Many details of the virion assembly remain unknown but the main sequence of events seems to be the following: synthesized vRNA is encapsidated by the N protein; the L protein attaches itself to the newly formed vRNPs. Gn and Gc proteins are co-translationally cleaved on the ER

membrane and transported to Golgi. The Gn protein possesses a Golgi-retention signal and, by forming a heterodimer with the Gc protein assists to localize it to Golgi, too. vRNPs interact directly with the membrane-associated viral glycoproteins. It was suggested that the initiation of virion assembly requires oligomerization of both components: the RNPs and the (Gn–Gc)4 spike complex (Hepojoki *et al*., 2010b). The RNP assembly is likely to precede the formation of the spike complex, but the Gn-CT, the indispensable mediator of the Gn-N interaction, seems to become available to the RNP only after spike complex has been formed.

4.4 ANALYSIS OF THE RNA-BINDING DOMAIN OF THE N PROTEIN (IV)

The primary function of the N protein is to encapsidate the virus genome for the purposes of RNA transcription, replication and packaging. Sequence analysis of hantavirus N protein reveals three conserved domains separated by two more variable regions. The RNA-binding domain, which includes aa residues 175–217, has been located in the central part of the molecule (Xu *et al*., 2002). In this study, using *in silico* approach, we developed a model for the initial step of RNA-encapsidation by the hantaviral N protein. We pursued a hypothesis that in the central part of the molecule hantaviral N protein possesses secondary structure elements capable of initiating the N-RNA interaction. The results of point mutagenesis together with the M2H assay were in good agreement with the model.

Analysis of regions forming the RNA-binding surface of the N protein. Multiple alignment of 6 partial sequences of hantaviral N proteins was performed. It is apparent from the data that the middle part of the N protein is highly conserved among different hantaviruses (**IV**: Fig. 1). The consensus secondary structure prediction results show that there are two strongly conserved putative RNA binding regions. They are both structured: the first one (aa 144-160) is predicted to form a beta sheet and the second region (aa 172-176) is predicted to form a loop (**IV**: Fig. 1) A model was built for TULV N residues 144-181 to analyse the 3D structure of the putative RNA binding region. It also shows a beta sheet formation for the aa 144-160 of TULV N, that presumably constitute the RNA binding sheet **(IV:** Fig. 3). In this model all the positively charged residues are pointing to the same direction. For the second putative RNA-binding region Rosetta program also predicts a small loop. It supposedly could fetch the RNA molecules (**IV:** Fig. 2 and 3).

Point mutagenesis of aa residues stabilizing the secondary structure of RNA-binding region. We used M2H assay for initial evaluation of the contribution of different N protein to the putative RNA binding. First, we tested the effect of RNase treatment on the interaction of non-mutated molecules of the N protein (**IV**: Fig. 4). We treated the HeLa lysates with RNase before measuring the luciferase activity and observed a substantial (50%) decrease in the reporter signal. The DNase treatment practically did not affect the reporter signal (**IV**: Fig. 4). These results showed that the protein-protein

interaction in M2H assay is RNA mediated and it proves useful for elucidation of RNA binding surface of the hantaviral N protein.

Subsequently we changed selected aa residues in both regions predicted to form beta sheets. To test the functionality of the first region we mutated aa residue G154. We created a deletion mutant (G154del), to test the importance of G154 to the whole fold. We also created a mutant G154I, because introducing a stiff, hydrophobic isoleucine in the turn should stiffen the main chain and affect the functionality of the first beta sheet. For the second putative RNA-binding region that might form a 'finger-like' structure, we applied an alanine scanning approach and mutated positively charged arginines to alanine (RR172,173AA). To see whether mutations have any cumulative effect of both regions of RNA binding we also tested triple mutants of TULV N protein, G154I+RR172,173AA and G154del+RR172,173AA. In the aa region 144-160 that supposedly forms the positively charged RNA binding surface we so far mutated one residue, K160.

