Hantavirus infection:

Insights into entry, assembly and pathogenesis

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Finland

Academic dissertation

Helsinki 2011

To be presented for public examination with the permission of the Faculty of Medicine, University of Helsinki in Lecture Hall 2, Haartman Institute, Haartmaninkatu 3, Helsinki on Friday 16.12.2011 at 12 noon.

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ISBN 978-952-10-7340-3 (Paperback)
ISBN 978-952-10-7341-0 (PDF, http://ethesis.helsinki.fi)
Unigrafia Oy, Helsinki University Print
Helsinki 2011

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Contents

Original publications 6
Abbreviations 7
Abstract 9
1 Review of the literature 11
1.1 Discovery and Classification 11
1.1.1 Discovery of hantaviruses 11
1.1.2 Classification of hantaviruses 12
1.2 Cell biology of hantaviruses 13
1.2.1 Virion 13
1.2.2 Entry 15
1.2.3 RNA synthesis 19
1.2.4 Progeny virus formation 25
1.3 Hantavirus pathogenesis 33
1.3.1 Clinical picture of hantavirus-mediated diseases 33
1.3.2 Vascular leakage 35
1.3.3 Renal failure in NE 40
1.3.4 Innate immunity responses 41
2 Aims of the study 44
3 Materials and methods 45
3.1 Materials 45
3.1.1 Cell cultures 45
3.1.2 Viruses 45
3.1.3 Peptide synthesis 46
3.1.4 Peptide array synthesis 46
3.1.5 Protein synthesis 47
3.1.6 Nucleic acid synthesis 47
3.1.7 Antibodies 48
3.1.8 Reagents 48
3.2 Methods 49
3.2.1 Immunoblotting 49
3.2.2 Immunoprecipitation 49
3.2.3 Pull-down assays 50
3.2.4 Virus inactivation 50
3.2.5 Sedimentation analysis 51
3.2.6 Cross-linking of RNA to proteins 51
3.2.7 Probing peptide arrays 51

3.2.8 Flow cytometry 52
3.2.9 Surface plasmon resonance 52
3.2.10 UV/visible spectroscopy 52
4 Results and discussion 54
4.1 Regulation of cell survival by hantaviruses (I) 54
4.1.1 Background 54
4.1.2 TULV regulates ERK1/2 cell survival pathway 54
4.1.3 Regulation of ERK1/2 and induction of apoptosis by TOPV, SEOV and PUUV 55
4.1.4 The effect of TNF-α on hantavirus-induced ERK1/2 inhibition 57
4.1.5 TULV regulates also EGFR and Akt (unpublished results) 57
4.1.6 Does anoikis play a role in TULV-induced apoptosis? 58
4.1.7 Does hantavirus-induced apoptosis occur *in vivo*? 59
4.2 The interaction of hantavirus glycoprotein-CTs with RNP (II, IV) 60
4.2.1 Background 60
4.2.2 Native Gn interacts with RNP through its CT (II) 60
4.2.3 Gn-CT binds nucleic acids (IV) 61
4.2.4 Mapping the binding sites of Gn-CT towards RNP, N protein and nucleic acids (II and IV) 62
4.2.5 The binding of Gn-CT to RNP, N protein and nucleic acids is primarily mediated by its C-terminal part (II and IV) 64
4.2.6 Significance of the interaction of Gn-CT with RNP and nucleic acids in virus life cycle 66
4.3 Hantavirus infectivity is dependent on free thiols (III) 69
4.3.1 Background 69
4.3.2 Hantaviruses are inactivated by thiol-blocking reagents 70
4.3.3 Inactivation of hantaviruses by NEM retains virion integrity 70
4.3.4 The possible role of the ZF in hantavirus entry 71
5 Concluding remarks 74
6 Acknowledgements 77
7 References 79

Original publications

This thesis is based on the following original publications which are referred to by their Roman numerals. In addition, this thesis includes some previously unpublished results.

I: Strandin T, Hepojoki J, Wang H, Vaheri A, Lankinen H. 2008. Hantaviruses and TNF-alpha act synergistically to induce ERK1/2 inactivation in Vero E6 cells. Virol. J. 29; 5; 110.

II: Hepojoki J*, Strandin T*, Wang H, Vapalahti O, Vaheri A, Lankinen H. 2010. Cytoplasmic tails of hantavirus glycoproteins interact with the nucleocapsid protein. J. Gen. Virol. 91(Pt 9); 2341-50.

III: Strandin T, Hepojoki J, Wang H, Vaheri A, Lankinen H. 2011. Inactivation of hantaviruses by N-ethylmaleimide preserves virion integrity. J. Gen. Virol. 92(Pt 5); 1189-98.

IV: Strandin T*, Hepojoki J*, Wang H, Vaheri A, Lankinen H. 2011. The cytoplasmic tail of hantavirus Gn interacts with RNA. Virology 418(1); 12-20.

* Equal contribution

Abbreviations

bacN	Baculovirus-expressed nucleocapsid protein
BHK-21	Baby hamster kidney cells-21
B-mal	Biotin-maleimide
CPE	Cytopathic effect
CPM	Counts per minute
CT	Cytoplasmic tail
CTL	Cytolytic T cells
cRNA	Complementary ribonucleic acid
DTNB	5,5-dithiobis-(2-nitrobenzoic) acid
DTT	Dithiothreitol
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular-signal regulated kinases 1 and 2
Gn	N-terminal part of bunyavirus glycoprotein precursor
Gc	C-terminal part of bunyavirus glycoprotein precursor
GC	Golgi complex
GSH	Reduced glutathione
GST	Glutathione S-transferase
HCPS	Hantavirus cardiopulmonary syndrome
HFRS	Hemorrhagic fever with renal syndrome
IAA	Iodoacetamide
L	RNA-dependent RNA polymerase of bunyaviruses
MOI	Multiplicity of infection
MNase	Micrococcal nuclease
N	Nucleocapsid protein
NE	Nephropathia epidemica
NEM	N-ethylmaleimide
NSRV	Negative-sense segmented RNA viruses
NTR	Non-translated region
ORF	Open reading frame
PARP	Poly (ADP-ribose) polymerase

PBS	Phosphate buffered saline
PDI	Protein disulfide isomerase
RIPA	Radioimmunoprecipitation buffer
RNP	Ribonucleoprotein
TNF-α	Tumor necrosis factor alpha
unRNA	Unrelated ribonucleic acid
VLP	Virus-like particle
vRNA	Viral ribonucleic acid
ZF	Zinc finger

Viruses in the Family *Bunyaviridae*

Genus *Hantaviruses*

ANDV	*Andes virus*
BCCV	*Black Creek Canal virus*
HTNV	*Hantaan virus*
NY-1V	*New York-1 virus*
PHV	*Prospect Hill virus*
PUUV	*Puumala virus*
SEOV	*Seoul virus*
SNV	*Sin Nombre virus*
TOPV	*Topografov virus*
TULV	*Tula virus*

Genus *Orthobunyaviruses*

BUNV	*Bunyamwera virus*

Genus *Nairoviruses*

CCHFV	*Crimean-Congo hemorrhagic fever virus*

Genus *Tospovirus*

TSWV	*Tomato spotted wilt virus*

Genus *Phleboviruses*

RVFV	*Rift valley fever virus*
UUKV	*Uukuniemi virus*

Abstract

Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) are enveloped viruses incorporating a segmented, negative-sense RNA genome. Each hantavirus is carried by its specific host, either a rodent or an insectivore (shrew), in which the infection is asymptomatic and persistent. In humans, hantaviruses cause Hemorrhagic fever with renal syndrome (HFRS) in Eurasia and Hantavirus cardiopulmonary syndrome (HCPS) in the Americas. In Finland, *Puumala virus* (genus Hantavirus) is the causative agent of NE, a mild form of HFRS. The HFRS-type diseases are often associated with renal failure and proteinuria that might be mechanistically explained by infected kidney tubular cell degeneration in patients. Previously, it has been shown that non-pathogenic hantavirus, *Tula virus* (TULV), could cause programmed cell death, apoptosis, in Vero E6 monkey kidney cell cultures. This suggested that the infected kidney tubular degeneration could be caused directly by virus replication. In the first paper of this thesis the molecular mechanisms involved in TULV-induced apoptosis was further elucidated. TULV-infected cells were analyzed by immunoblotting with antibodies specific for the activated, phosphorylated forms of extracellular signal-regulated kinases 1 and 2 (ERK1/2), which are known to promote cell survival. A virus replication-dependent down-regulation of ERK1/2, concomitantly with the induced apoptosis, was identified. In addition, this phenomenon was not restricted to TULV or to non-pathogenic hantaviruses in general since also a pathogenic hantavirus, *Seoul virus*, could inhibit ERK1/2 activity. However, TULV was clearly the strongest cell death inducer of the studied viruses, and this also correlated with its ability to grow more efficiently in cell culture compared to other hantaviruses. Interestingly TNF-α, expression of which is associated with more severe hantavirus-mediated disease and is found to be locally expressed in the kidneys of diseased individuals, markedly decreased ERK1/2 activity in hantavirus-infected cells. This was also seen in *Puumala virus*-infected cells where ERK1/2 inhibition was not observed solely by virus replication. In general, these data indicate that hantavirus infection in tubular kidney cells may have detrimental effects on cell viability, which can be the cause of renal failure in the course of HFRS.

Hantaviruses consist of membrane-spanning glycoproteins Gn and Gc, RNA-dependent RNA polymerase (L protein) and nucleocapsid protein N, which encapsidates the viral genome, and thus forms the ribonucleoprotein (RNP). Interaction between the

cytoplasmic tails of viral glycoproteins and RNP is assumed to be the only means how viral genetic material is incorporated into infectious virions. In the second paper of this thesis, it was shown by immunoprecipitation that viral glycoproteins and RNP interact in the purified virions. It was further shown that peptides derived from the cytoplasmic tails (CTs) of both Gn and Gc could bind RNP and recombinant N protein. In the fourth paper the cytoplamic tail of Gn but not Gc was shown to interact with genomic RNA. This interaction was probably rather unspecific since binding of Gn-CT with unrelated RNA and even single-stranded DNA were also observed. However, since the RNP consists of both N protein and N protein-encapsidated genomic RNA, it is possible that the viral genome plays a role in packaging of RNPs into virions. On the other hand, the nucleic acid-binding activity of Gn may have importance in the synthesis of viral RNA. Binding sites of Gn-CT with N protein or nucleic acids were also determined by peptide arrays, and they were largely found to overlap.

The Gn-CT of hantaviruses contain a conserved zinc finger (ZF) domain with an unknown function. Some viruses need ZFs in entry or post-entry steps of the viral life cycle. Cysteine residues are required for the folding of ZFs by coordinating zinc-ions, and alkylation of these residues can affect virus infectivity. In the third paper, it was shown that purified hantavirions could be inactivated by treatment with cysteine-alkylating reagents, especially N-ethyl maleimide. However, the effect could not be pin-pointed to the ZF of Gn-CT since also other viral proteins reacted with maleimides, and it was, therefore, impossible to exclude the possibility that other cysteines besides those that were essential in the formation of ZF are required for hantavirus infectivity.

1 Review of the literature

1.1 Discovery and Classification

1.1.1 Discovery of hantaviruses

Epidemic hemorrhagic fever that occurred among United Nation´s soldiers during the war between North and South Korea in 1951 initiated the search for an etiological agent of this disease (later called Korean hemorrhagic fever; KHF). Before the Korean War, the disease was already recognized in East Asia by Russian and Japanese scientists (Smadel, 1953), and was described not as a contagious disease between humans but as a "place disease" designating the site (specific geographic area) where humans acquired the disease. It was suspected already in the 1950´s that the disease was carried by rodents. However, it took 25 years to finally discover the microbe responsible for KHF, and in 1978 the hantavirus prototype *Hantaan virus* (referring to river Hantan at the border of North and South Korea) was isolated from field mouse *Apodemus Agrarius* (Lee *et al*., 1978).

By then a hemorrhagic fever with similar clinical symptoms had been recognized in Scandinavia where it was called Nephropathia Epidemica (NE). Its causative agent, *Puumala virus*, was isolated from bank voles (*Myodes glareolus*) in 1980 (Brummer-Korvenkontio *et al*., 1980; Lahdevirta, 1971; Yanagihara *et al*., 1984b). These two diseases, KHF and NE, with distribution from Europe to Far East were collectively called as hemorrhagic fever with renal syndrome (HFRS), and the causative agents were specified as a new genus, named *Hantavirus*, in the virus family *Bunyaviridae* of enveloped RNA viruses (Schmaljohn *et al*., 1985; Traub & Wisseman, 1978).

Thereafter it did not take long to detect a hantavirus-caused disease also in Northern America. However, the newly-discovered hantavirus did not cause HFRS as the hantaviruses of Eurasia, but an acute respiratory illness with very high mortality (Hughes *et al*., 1993; Nichol *et al*., 1993). This disease was originally named hantavirus pulmonary syndrome (HPS) and is today called hantavirus cardiopulmonary syndrome (HCPS) due to the involvement of cardiac depression. The prototype HCPS-causing hantavirus (*Sin Nombre virus*; SNV) is carried by a deer mouse (*Peromyscus maniculatus*) (Childs *et al*., 1994). This was, however, not the first time a hantavirus was discovered in the Americas

since in 1983 Prospect Hill hantavirus (PHV) was identified in meadow voles (*Microtus pennsylvanicus*) (Yanagihara *et al*., 1984a), but this virus has never been associated with any disease, and is, therefore, used as the primary model for a non-pathogenic hantavirus.

Currently, the hantavirus genus includes more than 30 species of which only a few are associated with HFRS and others either with HCPS or not associated with any disease. Each hantavirus species is maintained in nature in its own specific animal reservoir, rodents. Hantaviruses do not cause any clearly identifiable symptoms in their carrier hosts and infection of secondary host (e.g. human), which probably takes place through aerosols from rodent excreta, is thought to be a dead end for the virus (spillover infection). Recently, many shrew-borne hantaviruses has been discovered (Jonsson *et al*., 2010). However, shrew-borne hantaviruses are genetically distinct from rodent-borne hantaviruses and it is not known whether they cause a disease in humans (Kang *et al*., 2009).

1.1.2 Classification of hantaviruses

Based on genetic and antigenic determinants hantaviruses form a single genus in the family *Bunyaviridae* (Schmaljohn *et al*., 1985), which in addition to the *Hantavirus* genus include *Tospovirus*, *Phlebovirus*, *Nairovirus* and *Orthobunyavirus* genuses (Elliott *et al*., 2000). Viruses in the *Bunyaviridae* family possess similar morphological and genetic characteristics with each other including enveloped spherical virus particles and negative-sense, single-stranded RNA genome, which is divided into three segments of different sizes. The classification of different genuses among bunyaviruses is based on variation in their genomic 3′-terminal sequences, which are conserved within genus. Hantaviruses possess a 3′-terminal "AUCAUCAUCUG" sequence in each of their genome segments (Schmaljohn *et al*., 1985). Based on the segmented genome, bunyaviruses can be classified with *Orthomyxoviridae* (including influenzaviruses A, B and C) and *Arenaviridae* families to distinguish them from non-segmented negative-sense RNA viruses (Order *Mononegavirales*). A typical feature of the negative-sense, segmented ssRNA viruses (NSRV) is "cap-snatch" mechanism for mRNA synthesis. Virus "classification" is also possible by analyzing the transmitting agent. Virus members in

Hantavirus genus are carried by rodents or shrews while viruses in the other genuses within the family *Bunyaviridae* are arthropod-borne.

1.2 Cell biology of hantaviruses

1.2.1 Virion

Hantaviruses are enveloped RNA viruses in the family *Bunyaviridae*. They have a negative-sense genome divided into small (S), middle (M) and large (L) segments, which encode for a nucleocapsid protein (N; approx. 430 amino acids [aa]), envelope glycoprotein precursor (approx. 1180 aa) and RNA-dependent RNA polymerase (L protein; approx. 2530 aa), respectively (Plyusnin *et al.*, 1996). The N protein encapsidates the genomic RNA and forms three differently-sized ribonucleoproteins (RNPs), which are packaged inside the viral envelope (Fig. 1). The L protein is generally thought to be associated with the RNPs and is required for the replication and transcription of the virus in infected cells. The glycoprotein precursor is cleaved to Gn (approx. 630 aa N-terminal part of the precursor) and Gc (approx. 540 aa C-terminal part of the precursor) glycoproteins by a signal peptidase complex in the cells prior to virus assembly (Lober *et al.*, 2001). The Gn and Gc form a complex on the outer surface of the virion. Both glycoproteins span the viral membrane in such a way that their C-terminal parts (cytoplasmic tails [CT]) are towards the intraviral space (or cytoplasmic side) while the other parts of the proteins are in contact with the outer environment (ectodomains). The CTs of Gn and Gc are remarkably different in size probably reflecting their importance in various processes during the viral life cycle (Gn 110 aa vs. Gc 10 aa). The Gn-CT has a tandem CCHC-type zinc finger domain (ZF) of unknown function (Estrada *et al.*, 2009) and a conserved YxxL endocytosis motif. The Gn-CT ends in a membrane-spanning signal sequence for Gc but it is uncertain whether the signal sequence is actually incorporated in the virions and whether it continues to be embedded in the membrane after Gc cleavage.

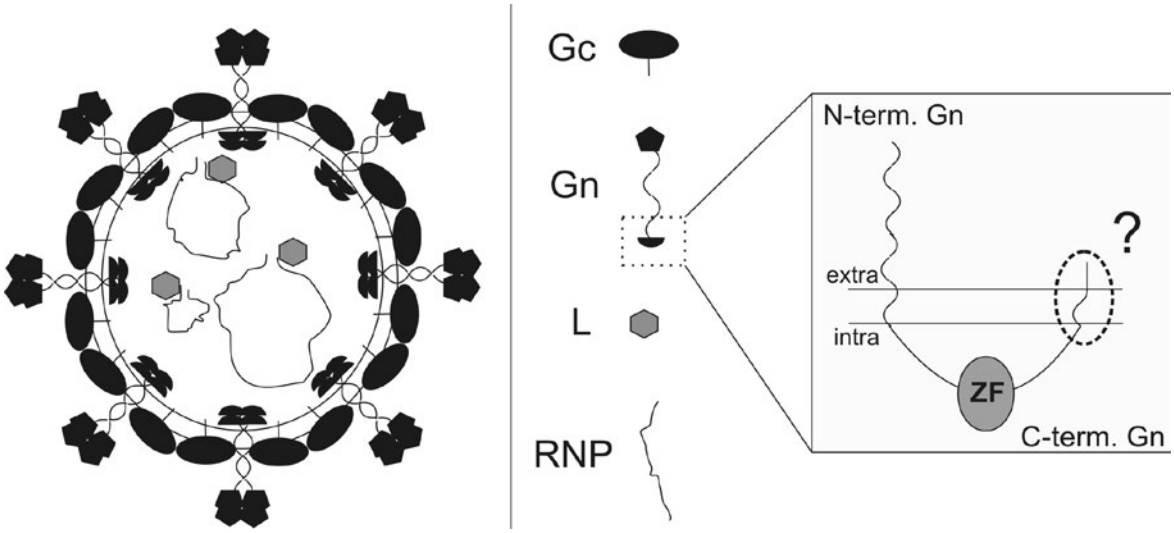


Figure 1. Virion structure. The hantaviral envelope consists of glycoproteins Gn and Gc. The genomic RNAs are encapsidated by nucleocapsid protein to form the ribonucleoprotein (RNP) complexes that are packaged inside the viral envelope. The genome is tripartite giving rise to three differently-sized RNPs. The cytoplasmic tail (CT) of Gn is exposed to interior of the virion and harbours a zinc finger (ZF) domain. It is unknown whether the C-terminal part of Gn-CT, containing the signal sequence for Gc, is actually incorporated into virions and whether it spans the viral envelope.

The structural organization of glycoproteins in the hantavirus particles is unique (Battisti *et al*., 2010; Hepojoki *et al*., 2010; Huiskonen *et al*., 2010). By analyzing the interaction of Gn and Gc (Hepojoki *et al*., 2010) it was shown recently that TULV Gn preferably forms homo-oligomers (dimers or tetramers) and Gc homodimers in the virion rather than heterodimers as was generally believed. The formation of heterodimers of Gn and Gc in infected cells has been shown for HTNV (Antic *et al*., 1992) but it is possible that glycoproteins rearrange to higher order complexes prior or during virus assembly. By chemical cross-linking of TULV particles it was also suggested that the homo-oligomeric Gn and Gc interact to form hetero-oligomeric complexes on the surface of TULV (spikes) and that Gn is more distal than Gc from the viral envelope due to its higher susceptibility to the applied cross-linking (Hepojoki *et al*., 2010). This has led to a model of glycoprotein organization on the surface of hantavirus particles where Gn glycoproteins form tetrameric complexes, which are interconnected to each other by dimers of Gc (depicted in Fig. 1). The existence of this type of glycoprotein organization in hantavirions was confirmed by electron cryo-tomography of TULV (Huiskonen *et al*., 2010) and HTNV (Battisti *et al*., 2010) particles, which indicated the presence of square-shaped spike complexes with four-fold symmetry at the virion surface and formation of

tetrameric Gn and/or Gc complexes. The grid–like four-fold symmetry of hantavirion surfaces is unique among enveloped viruses (Huiskonen & Butcher, 2007) and unlikely to be a general phenomenon among bunyaviruses since members of the *Phlebovirus* genus show pentagon- or hexagon-shaped glycoprotein organization, which is probably formed by Gn-Gc heterodimers (Huiskonen *et al*., 2009).

1.2.2 Entry

1.2.2.1 Receptors

Hantaviruses replicate primarily in the endothelium of infected patients, and endothelial cell cultures are susceptible to hantavirus infection *in vitro* (Schmaljohn & Nichol, 2007). Endothelial cells together with Vero E6 cells are the most widely employed models for hantavirus infectivity and cell biological studies. It is well established that integrins permit hantavirus infection in these cells probably through a direct interaction with the ectodomains of the viral envelope glycoproteins. Integrins are cell surface proteins playing a major part in cell adhesion to the extracellular matrix and, therefore, affect many aspects of cell and tissue functions including vascular haemostasis (Hynes, 2002). The initial findings that these proteins are hantavirus receptors came from cell infection studies in which infection of HCPS- or HFRS-causing hantaviruses was blocked by antibodies against integrin $\alpha_V\beta_3$ or its ligand, vitronectin. In contrast, PHV, which is regarded as a non-pathogenic hantavirus, was blocked by antibodies against integrin $\alpha_5\beta_1$ or its ligand fibronectin (Gavrilovskaya *et al*., 1998; Gavrilovskaya *et al*., 1999). The initial idea that pathogenic and non-pathogenic hantaviruses differ in their receptor usage was further supported by the fact that TULV, a rather non-pathogenic old-world hantavirus, was found to be unable to block endothelial cell migration on $\alpha_V\beta_3$ integrins in contrast to pathogenic hantaviruses (Gavrilovskaya *et al*., 2002). Typically $\alpha_V\beta_3$ recognize an arginine-glycine-aspartic acid (RGD) peptide motif present on their binding partners but the infectivity of pathogenic hantavirus is shown to be RGD-independent (Gavrilovskaya *et al*., 1998; Hall *et al*., 2007) and instead is mapped to plexin-semaphorin-integrin (PSI) domain present on inactive integrins (Matthys *et al*., 2010; Raymond *et al*., 2005). These findings suggested that pathogenic hantaviruses are capable of regulating vascular permeability, which is responsible for interstitial hemorrhage and development of shock in hantavirus-infected patients, through altering activity state of

integrin. The use of integrin β_3 as hantavirus receptor in cell cultures was further recognized by using peptides and peptidomimetics that competed for virus-integrin binding (Hall *et al*., 2007; Hall *et al*., 2010; Larson *et al*., 2005). Hantaviral inhibitory molecules were more effective against SNV than PHV emphasizing the link between integrin recognition and pathogenicity at least in the case of new-world hantaviruses. Platelets express another type of integrin, $\alpha_{IIb}\beta_3$, on their surface through which monolayers of pathogenic hantavirus-infected, but not TULV-infected, endothelial cells have been shown to recruit platelets (Gavrilovskaya *et al*., 2010). This is an interesting mechanism, which could explain the commonly observed platelet loss (thrombocytopenia; Table 1) in the bloodstream of infected patients (see 1.3.1).

The above-mentioned studies show hantavirus-integrin interaction in cell culture models but data indicating the importance of integrins for hantavirus infectivity *in vivo* are limited. Antibodies that recognize and block integrin $\alpha_V\beta_3$ have been shown to reduce viral loads in SNV-infected mice (Medina *et al*., 2007) and prolong survival of HTNV-infected newborn murine pups (Song *et al*., 2005). The latter study suggested that antagonist to $\alpha_V\beta_3$, action of which is analogous to RGD, was shown have a positive effect on survival, casting a doubt over the specificity of this study. The lack of appropriate animal model in hantavirus research has complicated the investigations on the role of integrins as *in vivo* receptors for these viruses.

The ability of integrin-specific antibodies or peptides in blocking of hantavirus infectivity in Vero E6 cell cultures is not highly robust (commonly an inhibition of 50-80% was obtained) and the existence of another cellular receptor to hantaviruses could not be ruled out by integrin-focused studies (Gavrilovskaya *et al*., 1998; Gavrilovskaya *et al*., 1999; Hall *et al*., 2007). Therefore, it was not surprising that another receptor for hantaviruses in cultured cells was found, namely decay accelerating factor (DAF)/CD55 (Krautkramer & Zeier, 2008). This cell membrane protein plays a part in inhibiting the complement system of the innate immunity. It acts in concert with integrin $\alpha_V\beta_3$ to facilitate entry of HFRS-causing hantaviruses into cultured cells but can also act as the sole cellular receptor for hantaviruses in the absence of integrin $\alpha_V\beta_3$ (Buranda *et al*., 2010). Another cellular protein of the complement system, C1qR/p32, has also been postulated to be involved as a co-receptor in hantavirus entry in Vero E6 cells (Choi *et al*., 2008) but its direct role in virus entry remains unclear.

1.2.2.2 Penetration

After binding to a cell surface receptor, enveloped viruses need to fuse with cell membranes in order to penetrate and release their genetic material into the host cell cytoplasm. The fusion can be accomplished directly with the plasma membrane or more commonly with inner membranes of the cell after endocytosis. Fusion with intracellular organelles as opposed to plasma membrane carries many advantages for viruses including exploitation of the endocytic transport machinery to reach the inner parts of the cell and utilization of the pH gradient in the endocytic pathway to activate fusion at specific subcellular locations. It is also beneficial that viral proteins are not exposed to the outer environment of the cell after fusion that would otherwise induce antiviral responses. The endocytosis pathways can be roughly divided into clathrin-dependent, clathrin-independent, caveosomal and macropinocytosis pathways. Clathrin-dependent pathway and low-pH induced fusion either at early endosomes (pH 6.0-6.5) or late endosomes (pH 5.5-6.0) are most often used by viruses (Marsh & Helenius, 2006). Clathrin-dependent pathway is also exploited by hantaviruses (Jin *et al*., 2002), and fusion is thought to occur at early endosome (Fig. 2) because hantavirus-infected cells fuse and form syncytia when exposed to pH 6.2-6.3 (Arikawa *et al*., 1985; McCaughey *et al*., 1999).

Fusion between viral and cellular membranes requires activation of viral fusion protein(s). These are viral surface proteins anchored on the viral envelope through a transmembrane domain and harboring a hydrophobic fusion peptide buried from the outer milieu in the native state of the virion. Fusion proteins can be divided into class 1 or 2 depending on the mechanism of how they exert the fusion process; this is accomplished either by receptor binding, exposure to low pH or both (Kielian & Rey, 2006). The hantaviral fusion protein (and bunyaviral in general) has been assumed to be the Gc envelope glycoprotein by computational modeling (Garry & Garry, 2004; Tischler *et al*., 2005) which was also used to predict the amino acid residues responsible for fusion (fusion peptide). Experimental evidence for this has been lacking until lately when it was shown that TULV Gc is subjected to conformational changes upon exposure to low pH at 6.2 (Hepojoki *et al*., 2010) and that mutations in the putative fusion peptide of Gc affected the fusion and entry of hantavirus glycoprotein pseudotyped-lentiviral particles (Cifuentes-Munoz *et al*., 2011).