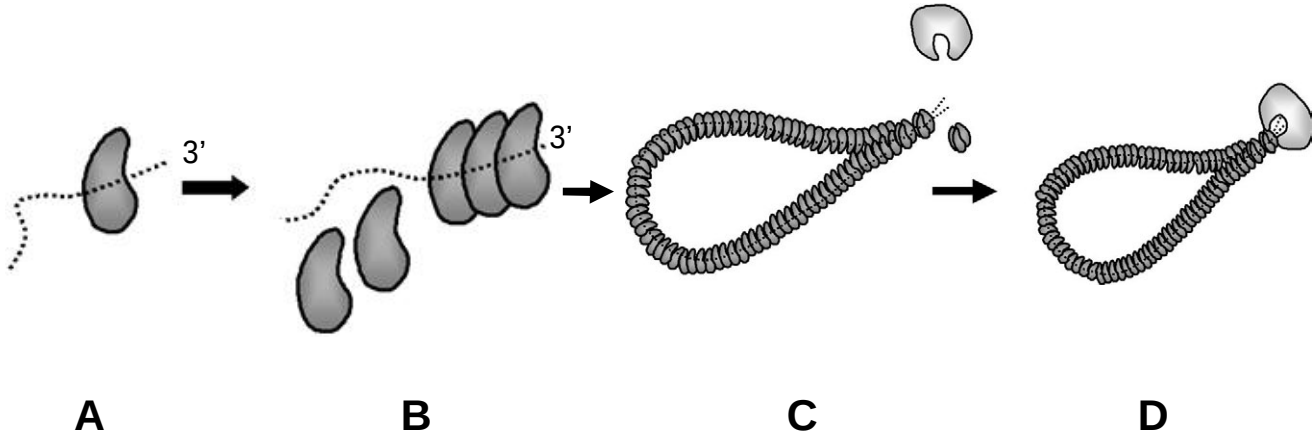
We then tested the functionality of the mutants in the mammalian two-hybrid assay and found that the N proteins carrying mutations in the RNA-binding region have substantially lower interacting efficiency compared to the wild type N proteins (**IV**: .5). Three mutants (RR172,173AA; G154I; and a triple mutant G154I,R172A,R173A) showed almost completely abolished N-N protein interaction. These data were in perfect agreement with the proposed model. The mutants G154del and triple mutant G154del,R172A,R173A showed approximately 50% of remaining interaction capacity. Surprisingly, interacting capacity of the triple mutant G154del,R172A,R173A was comparable to that of the mutant G154del, suggesting that, if the G154 is removed, the overall domain folding is changed. Thus further mutation of arginines at positions 172 and 173 does not affect the observed N-RNA-N interaction. The change of lysine at position 160 to alanine did not affect the oligomerization capacity of the N protein. We hypothesize, that the reason for the obtained result is that K160 is not a sole player in formation of the RNA binding surface.

We also investigated the interaction capacities of the mutants G154del and G154del,R172A,R173A with the non-mutated molecules of the N protein. Their oligomerization capacity was restored to the level observed with wtN (**IV**: Fig. 5). We speculate that the wtN protein molecules execute an initial N-RNA interaction, and the mutated-AD-fused partners bind the wtN without a need of direct interaction with RNA.

Next we investigated the effect of RNase on the interaction of aforementioned protein mutant combinations (G154del and G154del,R172A,R173A / wtN protein). The results showed that in both cases, after Rnase treatment, the restored interaction capacity decreases by approximately 30% (**IV**: Fig. 5). The effect of the treatment agrees with the hypothesis that N-RNA interaction can be disrupted by RNase, albeit not totally. Perhaps a portion of low molecular weight RNA segments survive the RNase treatment, being protected by the N protein, and are capable of mediating the N-RNA interaction.

In conclusion, we suggest that the hantavirus N protein possesses secondary structure elements that are located in the middle domain of the N molecule and are capable of initiating the N-RNA interaction. We speculate that aa residues 172-176 form a 'loop' structure that engages vRNA and the positively charged interaction surface formed by aa residues 144-160 enhances the N-RNA interacation. This initial contact brings the N protein molecules together; this is a prerequisite for the following N-N interaction. Since the initial contact between the N protein molecules is based on rather weak hydrophobic forces, the strong, charge-based, interaction of N with RNA is likely to preceed the N-N oligomerization. When the initial interaction has taken place, abundant N protein molecules supposedly continue to oligomerize covering the vRNA molecule (**Fig. 7**). Therefore the central region of the N protein seems to participate in RNA encapsidation and also brings the virion components together during the virion assembly by interacting with Gn-CT.

Fig. 7. *Model of vRNA encapsidation by the N protein. (A) Nascent vRNA coming out from a cRNA-L replication complex. The N protein initiates the N-vRNA interaction. (B) Initial N–vRNA interaction promotes the oligomerization of the N protein. (C) Encapsidation continues along the vRNA molecule and the terminal panhandle structure is recognized by the N trimer. (D) One copy of the L protein associates with the terminal panhandle of the new vRNPs.*



5. CONCLUDING REMARKS

This thesis summarizes our current knowledge of hantavirus life cycle with a special focus on the N protein. In the beginning of this work substantial amount of information was available about the N protein structure and functions. It is the most abundant viral component the primary function of which is to encapsidate the virus genome for the purposes of RNA-transcription, replication and packaging. However this molecule is not merely a structural genome encapsidating protein. It also coordinates viral activities by interacting with the L protein and the cytoplasmic tail of Gn protein during assembly of new virions. The N protein also functions as a key adapter molecule between virus and host cell processes by interacting with actin filaments, Daxx and SUMO-1 pathway components, transcription factor NF-κB, in infected cells. It is likely that the N protein also plays a role in combating cellular innate immune responses together with the nonstructural protein of hantaviruses.