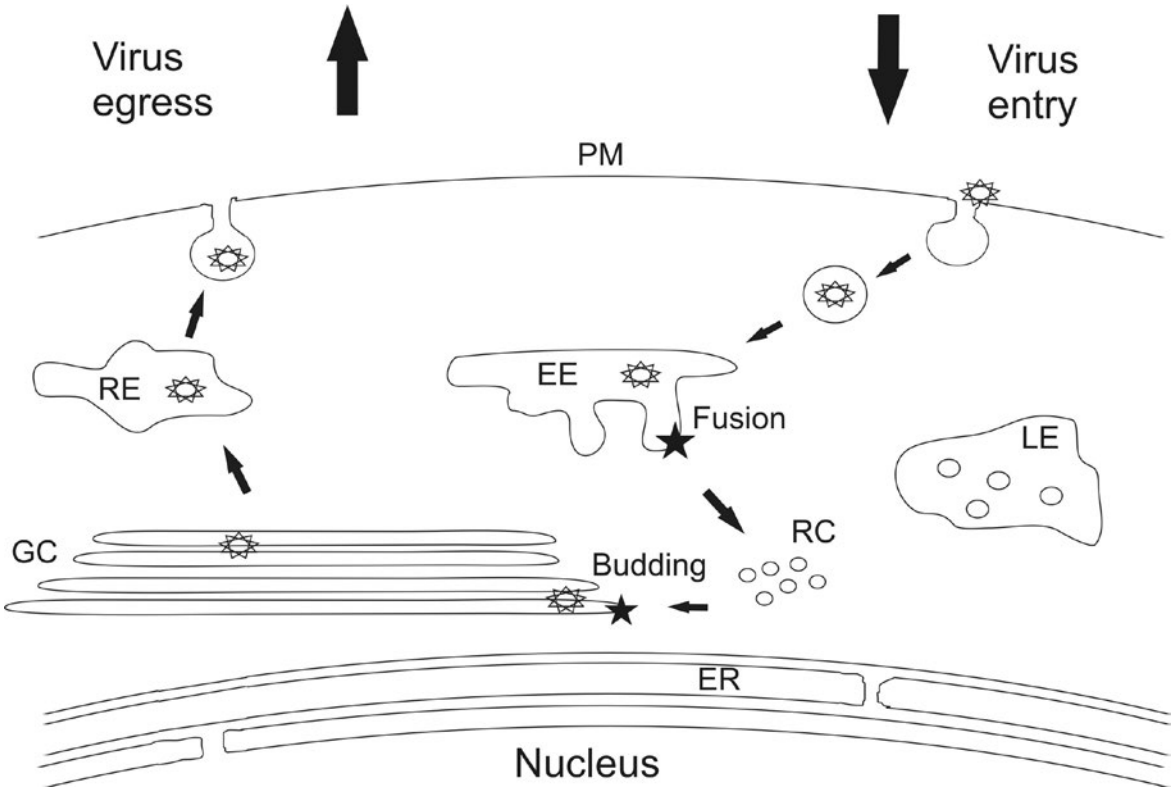


Figure 2. Life cycle of hantaviruses. Virus enters the cell by clathrin-mediated endocytic pathway, and fuses with the early endosomal membrane (EE) to release its genetic material into the cytoplasm. Replication complexes (RC) are formed close to endoplasmic reticulum (ER) and Golgi complex (GC). Assembled virions bud to the lumen of the Golgi. Viruses travel through the GC and enter recycling endosome (RE) from where they are released by exocytosis. PM = Plasma membrane, LE = late endosome.

The conformational changes in the viral fusion proteins required prior to fusion with cellular membranes may involve rearrangements of disulfide bridges as has been shown for a number of different virus families including class 1 and 2 fusion proteins (Sanders, 2000). These so-called thiol/disulfide exchange reactions are catalyzed either by a cellular disulfide isomerase (PDI) expressed on the surface of the cell or by a virally encoded PDI activity. The PDI activity is exerted through a CxxC motif (Freedman *et al.*, 1994). Intriguingly, hantaviral glycoprotein Gn and Gc ectodomains are rich in cysteine residues and they possess numerous CxxC motifs. However, the functions of CxxC motifs in virus-cell membrane fusion have not been elucidated.

1.2.3 RNA synthesis

1.2.3.1 Overview of the replication strategy of NSRV

After penetration into host cells, viral proteins are synthesized and genomes are replicated in order to produce progeny viruses. To achieve this, the RNPs need to be transported to the site of replication. While this step is poorly investigated for hantaviruses or other genera in the family *Bunyaviridae*, the replication strategy of these viruses is better understood. Negative-stranded segmented RNA viruses (NSRV) need their genome to be transcribed into positive-sense RNA (mRNA) to serve as template for viral protein synthesis on cellular ribosomes. Unlike positive-sense RNA viruses, which can use the genomic RNA directly as mRNA for protein synthesis, negative-sense RNA viruses have virus-encoded transcriptase associated with the viral ribonucleoprotein (RNP). The NSRVs harbor open reading frames on each individual RNA segments (on S, M and L segments in the case of bunyaviruses) whereas the genes of non-segmented viruses are transcribed from common RNA template. The reason for genome segmentation is unclear but could involve increased transcriptional regulation of individual viral genes with promoters with different transcriptional efficiency. On the other hand, there is a requirement to enclose at least one copy of each of the segments into the progeny viruses instead of just one RNA molecule as is the case for non-segmented viruses. In addition to mRNAs, negative-sense RNA viruses need to generate complementary RNA (cRNA) from the viral genomic strand, which then acts as a template for the synthesis of viral genomic RNA (vRNA). Like vRNA, cRNA is also encapsidated by the nucleocapsid and therefore active viral (N) protein synthesis is thought to be prerequisite for the replication process (Kolakofsky & Hacker, 1991; Schmaljohn & Nichol, 2007). A model suggesting a switch from transcription to replication as depicted in Fig. 3 is challenged with studies made on influenza (family *Orthomyxoviridae*) which suggested that the production of mRNA and cRNA occur concomitantly but cRNA is stabilized only by newly synthesized polymerase and nucleocapsid (Vreede *et al*., 2004; Vreede & Brownlee, 2007).

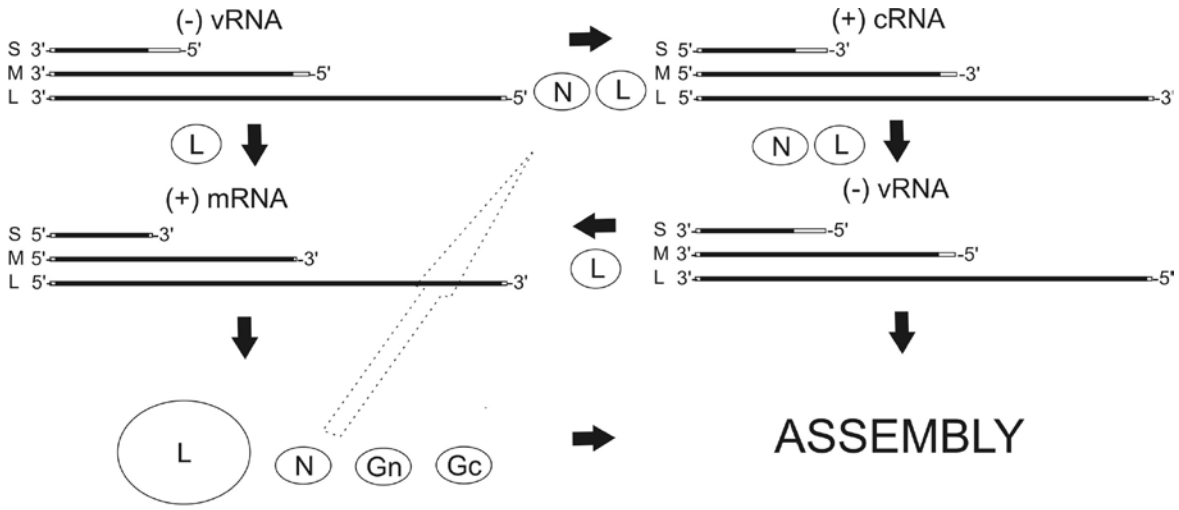


Figure 3. Outline of hantavirus replication and progeny virus production. Following the entry of the viral RNP into host cell cytoplasm, viral negative sense (-) RNAs (vRNA) is transcribed by the transcriptase activity of RNP-associated L protein into mRNAs, which are subsequently translated by host cell ribosomes to viral proteins. The newly-synthesized N protein is necessary for the replication of vRNA, in which the viral complementary RNA (cRNA) acts as template for the synthesis of more (-) vRNA. The cRNA and vRNA production is dependent on the replicase activity of L protein. The replicated vRNA is finally assembled together with L, N, Gn and Gc to form progeny virions. The protein coding sequences (open reading frames; ORFs) of the RNA are marked in black and non-translated regions of the RNA in white. The sizes of S, M and L segments together with their ORFs reflect their comparable size in nature.

1.2.3.2 The RNA synthesis mechanism of hantaviruses

The RNA synthesis mechanism in the case of hantaviruses is relatively poorly studied in comparison to other genera of *Bunyaviridae* mainly due to a lack of a feasible reverse genetics system. However, the mechanisms of RNA synthesis among bunyaviruses (and among other NSRV) are considered to be conserved and, therefore, studies made on other bunyaviruses are likely to be applicable for hantaviruses. Reverse genetics systems based on minigenomes that are active in transcription and replication have been utilized with orthobunyaviruses and phleboviruses. The successive replication of minigenomes is preliminary stage in the rescue of infectious viral clones solely from transfected plasmids encoding viral proteins and RNA (Bouloy & Flick, 2009). It is well established that 3′-terminal non-translated (NTR) sequences (Fig. 3) of the viral genomic RNAs act as promoters for the initiation of transcription. As mentioned before, these 3′-terminal nucleotides have perfect complementary to 5′-termini of the corresponding genomic RNA molecule (Fig. 4a) and base pair to form panhandle structures resulting in circularized

RNPs in the virion (Raju & Kolakofsky, 1989) and possibly in infected cells. The complementarity of the 5′-terminus is also important for the efficiency of the 3′-end transcriptional promoter as shown for orthobunyavirus prototype Bunyamwera virus (BUNV) (Barr & Wertz, 2004; Kohl *et al*., 2004) and Rift valley fever virus (RVFV) and Uukuniemi virus (UUKV) of the genus *Phlebovirus* (Flick *et al*., 2004; Flick *et al*., 2002; Gauliard *et al*., 2006).

The S segment-derived mRNA has been shown to be the most abundant species of the three mRNAs in SNV- (Hutchinson *et al*., 1996) and RVFV-infected cells (Gauliard *et al*., 2006). In the case of purified tomato spotted wilt virus (TSWV; genus *Tospovirus*) the S segment possessed the strongest transcriptional activity *in vitro* (van Knippenberg *et al*., 2002), which may reflect the requirement for newly synthesized N protein early in the replicative phase of the genome (Fig. 3). On the other hand, when analyzed in the context of reverse genetics systems, the strongest promoters were detected in M segment of BUNV and UUKV and L segment of RVFV (Barr *et al*., 2003; Flick *et al*., 2004; Gauliard *et al*., 2006; Kohl *et al*., 2004). There are indications that ongoing protein synthesis is compulsory for completion of transcription since premature termination of RNA transcripts in the absence of ribosomes *in vitro* or in the presence of protein synthesis inhibitors *in vivo* have been detected (Bellocq *et al*., 1987; Raju & Kolakofsky; 1987; Vialat & Bouloy, 1992).

A specific “corkscrew” structure has been shown to be required for the promoter activity of *Orthomyxoviridae* viruses (Flick & Hobom, 1999; Leahy *et al*., 1998; Leahy *et al*., 2001). Interestingly, the genomes of all viruses in the family *Bunyaviridae* with the exception of phlebovirus genomes can fold into this structure suggesting an analogous transcription initiation mechanism within these viruses (Fig. 4b) (Flick *et al*., 2002). All NSRVs use a process called “cap snatching” for the initiation of mRNA transcription. This was first described for influenza (Bouloy *et al*., 1978), and it revealed the use of host cell mRNA-derived capped primers for viral mRNA transcription by the viral polymerase. “Cap snatching” requires a virally encoded endonuclease activity, which produces around 10-20 oligonucleotides long capped primers from host cell mRNAs (Duijsings *et al*., 2001; Jin & Elliott, 1993; Patterson *et al*., 1984; van Knippenberg *et al*., 2005; Vialat & Bouloy, 1992). In contrast to transcription, replication of NSRVs initiates *de novo* by a complementary nucleotide triphosphate.

Prime and re-align mechanism for the initiation of the synthesis of genomic RNA and mRNA has been suggested for hantaviruses (Garcin *et al*., 1995). This is based on the observations that genomic 3′-ends of hanta- and nairoviruses consist of AMP, which requires replication to initiate with UTP on the opposite strand and result in a tri-phoshorylated U as the terminal 5′-end nucleotide of the newly synthesized RNA. However, pyrimidines (U and C) are not regarded to be efficiently utilized for initiation of RNA synthesis. The prime and re-align mechanism, therefore, gives an explanation of how hantaviruses overcome this obstacle by initiating replication with GTP (Garcin *et al*., 1995). It also explains why the synthesized mRNAs contain viral sequences upstream of the genome-templated sequence. It has also been suggested that the viral endonuclease specifically cleaves the host cell derived-primers after GMP and is required for trimming of the cRNA and vRNA 5′-termini to yield monophosphorylated U at the end. The existence of monophosphorylated hantaviral genomes were shown by (Garcin *et al*., 1995) for HTNV and recently for a subset of old-world hantaviruses (Wang *et al*., 2011) corroborating the postulated model of prime and re-align for RNA synthesis initiation in hantaviruses (Fig. 4c).

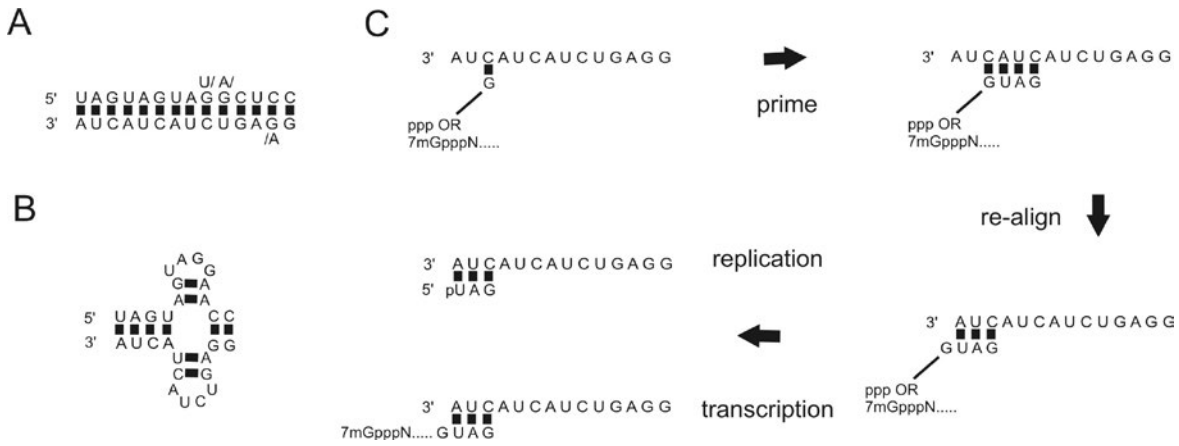


Figure 4. Initiation of transciption and replication by hantaviruses. (A) The consensus sequence of the complementary termini of the negative-sense hantaviral genomes forming a “panhandle” structure. (B) The predicted “corkscrew” structure in the panhandle with possible role in initiation of transcription (Flick *et al.*, 2002). (C) The prime and re-align mechanism as suggested for hantaviruses as the initiation mechanism of replication and transcription (Garcin *et al.*, 1995). Nucleotide triphosphate is indicated by ppp and monophoshate by p. The cap structure in the transcriptional primer is indicated by 7mGpppN… (N refers to any nucleotide residue).

Based on the reverse genetics systems developed for different genera of bunyaviruses it is clear that the expression of both L and N proteins is both essential and sufficient for viral RNA synthesis (Bouloy & Flick, 2009; Dunn *et al*., 1995).

1.2.3.3 L protein

Despite being the largest protein encoded by hantaviruses (approximately 2500 aa) or bunyaviruses in general, the L protein is probably the least-studied. The fact that L protein is expressed in very low amounts especially in virions but also in infected cells forms the major obstacle for elucidating its functions. Since there is high level of conservation between bunyaviral RNA-dependent polymerases, it is possible to draw conclusions of the functions of hantaviral L protein from studies made with other viruses. It is well known through sequence conservation that the polymerase activity of bunyavirus polymerases is defined by six motifs named pre-motif A, A, B, C, D and E (Muller *et al*., 1994; Poch *et al*., 1989) that are located in the central part of the L protein (approximately 900-1200 aa) and form a three-dimensional structure denoted palm, finger and thumb (Muller *et al*., 1994).

La Crosse orthobunyavirus was used as the model to demonstrate that the N-terminal part (1-180 aa) of the protein harbors a viral endonuclease activity involved in the initiation of mRNA transcription during the cap snatching process (Reguera *et al*., 2010). This domain is similar to the one found in the N-terminus of lassaviral (family *Arenaviridae*) L protein (Lelke *et al*., 2010; Morin *et al*., 2010) where it is shown to be dispensable for replicase activity (Lelke *et al*., 2010). There is also a level of conservation between N-terminus of La Crosse L and the orthomyxovirus polymerase complex subunit PA, which is the subunit shown to be responsible for endonuclease activity in the case of influenza (Dias *et al*., 2009). The orthomyxoviruses differ from bunya- and arenaviruses in the sense that their polymerase complexes are translocated to the nucleus of infected cells and are divided into three subunits: PA, PB1 and of PB2 (Boivin *et al*., 2010). Yet, as expected of the similar replication mechanism employed by all NSRVs (Fig. 3), bunya- and arenavirus L proteins should harbor the same functions that in the case of orthomyxoviruses are divided into different subunits. The middle part subunit PB1 harbors the polymerase activity and PB2 a cap-binding activity required for cap snatching (Guilligay *et al*., 2008). Interestingly, given the high degree of conservation among the

endonuclease and polymerase domains of these viruses, the bunyaviral L-protein does not express a clearly distinguishable cap-binding domain in its C-terminal part as seen for e.g. influenza by sequence prediction (Reguera *et al*., 2010), and there is even evidence, which suggests that this function is passed on to N protein of bunyaviruses (Mir *et al*., 2010). A recent model developed for influenza virus suggests that the transcription of vRNA to mRNA can initiate and proceed only by a RNP-resident polymerase complex (i.e in *cis*) in contrast to replication where a soluble polymerase complex (in addition to nucleocapsid) is needed for RNA synthesis (i.e. in *trans*) (Jorba *et al*., 2009; Vreede *et al*., 2004).

1.2.3.4 N protein

Hantaviruses and nairoviruses differ from other bunyaviruses in that their N protein is much larger (50 kDa vs. 25-30 kDa). The main function of the N protein is to encapsidate vRNA (and cRNA) but N protein is likely to possess other functions as well since it is the most abundant protein of bunyaviruses (Elliott *et al*., 2000). Hantaviral N protein binds RNA in a non-specific fashion (Gott *et al*., 1993), but it has higher affinity towards the NCRs of vRNA, especially the 5′-end NCR (Severson *et al*., 1999; Severson *et al*., 2001). The 5′-end of vRNA and cRNA may, therefore, act as an encapsidation signal. A similarly-located encapsidation signal has also been reported for BUNV of *Orthobunyavirus* genus (Osborne & Elliott, 2000). It is unclear why mRNA, which also contains this putative encapsidation signal, is not encapsidated by N protein. It has been reported that complete transcription requires ongoing protein synthesis (Bellocq *et al*., 1987), and it is possible that the cellular ribosomal translation apparatus outcompetes N-mediated encapsidation in the case of the capped mRNA.

N protein of hantaviruses readily forms oligomers (Alminaite *et al*., 2006; Alminaite *et al*., 2008; Kaukinen *et al*., 2005), and it has been reported that N binds the panhandle terminal structure of the viral RNA with higher affinity in trimeric vs. monomeric or dimeric form (Fig. 4a) (Mir & Panganiban, 2004). The N protein also exhibits RNA chaperone activity (Mir & Panganiban, 2006a; Mir & Panganiban, 2006b). This has also been demonstrated for influenza nucleoprotein (Baudin *et al*., 1994) and can, therefore, be shared by all nucleocapsid proteins. The RNA chaperone activity may either aid in the formation of terminal panhandles of vRNAs by melting RNA secondary structures or

unwind the pre-formed panhandle structures. N protein may, thus, act in transcription initiation together with L protein using 3′-terminus of vRNA as template. One striking feature of hantaviral N protein is its ability to recognize mRNA caps (Mir & Panganiban, 2008; Mir *et al*., 2008; Mir *et al*., 2010), which is commonly thought to be exerted by the polymerase and which is also shown for influenza PB2 (Guilligay *et al*., 2008). Hantaviral N protein localizes to cytoplasmic processing bodies (P bodies), to which cellular mRNAs are being sequestered (Mir *et al*., 2008), and from which it may "steal" capped mRNAs to be used as primers for transcription. In addition, N protein augments translation of viral mRNA (and non-viral to lesser extent) by binding to capped mRNA (to the actual cap and the 5′-end triplet sequence complementary to vRNA 3′-end transcriptional promoter) and ribosomal protein S19 (RPS19) (Cheng *et al*., 2011; Haque & Mir, 2010; Mir & Panganiban, 2010). In all, hantaviral N protein seems to be a very complex protein and have a variety of functions that promote the transcription process of vRNA. The N proteins of other bunyaviruses are unlikely to be any simpler although many of them are smaller in size. This is recently supported by the finding that mutations in BUNV N protein differentially regulate transcription and replication of orthobunyaviruses (Walter *et al*., 2011).

1.2.4 Progeny virus formation

1.2.4.1 Budding

Infection by enveloped viruses often results in rearrangement of host cell membranes that provides a membranous platform or microenvironment for viral replication (Mackenzie, 2005). This is possibly the way to couple the synthesis of vRNA to the budding of viruses into the lumen of intracellular membranes. In the case of hantaviruses not much is known about the actual replication sites of the virus. However, budding of all family *Bunyaviridae* viruses is thought to take place in the Golgi complex (GC) (Elliott *et al*., 2000) and for the bunyavirus prototype BUNV it is demonstrated in more detail that the virus exploits GC membranes to mechanistically combine viral replication and budding of the virus. BUNV-infection of host cells results in the formation of membraneous tubular structures, which are elongated inside Golgi stacks and are open towards the cytoplasm. The tubular structures harbor the RNA synthesis machinery of the virus, and RNPs

formed in the tubes associate with the Gn and Gc glycoproteins at nearby GC membranes before assembly and budding of the progeny virus. After budding, the virion is still immature but structural maturation takes place along virus egress from the cells through exocytosis (Fig. 2) (Fontana *et al*., 2008; Novoa *et al*., 2005; Salanueva *et al*., 2003).

There is evidence suggesting that hantaviruses also bud into GC. This is because glycoproteins predominantly localize to GC membranes. In addition, N and L proteins of hantaviruses have been shown to be GC-associated peripheral membrane proteins when expressed individually (Kukkonen *et al*., 2004; Ravkov & Compans, 2001). The glycoproteins Gn and Gc are synthesized from a common M segment-encoded ORF and are cleaved co-translationally into individual proteins (Lober *et al*., 2001). However, interaction of the two glycoproteins is needed for their transport to GC whereas when expressed individually they reside in the ER (Deyde *et al*., 2005; Ruusala *et al*., 1992; Shi & Elliott, 2002; Spiropoulou *et al*., 2003). As their name implies Gn and Gc glycoproteins are glycosylated proteins. Glycosylation takes place via N-linkage by high mannose-type oligosaccharides. The presence of only this type of sugars indicates that hantaviral glycoproteins are being transported not further than cis-Golgi, which suggests that cis-Golgi is the budding site for the virus (Shi & Elliott, 2004). There are five glycosylation sites in Gn and one in Gc. In the case of Gn, glycosylation sites play the major role in the correct folding and transport to GC. Like other bunyaviruses, hantaviruses are believed to exit the cells through exocytosis. There is only one study showing that recycling endosome is required for hantavirus egress from infected cells (Rowe *et al*., 2008) but the secretory pathway is not well defined for any member of the *Bunyaviridae* family. Interestingly, there are reports that two new-world hantaviruses, BCCV and SNV, bud from the plasma membrane (Goldsmith *et al*., 1995; Ravkov *et al*., 1997). In addition, SNV glycoproteins individually localize to plasma membrane and to late endosomes or lysosomes at least when over-expressed (Spiropoulou *et al*., 2003). If budding from plasma membrane is a unique phenomenon of new-world hantaviruses or in some particular circumstances a common feature of all hantaviruses, remains to be determined.

The signal that initiates the budding event of bunyaviruses is unknown. Budding of membraneous negative-sense RNA viruses commonly involves a viral matrix protein which function, in addition to facilitating budding, is to act as a bridge between the CTs of the surface glycoproteins and the viral core. However, in contrast to other members of

NSRV, bunyaviruses do not encode a matrix protein, which exerts this function. It is, therefore, assumed that the CTs of Gn and/or Gc proteins possess this activity and indeed there is evidence for this. The development of infectious virus like particle (VLP) generation systems for BUNV and UUKV where transfection of plasmids encoding for all viral structural proteins (Gn, Gc, N and L) together with plasmids producing either full-length or minigenomes are transiently expressed in cells (Lowen *et al*., 2004; Overby *et al*., 2006) has proved to be useful in elucidating the roles of different proteins in virus release. Firstly, mutagenesis of CTs of bunyavirus prototype BUNV suggested that both Gn- and Gc-CTs are required for VLP release (Shi *et al*., 2007). Secondly, studies with UUKV lead to identification of dileucine motif (Fig. 5) responsible for budding of VLPs. Mutation of this motif to alanines abolished budding of UUKV VLPs into GC while still enabling proper localization of glycoproteins on GC membranes (Overby *et al*., 2007b). Many viral matrix proteins contain late domains, which are typically four residues long amino acid stretches essential for virus budding (consensus sequences e.g. PT/SAP and YPxL). These motifs function to hijack the cellular budding machinery normally used by the host cell for the biogenesis of multivesicular bodies (Chen & Lamb, 2008). Interestingly some late domains resemble the canonical endocytosis motif Yxxθ (where θ is any hydrophobic amino acid) (Sorkin, 2004), which is fully conserved in Gn-CT of hantaviruses and also in CTs of some other bunyaviruses (Fig. 5, marked as green). Moreover, dileucine motif necessary for budding of UUKV is generally known to act as endocytosis signal (Sorkin, 2004). The putative bunyaviral endocytosis motifs, for which the actual functions in endocytosis have not been determined, may act similarly to late domains of matrix proteins and be responsible for budding. It was reported that the YxxL motif in hantaviruses is part of an immunoreceptor tyrosine-based activation motif (ITAM) consisting of tandem YxxL/I motifs (Geimonen *et al*., 2003b). However, the second C-terminally located YxxL motif identified in the study was only conserved among viruses of the new-world and by prediction it locates to a transmembrane domain (part of the signal sequence for Gc) indicating that this putative motif is unlikely to have any biological function *in vivo*. Instead, it is likely that the N-terminally located, fully conserved YxxL motifs possess a function in intracellular trafficking of hantaviruses.

Different bunyavirus genera are unlikely to share the same mechanism for budding since the expression of UUKV and RVFV (both are phleboviruses) glycoproteins in the absence of N or L proteins allowed the generation of VLPs (Overby *et al*., 2006; Piper *et*

al., 2011) which was not the case for e.g. hantaviruses. For hantaviruses the formation of VLPs require simultaneous expression of M and S segments indicating that N protein in addition to glycoproteins is necessary for budding to occur (Betenbaugh *et al*., 1995). The developed VLP generation systems for hantaviruses that would greatly ease studies concerning the molecular mechanisms of progeny virus formation (Flick *et al*., 2003) are not used to a large extent probably because of the difficulties in obtaining high enough virus titers.

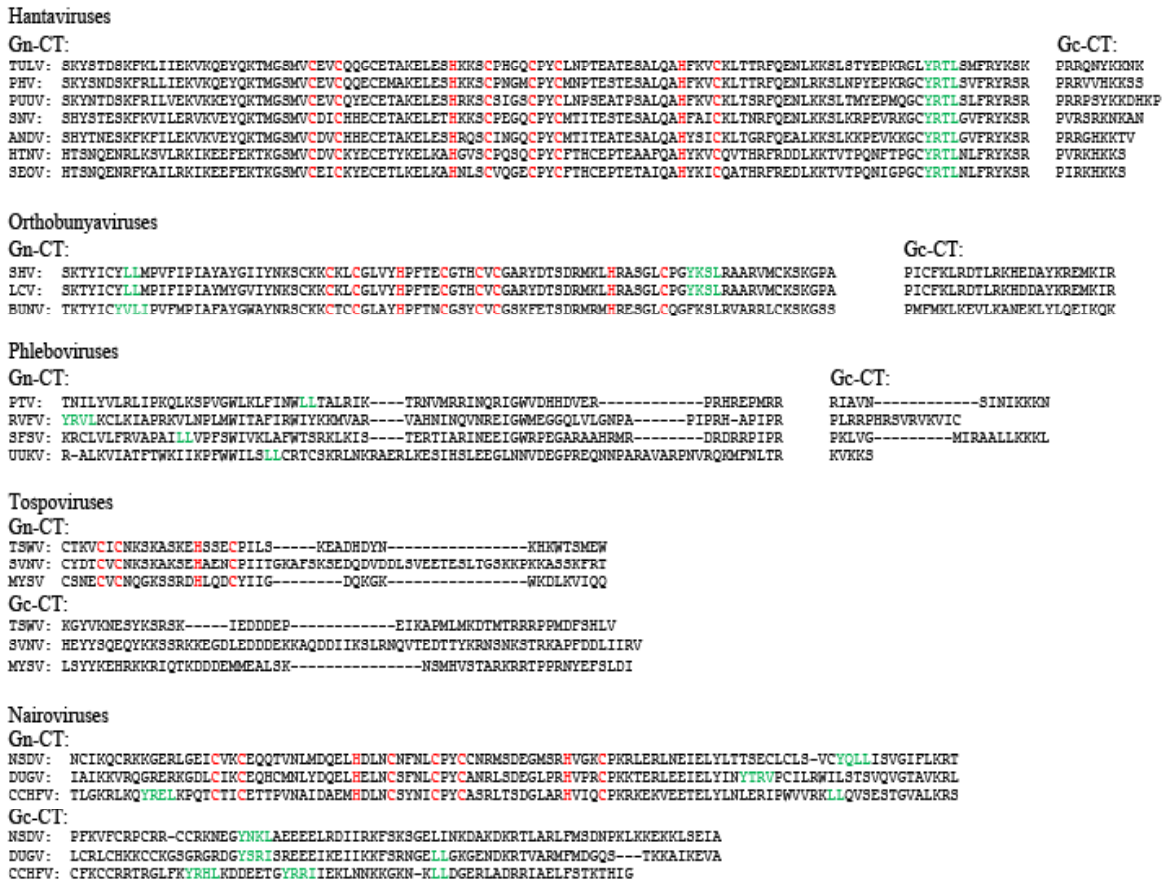


Figure 5. Alignment of cytoplasmic tails (CTs) of bunyaviral glycoproteins. Amino acids predicted to coordinate zinc-ions in a zinc finger fold are shown in red. A possible late-domain in hantaviral Gn-CT is shown in green. TULV = *Tula virus*, PHV = *Prospect Hill virus*, PUUV = *Puumala virus*, SNV = *Sin nombre virus*, ANDV = *Andes virus*, HTNV = *Hantaan virus*, SEOV = *Seoul virus*, SHV = *Snowshoe Hare virus*, LCV = *La Crosse virus*, BUNV = *Bunyamwera virus*, TSWV = *Tomato spotted wilt virus*, SVNV = *Soybean Vein necrosis virus*, MYSV = *Melon yellow spot virus*, PTV = *Punta Toro virus*, RVFV = *Rift velly fever virus*, SFSV = *Sandfly fever Sicilian virus*, UUKV = *Uukuniemi virus*, NSDV = *Nairobi sheep disease virus*, DUGV = *Dugbe virus*, CCHFV = *Crimean-Congo hemorrhagic fever virus*.