Structural organization of the N protein has also been studied extensively. Sequence analysis suggests three conserved domains separated by two variable regions. The RNA-binding domain has been mapped to the central part of the molecule. C- and N-terminal domains of the N protein contribute to the oligomerization. The N-terminal domain folds into a coiled-coil and the C-terminal domain adopts a helix–loop–helix structure. Unfortunately, except for the N-terminal coiled-coil domain, the 3D-structure of the hantavirus N protein has not been solved yet.

In this thesis, we focused on the interactions of the N protein with other viral components. The projects presented here regarded N protein in three aspects: (i) we investigated the role of N terminal coiled-coil in the process of N oligomerization and (ii) demonstrated N proteins ability to interact with the glycoprotein Gn and pointing out the the domain sufficient for this interaction, (iii) we also started to map the N-protein RNA-binding domain and found that conserved motifs in the domain contribute to the initial N-RNA interaction and RNA-binding surface formation. We pinpointed the amino acids that keep alpha-helices interacting via their hydrophobic seams and this way stabilizing the coiled-coil structure. We also found evidence that an intact coiled-coil structure is crucial for the oligomerization capacity of the N protein. Later on we evaluated the contribution of the charged aa residues within the N-terminal coiled-coil to N-protein oligomerization. We found out the aa residues forming the interaction surfaces of the monomers. And proposed a model in which the tips of the coiled-coils are the first to come into direct contact and thus to initiate tight packing of the monomers.

Finally, we demonstrate that the cytoplasmic tail of hantaviral Gn protein interacts with the N protein. This presents the mean(s) for a direct link between hantavirus RNPs and the surface spike structure during the virion assembly. We demonstrated that intact, properly folded zinc fingers in the Gn protein cytoplasmic tail as well as the middle domain of the N protein (that also carries the RNA-binding domain) are essential for the interaction. It is of high importance to study N protein interactions since all N protein functions during the viral life cycle are based on its capability to maintain these contacts with other N protein molecules and also other viral components and cellular proteins.

The next step in structure-functional studies of the N protein would be to gain more insight on the N interaction with viral RNA. It would be interesting to see how all three domains perform their functions within the RNP complex and whether this interaction alters the overall folding of the N protein since conformational change(s) of the nucleocapsid protein molecule upon binding the RNA is probably essential for the RNA encapsidation.

Notably, our studies of the N-Gn interaction confirmed that not only N protein molecule but also its domains are multifunctional. We showed that besides serving as RNA-binding domain, the central domain of the N protein is essential for the N-Gn interaction. Therefore it is of interest which aa residues from the central domain of the N protein form the RNA binding interface and which ones take part in forming interaction surface with the glycoprotein Gn. Undoubtedly, solving the crystal structure of the hantaviral N protein would provide answers at least to some of these questions. However, the 3D structure of the whole N is not yet available. The main obstacle here is the tendency of the N protein to form aggregates. Nevertheless based on the current knowledge of the structural organization of the N protein domains it might be possible to mutate the aa residues responsible for the 'stickiness' and finally resolve the 3D-structure of the N protein.

Hantavirus N-protein interaction with the polymerase is another interesting target for investigation. Hantavirus L and N proteins do colocalize in infected cells, but it remains unknown whether the interacting N- protein molecules belong to RNPs or if the L and N proteins are in contact with the cellular membranes and which regions of N are responsible for interacting with the polymerase.

Another promising object of studies is the interactions of the N protein with the cellular components. In the course of infection various hantaviruses (Old World/New World; Pathogenic/Apathogenic) differently utilize the host cell machinery for efficient replication. Uncovering the details of this process may provide important clues for understanding the differences between pathogenic and apathogenic hantaviruses and may help prevent the diseases caused by hantaviruses. The role of N protein in blocking the innate immune response also seems likely. Some of the N protein is colocalized with the NSs protein. N protein is found abundantly in P-bodies that are the loci of mRNA degradation. Notably, additional important functions associated with P bodies include RNA silencing by micro- and small interfering RNAs. It would be interesting to know if N interacts with the NSs in the P-bodies and has functions in combating antiviral response at the RNA level.

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Science is an amazing opportunity to report a million year old events as a top story!

Thank you for this opportunity,

Agnė Aliminaitė

Helsinki, December 2010

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