1.2.4.2 Assembly

In order to produce infectious viruses all NSRVs need to pack at least one copy of each of their segments into progeny virions during budding. For influenza viruses two different hypotheses of how this occurs has been proposed. According to the first one each individual genomic RNA segment has its own specific packaging signal that directly interacts with proteins associated with the viral membrane (e.g. matrix protein) or mediates inter-segment specific interactions resulting in a complex of the differently-sized RNPs that is ready to be packaged. The second, less-favored hypothesis assumes random packaging of segments. In this scenario the packaging signal is conserved between the segments. Random packaging scheme of segmented viruses would less efficiently result in infectious viruses with at least one copy of each individual segment than the specific packaging scheme. This drawback could be overcome by packaging excess number of segments into a virus particle in order to increase the likelihood of obtaining at least one copy of an individual segment into the progeny virion. The segment-specific packaging scheme is currently more favored in the case of influenza viruses since it has been shown that the RNA sequences that mediate packaging are different for the different segments. These regions consist of not only NTRs but also ORFs of individual RNA segments (Hutchinson *et al*., 2010). However, bunyavirus minigenomes, which consist only of NTRs and lack ORFs of viral origin, can be packaged into recombinant virions by superinfecting the minigenome-transfected cells with wild-type virus (Blakqori *et al*., 2003; Flick *et al*., 2003; Flick *et al*., 2004; Flick *et al*., 2003; Kohl *et al*., 2006). As shown for BUNV and UUKV, the promoter strengths of these minigenomes are in the order of M > L > S but packaging efficiencies are in the order of L > M > S showing that the NTRs of each segment play their specific roles in packaging stage (Flick *et al*., 2004; Kohl *et al*., 2006). Different packaging efficiencies of segment-specific NTRs may relate to the different sizes of the individual native segments; the largest L segment needs the strongest packaging signal to be efficiently incorporated into progeny viruses. When studying packaging of RVFV RNA segment, conflicting results have been obtained. In one study it was shown that the packaging of L and S segment RNAs were dependent on wild-type M segment NTRs (Terasaki *et al*., 2011). Contradictory to this, a viable two-segmented recombinant form of RVFV without the M segment RNA was recently obtained. In this arenavirus-like RVFV, glycoproteins were cloned into the S segment replacing the non-essential NSs protein (Brennan *et al*., 2011). A study performed with BUNV indicated that when the NTRs of L segment were

replaced by those of M segment, the recombinant virus was infectious (Lowen *et al*., 2005). This in turn indicated that at least two copies of M segment NTRs can be packaged into progeny virus showing a degree of randomness in the packaging. While it is well-established that NTRs are required for packaging of bunyaviral RNA segments, the actual sizes of the RNA segments may contribute to the specificity of packaging of each individual RNA segments.

The ability to produce infectious VLPs directly from cloned viral proteins allows mutational analysis of viral proteins related to virus assembly. By these means it has been demonstrated that Gn-CT is necessary the packaging of RNP in UUKV (Overby *et al*., 2007a) and RVFV (Piper *et al*., 2011). For UUKV, the most C-terminal residues of Gn-CT are essential for RNP incorporation into VLPs whereas for RVFV it is the N-terminal part of Gn-CT. In the case of RVFV it was shown that Gn-CT can bind and package L and N proteins independently but for efficient release of VLPs the N-encapsidated genomic RNA, but not L protein, is required. Since N protein of phleboviruses has no sequence-specificity in its RNA binding activity, it was hypothesized that it is the Gn-CT that recognizes a packaging signal in the genomic RNA thus playing an indispensable part in the formation of infectious virions (Piper *et al*., 2011). In contrast to phleboviruses where Gc-CT does not seem to play major role in virus assembly, the N protein of TSWV tospovirus can interact with both Gn and Gc (Ribeiro *et al*., 2009; Snippe *et al*., 2007). However, the predicted Gc-CT of TSWV is much larger than the corresponding Gc-CTs of phleboviruses (or hantaviruses) and it is, therefore, likely that it has some functions, which are not analogous to other bunyaviruses.

The Gn-CTs of hantaviruses (ANDV) and nairoviruses (CCHFV) are shown by NMR analysis of recombinant Gn-CTs to possess a unique dual CCHC-type ZF-fold (Estrada *et al*., 2009; Estrada & De Guzman, 2011). The structural analysis of this domain indicates that it has two individual zinc fingers (coordinating one zinc(II)-ion each), which both fold into a conventional ββα conformation. However, unlike typical ZFs of this type, the hanta- and nairovirus ZFs associate tightly with each other to form a globular domain. The cellular ββα-type ZFs usually form arrays of multiple ZFs as a so called “beads on a string” orientation and act commonly as transcriptional regulators. Given the common role of cellular ZFs in regulation of RNA or DNA functions it is hypothesized that bunyaviral ZFs would recognize viral RNA. Although the ZF domains of CCHFV and ANDV have similar structures they differed in the sense that CCHFV ZF was capable of

binding viral RNA while ANDV was not (Estrada & De Guzman, 2011). On the other hand, the ZF domain of TULV is shown to bind N protein (Wang *et al*., 2010). Taken together these results suggest a role for the ZF domain in the assembly of RNPs into progeny viruses. By sequence prediction it can be assumed that orthobunyaviruses and tospoviruses also have ZFs in their Gn-CTs (Estrada *et al*., 2009) (Fig. 5) although those are likely to represent different types of ZFs and may, therefore, exert different functions. Interestingly, phleboviruses do not contain a predicted ZF fold in their Gn-CT (Fig. 5) or anywhere else in the viral proteins (personal observation). Intriguingly, phleboviruses are also the only members of *Bunyaviridae*, which are known to form VLPs in the absence of N or RNP (Overby *et al*., 2006; Piper *et al*., 2011).

1.2.4.3 Budding and assembly of matrix-protein containing NSRVs

Irrespective of the packaging mechanism, the influenza matrix protein M1 is most likely required for incorporation of the genetic core into progeny virions. The M1 has been shown to interact with the viral RNP through direct binding of the nucleocapsid protein (Baudin *et al*., 2001; Noton *et al*., 2007). It has also ability to bind single-stranded RNA nonspecifically (Elster *et al*., 1997; Wakefield & Brownlee, 1989; Ye *et al*., 1999; Ye *et al*., 1989) but the role of the RNA-binding activity in RNP recognition is still unclear. M1 inhibits transcription of purified RNPs *in vitro* and the activity of influenza transcription system *in vivo* (Perez & Donis, 1998). It is hypothesized that this mode of action of the matrix protein is a signal to the viral polymerase to stop producing new viral proteins or genomes and to engage in packaging of progeny viruses. The transcription inhibition is in many studies attributed to the RNA-binding motif of M1 (Perez & Donis, 1998; Watanabe *et al*., 1996; Ye *et al*., 1989), which on the other hand is pin-pointed to the nuclear localization signal RKLKR of the protein (Elster *et al*., 1997) (orthomyxoviruses replicate in the nucleus). There is also a report suggesting that a zinc finger-containing domain of M1 is able to bind RNA (Ye *et al*., 1989). Peptides encompassing this zinc finger are potent inhibitors of viral transcription *in vitro* and also harbor antiviral activity when introduced into infected cells (Nasser *et al*., 1996; Wang *et al*., 2011).

The influenza M1 is not designated for budding, and VLPs can form in the absence of M1 (Chen *et al*., 2007). In contrast, the matrix protein Z of arenaviruses, like many other matrix proteins of enveloped RNA viruses, is essential for virus budding (Perez *et al*.,

2003; Perez *et al*., 2004). Many arenavirus Z proteins also contain proline-rich late domains that mediate budding of VLPs (Perez *et al*., 2003) and it can bud into VLPs even in the absence of other viral proteins. It recruits the nucleoprotein into progeny viruses presumably by direct protein-protein interactions (Capul *et al*., 2011; Casabona *et al*., 2009; Eichler *et al*., 2004; Groseth *et al*., 2010). It is of interest to note that tacaribe arenavirus (TACV) Z protein does not have the conventional proline-rich late domain like many other arenaviral Z proteins and, therefore, cannot bud efficiently. However, VLP formation of this virus is greatly enhanced by co-expression of the nucleoprotein and this phenomenon is dependent on YxxL motif (similar to what is found in hantaviruses) in Z protein (Groseth *et al*., 2010). Like influenza M1, the Z proteins inhibit viral RNA synthesis. This has been shown *in vivo* with reverse genetics systems and in infected cells that express Z proteins transiently (Cornu & de la Torre, 2001; Cornu *et al*., 2004; Lopez *et al*., 2001). For TACV Z protein, the ability of Z protein to bind the viral polymerase has been shown to be essential for the inhibitory effect of RNA synthesis (Jacamo *et al*., 2003; Wilda *et al*., 2008). Like influenza matrix protein and most bunyaviral Gn-CTs the Z protein contains a zinc finger domain. However, the Z protein zinc finger is different from the others, and it is predicted to fold into a RING finger structure (Djavani *et al*., 1997). Cellular homologues with RING finger structures function as E3 ubiquitin ligases (Matthews & Sunde, 2002), but this activity has not been detected for the RING finger domain of Z protein. Conversely it is shown to be required for packing of RNPs into VLPs (Casabona *et al*., 2009) and for the repression RNA synthesis by partially mediating interaction with the polymerase (Cornu & de la Torre, 2001; Jacamo *et al*., 2003). Cysteine-alkylating zinc finger inactivating chemicals are shown to block arenavirus infectivity when applied to virions prior to virus adherence to the host cells (Garcia *et al*., 2000; Garcia *et al*., 2002; Garcia *et al*., 2006; Garcia *et al*., 2009). This indicates that the RING finger of Z is required in early stages of arenavirus infectivity similarly to what has been shown for retroviruses in which cysteine-alkylating chemicals target the zinc finger of the viral nucleocapsid and presumably block the post-entry reverse transcription step (Guo *et al*., 2000; Johnson *et al*., 2000; Rice *et al*., 1993; Rice *et al*., 1995). The only evidence for the requirement of Z protein for arenavirus transcription comes from a study in which Z protein depletion from infected cells markedly reduced mRNA transcription when the depletion was performed in the early stage of infection (Garcin *et al*., 1993).

For bunyaviruses that lack a matrix protein these above-mentioned functions need to be executed by other viral proteins and a most likely candidates for this are the CTs of glycoproteins. When comparing the functions of matrix proteins between orthomyxoviruses and arenaviruses it is evident that they differ to large extent even between themselves but some analogies to bunyaviral Gn-CTs can be detected. Firstly, predictions suggest that all members of family *Bunyaviridae* except for phleboviruses harbor a ZF, which has been shown in the case of orthomyxo- and arenaviruses to inhibit viral RNA synthesis. This is probably a signal for the polymerase to switch from replication to progeny virus formation. The findings that bunyaviral ZFs can bind RNA and N protein certainly support the idea that the ZFs share a crucial function in packaging of all NSRVs. Secondly, sequences resembling late domains of arenaviral Z protein, which are responsible for the recruitment of a cellular budding apparatus to the site of virus budding, can be predicted also in the sequences of CTs of bunyaviruses (e.g. the totally conserved YxxL in hantaviruses).

1.3 Hantavirus pathogenesis

1.3.1 Clinical picture of hantavirus-mediated diseases

Hantaviruses cause HFRS in Eurasia (old-world hantaviruses) or HCPS in the Americas (new-world hantaviruses). In general, the incubation period between virus encounter through rodent excreta and the first signs of a disease is around three weeks. The severity of HFRS depends on the causative virus and can be divided into severe (*Hantaan virus*; HTNV), moderate (*Seoul virus*; SEOV) or mild (*Puumala virus*; PUUV) disease. The clinical course of HFRS can be separated into febrile, hypotensive, oliguric, diuretic and convalescent stages. However, it is not uncommon that these different stages are indistinguishable and especially with milder forms of HFRS some stages can also be absent. The febrile phase is manifested with fever, headache and backache with frequent hemorrhages (Table 1). After approximately 4 days it progresses to the hypotensive phase, which can last from hours to two days, where the symptoms intensify and nausea together with vomiting are more common. In the most severe cases, the hypotensive phase can already develop to fatal shock. The subsequent oliguric phase (1 to 16 days)

accounts for about half of the deaths seen in HFRS, which are mostly due to renal insufficiency, shock and hemorrhages. Dialysis is sometimes required at this stage. Patients who progress into the polyuric stage show improved renal function, and full recovery is achieved during the convalescent phase, which can last for months (Jonsson *et al*., 2010; Schmaljohn & Hjelle, 1997).

In Finland, there are 2000-3000 cases of NE (mild HFRS) diagnosed annually. There is considerable variability in the disease manifestation from subclinical to lethal infections (mortality 0.1%), and it is believed that most infections are subclinical. Genetic susceptibility studies in Finnish population indicated a major histocompatibility complex haplotype (human leukocyte antigen-B8-DR3) as an important risk factor of a more severe course of NE (Makela *et al*., 2002; Mustonen *et al*., 1996). The actual mechanism of how this genetic variability of individuals affects the disease outcome is unknown, but interestingly the same haplotype is associated with many autoimmune diseases (Price *et al*., 1999).

In contrast to HFRS with disease incidence of up to 50,000 each year in Eurasia, there have been only around 500 cases of HCPS in North America altogether but dramatically with over 40% mortality. In South America the incidence rate of HCPS seems to be higher than in North America but the mortality rate of South American HCPS in is not well-defined. The North American HCPS is mainly caused by SNV and is manifested by a rapid onset of respiratory failure and cardiogenic shock. A similar febrile phase that is associated with HFRS can be identified also in HCPS as well as laboratory findings of thrombocytopenia and leukocytosis (Table 1) but a major difference is that vascular leakage affects predominantly lungs and in contrast to HFRS kidneys are largely unaffected. The average duration of illness is 3-4 days prior to admission, which after death often occurs in 1-3 days (Jonsson *et al*., 2010; Peters *et al*., 1999; Schmaljohn & Hjelle, 1997). In general, the allocation of hantaviruses to old- and new-world hantaviruses with different and clear-cut disease manifestations is somewhat obscure since predominant renal involvement has also been occasionally associated in HCPS caused by other new-world hantaviruses than SNV and also in one case of SNV-caused HCPS (Passaro *et al*., 2001). On the other hand, pulmonary involvement has been recognized in PUUV-infected patients (Kanerva *et al*., 1996; Rasmuson *et al*., 2011). One striking difference between HCPS and HFRS is that even mild HFRS is frequently associated with eye pain or myopia (especially in PUUV-infection) whereas in HCPS this manifestation is not encountered at all (Schmaljohn & Hjelle, 1997).

Table 1. Symptoms and laboratory findings of hantavirus-associated diseases (% of patients)

disease	HTNV severe HFRS	SEOV moderate HFRS	PUUV mild HFRS (NE)	SNV HCPS
Symptoms				
fever	100	100	98	95-100
nausea	72-82	61	58-84	71
cough	44	14	14-32	71
headache	83-86	42-89	62-90	71
backache	94-95	85-90	54-82	29
abdominal pain	23-25	65	43-67	24
myalgia	69-93	52-73	20-68	100
dizziness	41-100	7-52	9-33	41
diarrhea	11-39	24-40	12-20	59
hemorrhage	31-77	26-48	12-37	0
Laboratory findings				
thrombocytopenia	54-100	70-100	50-80	98
leukocytosis	88-92	41-69	23-57	76
proteinuria	96	94	94-100	40
hematuria	27-86	27-73	58-85	57
oliguria	59-74	37-46	54-83	n.d
polyuria	87-92	63	96-97	n.d
Mortality	5-15	< 1	< 0,1	> 40

Data are deduced from Kim *et al*., 1995 and Lee, 1989 in the case of HTNV and SEOV; Lahdevirta, 1971, Mustonen *et al*., 1994 and Settergren *et al*., 1989 in the case of PUUV; and Duchin *et al*., 1994 and Khan *et al*., 1996 in the case of SNV. n.d. = not determined.

1.3.2 Vascular leakage

The hallmark of hantavirus-caused HFRS and HCPS is vascular permeability, which in the case of HFRS leads to microvascular hemorrhage often associated with kidney failure and in the case of HCPS to acute pulmonary edema. The endothelial cells lining the walls of the microvasculature are known to be the prime targets of the virus. Although there is no evidence for cytopathic effects (CPE) either in the infected endothelium *in vivo* or in experimental infections of endothelial cell cultures (Chen & Cosgriff, 2000; Mackow & Gavrilovskaya, 2009) several causes for the increased vascular permeability in HFRS and HCPS have been suggested (see later sections). The studies of hantavirus pathogenesis have been greatly hampered by the lack of appropriate animal models. Infection of Syrian hamsters with ANDV induces a disease resembling HCPS in humans and could,

therefore, be a model virus for animal studies. However, SNV is not infectible in hamsters (Hooper *et al*., 2001; McElroy *et al*., 2004; Wahl-Jensen *et al*., 2007).

1.3.2.1 Thrombocytopenia

Acute thrombocytopenia (low platelet counts; Table 1) is a common feature to both HFRS and HCPS. Since platelet function is required for normal blood coagulation and inhibition of internal hemorrhage due to vascular injury, thrombocytopenia may be directly involved in pathogenesis of hantavirus-caused diseases. Thrombocytopenia can be caused either by diminished platelet production due to defects in mature megakaryocytes or abnormally high consumption of platelets in the bloodstream. Partly because increased levels of normally functioning megakaryocytes and partly because platelets with low survival times have been detected in HFRS-patients, it is believed that the low platelet counts associated with hantavirus-mediated diseases are due to their excessive consumption (Chen & Cosgriff, 2000). One reason for the excessive consumption of coagulation factors (e.g. platelets) is disseminated intravascular coagulation (DIC) but this is only present in the more severe cases of HFRS (Chen & Cosgriff, 2000). Since thrombocytopenia is a common finding in hantavirus-infected patients (Table 1), other explanations for its development are needed. Recently, it was demonstrated that endothelial cell cultures infected with pathogenic hantaviruses adhere quiescent platelets to their surface (Gavrilovskaya *et al*., 2010). The interaction between platelets and the infected-cell surface can be explained mechanistically by the fact that platelets express integrin β_3, which is shown to be a receptor for pathogenic hantaviruses (see 1.2.2.1), on their cell surface and could, therefore, bind directly to endothelial cell surface-expressed viral glycoproteins (Mackow & Gavrilovskaya, 2009). A different explanation for the high platelet consumption could be the presence of circulating virus-specific immune complexes, which were shown to be attached to platelets during the onset of HFRS (Chen & Cosgriff, 2000). Immune complexes bind platelets either directly or via virus, which are attached to the circulating platelets through integrin β_3 (Gavrilovskaya *et al*., 2010). Immune complex-bound platelets may then be destined for destruction by complement attack or phagocytosis (Chen & Cosgriff, 2000). Thrombocytopenia is also detected in NE, where systemic hemorrhage is not that common (Table 1). This suggests that while thrombocytopenia could be a contributing

factor to hantavirus pathogenesis, it is definitely not the only reason for the development of the disease.

1.3.2.2 Cytokine storm

In addition to thrombocytopenia, another common feature of HFRS and HCPS pathogenesis is a widespread activation of pro-inflammatory responses sometimes referred to as "cytokine storm" (Mackow & Gavrilovskaya, 2009). It involves marked up-regulation of various cytokines that could have detrimental effects on microvascular functions. Up-regulation of TNF-α seems to be one factor that correlates with disease severity (Chen & Cosgriff, 2000; Linderholm *et al*., 1996b; Mori *et al*., 1999). By analyzing genetic susceptibilities to more severe hantavirus-mediated diseases an association with TNF-α gene (TNF2 gene) polymorphism was detected. Individuals with the TNF2 allele have a stronger TNF-α promoter and thus higher levels of TNF-α production (Borges *et al*., 2010; Kanerva *et al*., 1998; Maes *et al*., 2006; Makela *et al*., 2001). The persistence of SEOV in rat host may be due to lower level of pro-inflammatory responses to infection as compared to humans (Easterbrook *et al*., 2007; Easterbrook & Klein, 2008), and more specifically the persistence of PUUV in bank voles in endemic areas may be due to TNF-α promoter mutations resulting in lower level of TNF-α production in the rodents (Guivier *et al*., 2010).

Interleukin (IL)-6 is another cytokine with high importance in hantavirus pathogenesis. IL-6 is invariably up-regulated in hantavirus-associated diseases (Klingstrom *et al*., 2002; Linderholm *et al*., 1996b; Mori *et al*., 1999), and has been linked to a more severe disease outcome in both NE and HCPS (Borges *et al*., 2008; Makela *et al*., 2004; Outinen *et al*., 2010). Besides having direct effects on vascular endothelium, TNF-α and IL-6 are known to induce the production of nitric oxides (NO). Elevated levels of NO have been detected in HCPS (Davis *et al*., 2002) and NE (Klingstrom *et al*., 2002; Linderholm *et al*., 1996a) and they have negative, inotropic effects on cardiac functions (Finkel *et al*., 1992). Increased NO could play a role in the development of hypotension by promoting cardiac dysfunction detected in HCPS (Hallin *et al*., 1996; Saggioro *et al*., 2007) and also in NE albeit at a clearly lower level (Makela *et al*., 2009).

1.3.2.3 Immune system

It is well established that immune responses play a role in the pathogenesis of HFRS. The severity of HTNV-caused HFRS clearly correlates with increased amounts of virus-specific immune complexes peaking at the hypotensive phase of the syndrome. Immune complexes activate complement, which can promote vascular leakage by activating cytokine-producing monocytes or by direct attack on the endothelium (Chen & Cosgriff, 2000). The level of complement activation is associated with severity of NE also in PUUV-infected patients, but conflicting results have been obtained indicating that either the classical (Paakkala *et al*., 2000) or the alternative route (Sane *et al*., 2011) act as the primary mode of complement activation. A recent finding indicates that elevated levels of a Mac-2-binding protein (Mac-2-BP; 90K) correlate with complement activation and, thus, disease severity in PUUV-infected patients. Interestingly the same protein has been suggested to bind hantaviruses through glycoprotein Gn (Hepojoki *et al*., personal communication). This is a protein that possesses immunostimulatory activity (Ullrich *et al*., 1994), but its significance in hantavirus pathogenesis is still unknown.

Cytotoxic CD8-positive T cells (CTLs) may also play a role in hantavirus pathogenesis especially in HCPS. The amount of CTLs correlate with the severity of HCPS (Kilpatrick *et al*., 2004) and are also elevated in the acute phase of HFRS (Chen & Yang, 1990; Tuuminen *et al*., 2007). There is genetic susceptibility to more severe HCPS or NE, namely HLA-B*3501 or HLA-B8-DR3 haplotypes, respectively (Kilpatrick *et al*., 2004; Mustonen *et al*., 1996). These haplotypes may have a link the more severe disease outcome via antigen presentation and, thereby, possibly to the activation of CTLs. Another factor that may have an impact on the levels of CTLs is down-regulation of immunosuppressant transforming growth factor (TGF)-β, which is observed in HCPS (Borges *et al*., 2008). The elevated amounts of CTLs could increase vascular leakage in patients (Terajima *et al*., 2007) as has been shown *in vitro* for hantavirus-infected endothelial cells (Hayasaka *et al*., 2007). On the other hand, it was recently shown that proliferating immune cells (T and B cells) do not contribute to ANDV pathogenesis in Syrian hamsters (Hammerbeck & Hooper, 2011).

1.3.2.4 Direct virus infection-caused dysregulation of endothelial cell functions

Recent evidence shows that ANDV-infection in capillary lung endothelial cells causes an increase in cellular permeability that is exerted through up-regulation of vascular endothelial growth factor (VEGF), which subsequently promotes down-regulation of vascular endothelial (VE)-Cadherin, a major component of endothelial cell adherent junctions (Shrivastava-Ranjan *et al*., 2010). It has also been shown that exogenous VEGF increases the permeability of pathogenic hantavirus-infected human umbilical vein endothelial cells (HUVECs) through VE-Cadherin down-regulation (Gavrilovskaya *et al*., 2008; Gorbunova *et al*., 2010; Gorbunova *et al*., 2011). Interestingly, pharmacological inhibitors to VEGFR2 and Src kinase inhibit permeabilization of VEGF-induced, virus-infected endothelial cells (Gorbunova *et al*., 2011). It has been proposed that the VEGF-induced vascular hyperpermeability facilitated by hantavirus infection is due to inactivation of β_3 integrins by the virus and subsequent deregulation of VEGFR2 on the cell surface (Mackow & Gavrilovskaya, 2009).

1.3.2.5 Integrins

It has been suggested that pathogenic and non-pathogenic hantaviruses differ in their receptor usage by binding β_3 or β_1 integrins, respectively (Gavrilovskaya *et al*., 1999; Gavrilovskaya *et al*., 2002). If this is the case, it underlines the role of β_3 integrin in hantavirus pathogenesis. SNV has been found to bind human and hamster but not murine or bovine β_3 integrins (Matthys *et al*., 2010) making it possible that hantavirus persistence in rodents could be due to their inability to regulate the activity of rodent β_3 integrin. As discussed above, the inhibition of β_3 integrin functions by hantaviruses could be linked at least to thrombocytopenia and direct endothelial cell permeabilization by virus infection. However, the β_3 interaction does not explain why even pathogenic hantaviruses dramatically differ in their potential to cause disease in man. On the other hand, while TULV is often considered non-pathogenic and is suggested to have β_1 integrin specificity (Gavrilovskaya *et al*., 2002); there is an implication linking it to a mild HFRS-type disease (Klempa *et al*., 2003) making the clear-cut categorization of hantaviruses to pathogenic and non-pathogenic viruses with different receptor usage somewhat obscure. Furthermore, the pathogenesis of ANDV in Syrian hamsters is not attributed to its glycoproteins since a reassortant virus containing L and S segments of ANDV and M segment of SNV (non-pathogenic in Syrian hamsters) was still capable of causing the

disease (McElroy *et al*., 2004). This result is contradictory to the idea that vascular leakage caused by hantaviruses could be due to virus glycoprotein-mediated regulation of integrins.

1.3.3 Renal failure in NE

Kidney dysfunction is the hallmark of HFRS but is not often found in HCPS. Proteinuria is almost always present in HFRS-patients and can be caused either by lowered glomerular filtration rate or damage in the proximal tubular cells of the kidneys. Both of these cases seem to contribute to acute renal failure in NE (Ala-Houhala *et al*., 2002; Settergren *et al*., 1990). The tubular proteinuria is even found to persist years after the acute disease (Miettinen *et al*., 2009). Tubulointerstitial nephritis involving cytokines (e.g. TNF-α) and cell infiltrates is typical in NE (Mustonen *et al*., 1994; Temonen *et al*., 1996). Interestingly, NE can be mimicked in a macaque model to a great extent where degeneration of the tubular epithelial cells co-localize with PUUV antigens and virus-specific CD8-positive T cells (Klingstrom *et al*., 2002; Sironen *et al*., 2008). The role of CTLs in the pathogenesis of NE is also suggested by the fact that there is up-regulation of perforin, granzyme B and markers for epithelial cell apoptosis in serum of acute NE-patients (Klingstrom *et al*., 2006). The detection of hantavirus antigens in tubular epithelial cells is not restricted to animal models since both HTNV and PUUV antigens have been found in renal tubular cells of HFRS-patients (Groen *et al*., 1996; Hautala *et al*., 2002; Kim *et al*., 1993; Sironen *et al*., 2008) strengthening the idea that they may directly cause degenerative effects or attract T cells to attack the renal tubular system. A recent study indicated that pathogenic old-world hantaviruses can infect, in addition to tubular epithelial cells, also glomerular endothelial cells and podocytes of human kidney, and disrupt cell-to-cell contacts in all of these cell types (Krautkramer *et al*., 2011). This was suggested to decrease the barrier functions of the kidney and be therefore the direct cause of proteinuria.

As previously discussed, hantaviruses do not readily cause CPE in cellular monolayers *in vitro* (Hardestam *et al*., 2005). However, in some kidney epithelial cell lines (Vero E6 and HEK293) apoptosis has been reported in response to hantavirus infection (Kang *et al*., 1999; Markotic *et al*., 2003). The best studied virus in this respect is TULV, which

causes apoptosis in Vero E6 cells through a mechanism involving ER stress, caspase-8 activation and Poly(ADP-ribose) polymerase **(**PARP**)** cleavage (Li *et al*., 2004; Li *et al*., 2005). It was also shown that exogenously applied TNF-α elevates the amount of N protein and apoptosis in infected cells (Li *et al*., 2004). TNF-α is a pro-inflammatory cytokine known to exert its functions through up-regulation of Nuclear factor (NF)-κB transcriptional regulator and causes apoptosis in cells where normal NF-κB activation is impaired (Liu, 2005). Intriguingly, transiently expressed hantaviral N protein is capable of blocking TNF-α mediated NF-κB activation (Taylor *et al*., 2009a; Taylor *et al*., 2009b). The N protein does not cause apoptosis by itself even in a TNF-α treated cells, but in the natural virus infection, in the presence of ER stress, or during possible up-regulation of other pro-apoptotic factors, the situation may be different. In SEOV-infected macrophages of rat origin NF-κB mediated responses are blocked, which in that context was suggested to be one of the mechanism how the virus counteracts pro-inflammatory state of the host in order to promote the persistence in its natural reservoir (Au *et al*., 2010). Taken together renal dysfunction associated with hantavirus-infections involves epithelial cell damage, which may occur via virus-induced apoptosis or induced the recruitment of CTLs to the tubular epithelium.

1.3.4 Innate immunity responses

Innate immunity is highly important in combating virus infections. Interferons (IFN) are cytokines that stimulate anti-viral state of host cells by up-regulating various genes related to restriction of viral replication. Of these genes MxA is probably of highest importance. IFNs also promote humoral and cellular immunity of the host organism, which generally counteract virus infections. Although immune responses contribute to hantavirus pathogenesis (1.3.2.3), efficient immune responses are necessary for the host to achieve virus clearance. Infected cells use pattern recognition receptors (PRRs) to detect the invading virus and to induce signaling pathways leading to e.g. type I IFN (IFN-α and -β) production (Brennan & Bowie, 2010). Viruses need to inhibit these pathways in order to be viable, and hantaviruses make no exception to this rule. All hantaviruses studied so far induce type 1 IFNs in infected cells but they vary in the extent and duration of IFN production and subsequent innate immunity response. Clearly, non-

pathogenic PHV differs from other hantaviruses in its inability to block early IFN production, which leads to early restriction of PHV replication in immune-competent cells (Alff *et al*., 2006; Geimonen *et al*., 2002; Handke *et al*., 2009; Matthys *et al*., 2011; Spiropoulou *et al*., 2007). Furthermore, TULV is a more potent inducer of MxA expression than HTNV in HUVECs (Kraus *et al*., 2004), suggesting that the ability of hantaviruses to inhibit innate immunity may actually relate to their various degrees of pathogenicity.

The viral determinants resulting in inhibition of innate immunity have been widely sought thereafter. The CT of hantaviral Gn, when transiently expressed, has been associated with inhibition of IFN regulatory factor 3 (IRF-3)-mediated IFN-β production. This effect has been shown for Gn-CTs of NY-1V and TULV, but not for PHV, and is attributed to the C-terminal part of the protein (Alff *et al*., 2006; Alff *et al*., 2008; Matthys *et al*., 2011). A possible drawback of the studies involving transient expression of Gn-CT is its localization in cells that may drastically differ from wild-type Gn in infected cells, although transient expression of full-length glycoprotein precursor in the case of SNV has also been shown to block IFN-β induction (Levine *et al*., 2010). The innate immunity induction in studies involving Gn-CT was achieved by over-expressing Retinoic acid receptor I (RIG-I). RIG-I is a PRR that recognizes double-stranded RNA or tri-phosphorylated termini of RNA molecules. The hantaviral genomic RNA termini are monophoshorylated (Garcin *et al*., 1995, Wang *et al*., 2011) and instead of genomic RNA it was found that double-stranded secondary structures in N protein ORF RNA are responsible for activation of RIG-I during hantavirus replication (Lee *et al.*, 2011). In any case, recent evidence indicates that TULV Gn-CT could also block IFN-β production stimulated by other factors than RIG-I (Wang *et al.*, personal communication). The same group that performed the initial studies showing that Gn-CT inhibits innate immunity also reported that the Gn-CT of pathogenic hantaviruses are destined for degradation by the proteasome; a phenomenon, which may have relevance in viral antigen presentation by infected cells to the immune system (Geimonen *et al*., 2003a; Matthys *et al*., 2011; Sen *et al*., 2007). However, a later study showed that the Gn-CTs of the less virulent PHV and TULV are also degraded (Wang *et al*., 2009). Degradation was shown to be dependent on the C-terminal transmembrane domain, which is proposed to act as signal sequence for Gc. In addition to glycoproteins, a nonstructural protein expressed from the S segment (NSs) of PUUV and TULV can also inhibit the IFN-β promoter (Jaaskelainen *et al*.,

2007). Furthermore, hantavirus infection or transient expression of its glycoproteins can block later stages of innate immunity following IFN-β production (Levine *et al*., 2010; Spiropoulou *et al*., 2007).

Hantaviruses are typically propagated in Vero E6 cells prior to their usage in downstream experiments. Vero E6 cells are considered immuno-deficient since they carry a defect in their type 1 IFN genes (Diaz *et al*., 1988; Mosca & Pitha, 1986), which is a likely reason for their high susceptibility to hantavirus (and other virus) infections. However, recent studies have shown that the supernatants of hantavirus-infected Vero E6 cells contain, in addition to infectious viruses, also type III IFNs (IFN-λ), which can elicit similar antiviral responses than type 1 IFNs. An IFN-λ mediated antiviral response was shown in epithelial cells which were infected with Vero E6-propagated hantaviruses. However, a similar response to hantavirus-containing supernatants was not detected in endothelial cells since these cells lack the IFN-λ receptor (Prescott *et al*., 2010). This finding makes the studies and conclusions of induction of innate immunity by hantaviruses at least in epithelial cells debatable. The validity of research around hantavirus-induced innate immunity is also debated by the fact that different sub-strains of PUUV are remarkably different in eliciting innate immunity induction in cells, which indicates that spontaneous mutations in the viral genome obtained through passaging of the viruses in cell cultures are likely to affect the outcome of immune responses (Sundstrom *et al*., 2011)

2 Aims of the study

- To study the signaling pathways involved in TULV-induced apoptosis and to investigate the potential of other hantaviruses to cause apoptosis *in vitro*
- To identify and characterize the interactions between hantaviral glycoproteins Gn and Gc with ribonucleoprotein
- To study the role of free thiols in hantavirus entry into host cells
- To explore the functions of the zinc finger domain in Gn glycoprotein during virus entry and assembly

3 Materials and methods

3.1 Materials

3.1.1 Cell cultures

Vero E6 cells (green monkey kidney epithelial cell line; ATCC: CRL-1586) and BHK-21 (baby hamster kidney cell line; ATCC: CCL-10) were grown in minimal essential medium supplemented with 10% or 2% heat-inactivated fetal calf serum, respectively, and 2 mM glutamine, 100 IU/ml of penicillin and 100 µg/ml of streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

3.1.2 Viruses

TULV (Moravia strain 5302), TOPV, SEOV and PUUV (Sotkamo strain) were propagated in Vero E6 cells at titers of 10^4-10^7 focus forming units (FFU)/ml conditioned medium. Viral titers in supernatants of infected cells were determined as previously described (Kallio *et al*., 2006). Briefly, 10-fold diluted supernatants were applied onto Vero E6 cells in a 10-well microscopic slide and stained with virus-specific antibodies 3 days post infection (d.p.i.). To radiolabel the viral proteins, infected Vero E6 cell cultures were starved for 1 h at 37 °C in methionine- and cysteine-depleted medium, and propagated with a 1 mCi mixture of [^{35}S]-cysteine and [^{35}S]-methionine (Wallac Perkin-Elmer) for 3 days at 37 °C. For purification and concentration of viruses, cell culture medium passed through a 0.22 µm filter (Millipore) was concentrated by pelleting through a 30% (w/v) sucrose cushion (27,000 r.p.m., 2 h, 4 °C, in a Beckman SW28 rotor) and suspended in TBS (50 mM Tris pH 7.5, 150 mM NaCl) or HBS (25 mM HEPES pH 7.5, 150 mM NaCl). UUKV was propagated in BHK-21 cells, conditioned medium was collected at 3 d.p.i. and stored in aliquots at –70 °C for later use. UUKV stocks (~10^8 plaque forming units {p.f.u.}/ml) were diluted 10^4-fold for inactivation analysis with thiol-reacting reagents in BHK-21 cells or tenfold in Vero E6 cells.

3.1.3 Peptide synthesis

N-terminally biotinylated Gn_N, Gn_M, Gn_C and Gc-CT peptides together with the ZF domain (Table 2) were synthesized in a 25 µmol scale on a MultiPep synthesizer according to the manufacturer's protocol (Intavis AG) with standard Fmoc chemistry. The full-length Gn-CT peptide was synthesized using Applied Biosystems peptide synthesizer 433A with Fmoc chemistry. Salt removal from peptides was done in a C18 reversed-phase column (Supelco Discovery; Wide Pore) using a steep 0–70 % acetonitrile–H_2O gradient in 0.1 % TFA, and by collecting the main peak. The fraction containing the peptide of interest was verified by mass spectrometry (MALDI-TOF; AutoFlex III, Bruker) for Gn_N, Gn_M, Gn_C and Gc-CT. The correct size of Gn-CT peptide was verified by Tris/Tricine SDS-PAGE (result not shown).

Table 2. Sequences of peptides used in this study.

Peptide name	Peptide sequence
Gn-CT	SKYNTDSKFRILVEKVKKEYQKTMGSMVCEVCQYECETAKELESHRKSCSIGSCPYCLNPSEATPSALQAHFKVCKLTSR ENLKKSLTMYEPMQGCYRTLSLFRYRSR
GnN	KVKKEYQKTMGSMVCEVC
GnM	QAHFKVCKLTSRFQENLKK
GnC	EPMQGCYRTLSLFRYRS
ZF	MVCEVCQYECETAKELESHRKSCSIGSCPYCLNPSEATPSALQAHFKVCKL
Gc-CT	PRRPSYKKDHKP

3.1.4 Peptide array synthesis

MultiPep synthesizer was used for synthesis of SPOT peptide arrays by Fmoc chemistry according to the manufacturer's instructions. Hantavirus Gn-CTs were synthesized on an amino-functionalized cellulose membrane as 16- or 18-residue long overlapping peptides with a three-residue shift. The 18 carboxyl-terminal residues containing the predicted Gc-CT were also synthesized. Sequences corresponding to Gn-CTs of TULV and PUUV were also synthesized in CelluSpot™ format as 20-residue long peptides on MultiPep (Intavis Ag) and the slides were printed with SlideSpotter (Intavis Ag), all according to the manufacturer's instructions as described (Beutling *et al*., 2008).

3.1.5 Protein synthesis

The N protein of PUUV and TULV was expressed and labelled radioactively ([^{35}S]methionine; Wallac, Perkin-Elmer) in rabbit reticulocyte lysates (TnT Quick; Promega) using plasmids harbouring the virus-specific S segment under the T7 promoter. Recombinant, baculovirus-expressed PUUV N protein was a kind gift from Reagena Ltd., Finland. The RNPs of PUUV and TULV were purified by gel permeation from virions pelleted through a sucrose cushion as described recently (Hepojoki *et al*., 2010). The Gn-CTs of PUUV and TULV were expressed as glutathione S-transferase (GST) fusion proteins in *E. coli* according to protocols of the manufacturer (GST Gene Fusion System Handbook, Amersham Bioscience 18-1157-58 Edition AA). The expression plasmids for GST-tagged Gn-CTs were constructed by inserting respective Gn-CT fragments (residues 526-637 of PUUV and 521-632 of TULV) into the pGEX-4T-3 vector (GE Healthcare). GST-fusion proteins were purified by glutathione (GSH) Sepharose 4B beads (Amersham Pharmacia, GE Healthcare).

3.1.6 Nucleic acid synthesis

The genomic PUUV S segment was *in vitro* transcribed (TranscriptAid™ T7 High Yield Transcription Kit; MBI Fermentas) from a pGEM-T plasmid containing the respective sequence information under a T7 promoter. The PUUV S segment together with an unrelated RNA (unRNA) of similar size (included in TranscriptAid™ T7 High Yield Transcription Kit; MBI Fermentas) were radioactively labeled with ^{32}P-UTP (Wallac PerkinElmer) during transcription and purified using Tripure (Roche). The 42-mer single-stranded DNA represented the 3´-end of the genomic PUUV S segment: 5´-TCCAGACTTTCTCGTAGTAGCTTTTCAAGGAGTCTACTACTA-3´. An IRdye800-moiety was conjugated to the 5´-end of the oligonucleotide, which was synthesized by Oligomer Oy, Finland.

3.1.7 Antibodies

PUUV-neutralizing bank vole mAbs 5A2 (Gn-specific) and 4G2 (Gc-specific), human mAb 1C9 (Gc-specific) and bank vole mAbs 5E1 and 3C11 (N-specific) have been described previously (Lundkvist & Niklasson, 1992; Lundkvist *et al*., 1993; Lundkvist *et al*., 1996a; Lundkvist *et al*., 1996b) and were kindly provided by Professor Åke Lundkvist, Swedish Institute for Infectious Disease Control and Karolinska Institutet, Stockholm, Sweden. Polyclonal antisera raised against GST fusion proteins of PUUV Gn, Gc and N have been described previously (Vapalahti *et al*., 1995). A mixture of rabbit polyclonal antibodies K3 and K4, recognizing UUKV N protein (Kuismanen *et al*., 1982), was kindly provided by Anna Katz (Department of Virology, University of Helsinki, Finland). Mouse monoclonal antibodies against phosphorylated form of epidermal growth factor receptor (EGFR; cat. # sc-12351) and extracellular signal-regulated kinase (ERK) 1/2 (cat. # sc-7383) were from Santa Cruz Biotechnology Inc. Mouse monoclonal antibody against cleaved poly-ADP ribose polymerase (PARP; cat. # 92679546) and rabbit polyclonal antibodies against ERK1/2 (Cat. # 9102) and phosphorylated Akt (Cat. # 9267) were from Cell Signaling Technology. The anti-HA mAb was from Abcam. The mouse mAb against actin was from Sigma and the IRDye800-conjugated streptavidin and secondary antibodies were from LI-COR. Alexa Fluor 680-conjugated secondary antibodies were from Invitrogen.

3.1.8 Reagents

Recombinant human tumor necrosis factor-α (TNF-α) was from R&D Systems. Thiol-reacting reagents N-ethylmaleimide (NEM) and iodocatemide (IAA) were from Sigma. Biotin-malemide (B-mal) and 5, 5-dithiobis-(2-nitrobenzoic) acid (DTNB) were from Thermo Scientific and Calbiochem, respectively. Micrococcal nuclease (MNase) and RNase A were from MBI Fermentas.

3.2 Methods

3.2.1 Immunoblotting

Viruses were adhered to confluent cell monolayers for 1 h at 37 ºC. Infected and mock-infected cells were scraped off into medium, washed twice with phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 3 mM EDTA, 1% NP-40, and EDTA-free cocktail of protease inhibitors (Roche). For analysis of protein phosphorylation, RIPA was supplemented with 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4 and 20 mM NaF. The protein concentrations of the cell lysates were determined using BCA Protein Assay Kit (Pierce). Laemmli gel loading buffer was added into samples, which were denatured at 95 °C for 5 min and stored at -20 °C. Samples were analyzed by immunoblotting according to standard protocols using SDS-PAGE and detection by enhanced chemiluminescence or infrared-dye based detection system (Odyssey, Li-COR).

3.2.2 Immunoprecipitation

Immunoprecipitations were performed using lysates of purified PUUV and TULV as the source for viral proteins. MAbs were used at 10 µg of antibody and polyclonal antisera were used at 20 µl of serum per 5 µl of virus preparation. After overnight incubation at 4 °C or 1 h at RT in an end-over-end shaker, pull-down of immune-complexes was carried out for 2 h with protein G–Sepharose beads (Amersham). Precipitated proteins were separated by SDS-PAGE and detected by immunoblotting or alternatively by autoradiography when radioactive samples were used. When exogenously applied radiolabeled RNA was used substrate for immunoprecipitated proteins, its binding to proteins was detected directly from the beads by scintillation counting.

3.2.3 Pull-down assays

CT-N interactions: The Gn-CT peptide was coupled to thiopropyl Sepharose 6B (Amersham Pharmacia) through free thiol groups and peptides Gn_N, $Gn_{M,}$ Gn_C and Gc-CT were coupled to monomeric avidin beads (Pierce Biotechnology) through their biotin-moiety. The recombinant N or native RNP was incubated overnight in end-over-end shaking at 4 °C with 10 µl of peptide-coupled or control beads in 500 µl HBS. Laemmli sample buffer was added, samples boiled and separated by SDS-PAGE. The precipitated N protein was detected by either immunoblotting or autoradiography.

CT-RNA interactions: The glutathione beads-coated GST-fusion proteins and avidin beads-coated biotinylated peptides were used in the pull-down experiments of the radiolabeled S segment RNA (400 CPM) or unRNA (500 CPM). After 1 h incubation at RT and subsequent washes the amount of bead-bound RNA was detected by scintillation counting. For the binding of DNA, the IRdye800-conjugated oligonucleotide (100 nM) was incubated for 1 h at RT and after two washes the bead-bound DNA was eluted by boiling in Laemmli sample buffer. 1 µl of LSB eluate (corresponding to 1 µl bead volume) was dot-blotted on nitrocellulose, cross-linked by UV and the amount of IRdye800-conjugated oligonucleotide quantified using Odyssey.

Virus-cell interactions: To assess virus attachment to host cells, confluent Vero E6 monolayers were incubated with radiolabeled TULV for 30 min at 37 °C. Cells were washed twice with PBS and lysed in RIPA buffer for 15 min RT. The cell-bound material was separated by SDS-PAGE and the radiolabeled viral proteins were detected by autoradiography.

3.2.4 Virus inactivation

Virus preparations were incubated with NEM, B-mal, IAA or DTNB for 1 h at RT. In the case of NEM and IAA treatments, the residual reagent reactivity was quenched with 5 mM GSH for 10 min at RT. UV-inactivation of stock virus was performed on ice in a lid-less 3 cm diameter culture dish, which was irradiated at 254 nm using a 30 W UV lamp at a distance of 10 cm with an exposure time of 30 min.

3.2.5 Sedimentation analysis

The conventional medium of TULV-infected Vero E6 cells was changed to growth medium supplemented with 2.5% FCS (pre-filtered through a 100 kDa cut-off filter; Millipore) at 3 d.p.i.. At 5 d.p.i., the conditioned medium was concentrated approximately 100-fold. Viruses were sedimented (25,000 r.p.m., 16 h, +4 °C, in a Beckman SW41 rotor) in a 0–70% sucrose gradient (in 10 mM HEPES pH 7.4, 100 mM NaCl) and the gradient was collected from the bottom in ~600 µl fractions. The protein composition of fractions was determined by non-reducing SDS-PAGE with silver staining (PageSilver silver staining kit; Fermentas). The sucrose concentration of each fraction was determined at 22 °C using a manual Zeiss Opton Abbe refractometer.

3.2.6 Cross-linking of RNA to proteins

Lysates of purified PUUV or TULV were treated with 0.1 U/µl MNase in 50 mM Tris-HCl pH 7.5 with 5 mM $CaCl_2$ for 1 h at 37 °C. MNase was inactivated with 10 mM ethylene glycol tetra-acetic acid (EGTA) for 15 min at RT prior to addition of ^{32}P-labeled RNA. After 2 h incubation at RT the RNAs were cross-linked to proteins under a UV lamp (similarly to virus inactivation) and RNA cleaved with 10 µg RNase A for 1 h at 37 °C. The enzyme was inactivated by addition of Laemmli sample buffer, samples boiled and proteins separated on 6% SDS-PAGE. To visualize RNA-interacting proteins, the gels were subjected to autoradiography or immunoblotting.

3.2.7 Probing peptide arrays

The SPOT peptide array membrane was probed with bacN or PUUV lysate overnight at 4 °C, washed and peptide-bound proteins detected by N protein-specific mAb 5E1. All SPOT peptide reactions were recorded on X-ray film by enhanced chemiluminescence. The CelluSPOT glass slides were probed with IRdye800-conjugated DNA for 1 h at RT, washed, and bound DNA was detected with Odyssey.

3.2.8 Flow cytometry

To quantify the proportion of apoptotic cells, infected and mock-infected Vero E6 cells (grown in 25 cm^2 flasks) were scraped off with a rubber policeman), washed once with PBS, fixed and stored in 70% ethanol at -20 °C. Cells were washed once with PBS and incubated with 10 mg/ml RNase A for 10 min at RT. To stain DNA, propidium iodide (PI) was added at 25 µg/ml in PBS and samples were stored at +4°C overnight. Cell cycle and apoptosis analysis was performed with 20,000 cells by FACSarray flowcytometer (BD Biosciences) and the resulting data was analyzed by FACSdiva software (BD Biosciences) at the FACS core facility, Biomedicum Helsinki. The equivocal low intensity cell debris was not included in the analysis.

3.2.9 Surface plasmon resonance

Peptide-N protein interactions were analyzed by surface plasmon resonance with Biacore 2000™ (Biacore, GE Healthcare). The CT-peptide was coupled to a CM5 sensor chip either through cysteine or lysine residues and biotin-conjugated peptides were captured on streptavidin coated sensor chips (SA-chip) following manufacturer's recommendation. The levels of peptide coating were comparable in molecular density of 5000 response units (RU) for CT and 1000 RU for biotin-conjugated peptides. The bacN protein diluted in running buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 1 mM EDTA) was used as analyte at various concentrations (20-1000 nM). In the case of ZF-peptide running buffer devoid of EDTA and supplemented with 100 µM $ZnCl_2$ was used. Sensorgrams obtained from empty reference channel were subtracted from the sensorgrams obtained from peptide-coated channels.

3.2.10 UV/visible spectroscopy

The ZF-peptide at 70 µM in 50 mM Tris-HCl (pH 7.0) was mixed with excess of Co(II) (1 mM $CoCl_2$). After one minute in Co(II), sulfhydryl-reactive chemicals NEM or IAA (1 mM) or alternatively EDTA (2 mM) as a control were added, and after reaction time of

one minute absorption spectrum of 550-750 nm was recorded in Ultrospec 3000 spectrometer (Amersham-Pharmacia). The NEM and IAA concentrations were two-fold to cysteine residues in the ZF-peptide.

4 Results and discussion

4.1 Regulation of cell survival by hantaviruses (I)

4.1.1 Background

Hantavirus infections are not commonly associated with cytopathic effects. However, signs/symptoms of tubular epithelial cell degeneration is seen in kidneys of NE-patients and in monkeys with NE-like diseases, which could be the underlying cause of acute renal failure associated with this disease. Previous studies in our laboratory showed that TULV, supposedly non-pathogenic hantavirus, induces apoptosis in Vero E6 cells (Li *et al*., 2004; Li *et al*., 2005). Apoptosis was confirmed by cell cycle inhibition, caspase activation, DNA fragmentation and PARP cleavage. In the present study (paper I) the molecular mechanisms of TULV-induced apoptosis were investigated. In addition, since apoptosis has thus far only been detected in TULV-infected cells, the cell death-inducing potential of a panel of different hantaviruses including NE-causing PUUV, HFRS-causing SEOV and the apparently non-pathogenic TOPV was studied.

4.1.2 TULV regulates ERK1/2 cell survival pathway

In the present study the effects of hantavirus replication on the activity of extracellular signal-regulated kinases (ERKs), which are ubiquitous proteins belonging to the mitogen-activated protein kinase (MAPK)-family and constituents of Ras/Raf/MAPK signaling pathway were investigated. ERKs consist of two homologous isoforms ERK1 and ERK2 with molecular weights of 44 and 42 kDa, respectively (referred hereafter as ERK1/2). The ERK1/2 activation proceeds through its phosphorylation and is commonly involved in cell proliferation and cell survival whereas its inhibition may lead to cell death (Mebratu & Tesfaigzi, 2009; Ramos, 2008). ERK1/2 activity was assayed in TULV-infected Vero E6 cells by immunoblotting using antibodies specific for its phoshorylated form. In addition, apoptosis was analyzed by the extent of PARP cleavage and virus replication by N protein expression levels both with immunoblotting. Virion production

was quantified from the conditioned cell culture supernatant with FFU assay (I, Fig. 1a). Reduced activity of ERK1/2 in response to TULV was observed that correlated with increasing multiplicity of infection (MOI). As judged by PARP cleavage, apoptosis was also induced by the high MOI of virus. However, the reduced activity of ERK1/2 was not a consequence of apoptosis since it was also observed in the absence of apoptosis when low MOIs were used. These results showed that TULV is capable of inhibiting cell survival pathways in Vero E6 cells and that this is likely to be associated with its potential to induce apoptosis. The link between apoptosis and inhibition of ERK1/2 phosphorylation was substantiated in non-infected Vero E6 cells by the use of apoptosis-inducing concentration of exogenous TNF-α (100 ng/ml), which in addition to inducing PARP cleavage also inhibited ERK1/2 activity (I, Fig. 1b). Moreover, UV-inactivated TULV did not inhibit ERK1/2 activity showing that its regulation by TULV is probably replication-dependent (I, Fig. 2). This was further alleviated by the observation that ERK1/2 inactivation by TULV seemed to be reversible; decreased replication of TULV by prolonged infection resulted in concomitant relief of TULV-mediated ERK1/2 inhibition (I, Fig. 2).

4.1.3 Regulation of ERK1/2 and induction of apoptosis by TOPV, SEOV and PUUV

Having established that ERK1/2 can be used as a marker of cell survival in hantavirus-infected cells the ability of TOPV, SEOV and PUUV to cause ERK1/2 down-regulation in Vero E6 cells were tested (I, Fig. 3). TOPV and SEOV inhibited ERK1/2 only at a relatively low level and PUUV did not inhibit ERK1/2 at all. However, the amount of ERK1/2 inhibition with TOPV and SEOV directly correlated with the level of virion production similarly to TULV (I, Fig. 2). TULV differed from other hantaviruses also in that it caused marked CPE as observed by light microscopy (not shown) with concomitant total ERK1/2 inactivation in highly prolonged infection of 25 days. The observed CPE was most probably a result of extensive apoptosis in TULV-infected cells. Since PARP cleavage was not repeatedly detected in cells infected with TOPV, SEOV or PUUV; these cells were also analyzed by staining with propidium iodide (PI). PI is a fluorescent dye, which binds the cellular genomic DNA facilitating quantitative analysis of DNA fragmentation and thus apoptosis by flow cytometry. By this method, apoptosis was

evident in TOPV- and SEOV-infected cells but not in mock- or PUUV-infected cells (unpublished results, Fig. 6), consistent with the earlier observed inhibition of ERK1/2 by TOPV and SEOV. Taken together these results demonstrated that also other hantaviruses than TULV can cause apoptosis in Vero E6 cells accompanied by ERK1/2 down-regulation. However, it seems that only TULV is capable of inducing massive 100% apoptosis, which is easily detected by light microscopy.

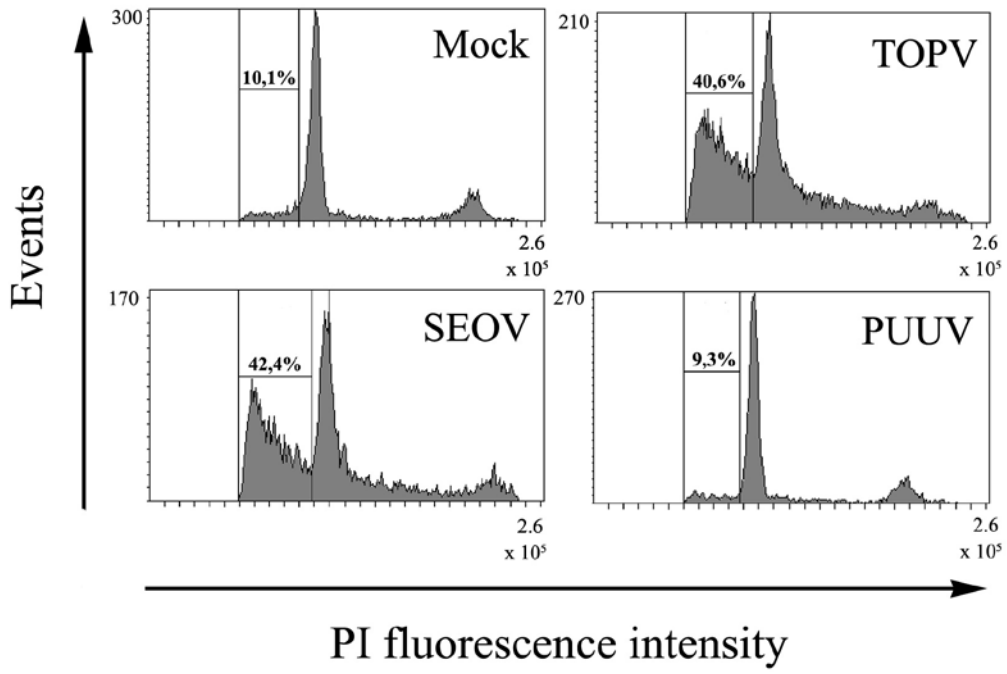


Figure 6. Cell cycle analysis of hantavirus-infected Vero E6 cells. Cells were either mock-infected or infected with TOPV, SEOV or PUUV and collected 21 d.p.i. for propidium iodide (PI) staining and flow cytometric analysis. The amount of cells in sub-G1 phase correspond to apoptotic cell population and are indicated as percentages of total cell population.

When comparing the ability of hantaviruses to produce infectious virions it was evident that TULV was produced about 100-times more efficiently than TOPV, SEOV or PUUV (I, Fig. 3b). This suggested that the amount and kinetics of virus replication is likely to play a significant role in hantavirus-induced apoptosis. It is possible that also other hantaviruses than TULV are capable of inducing more pronounced apoptosis if they

replicated more efficiently. The reason for the observed differences in replication efficiencies of various hantaviruses is not currently known.

4.1.4 The effect of TNF-α on hantavirus-induced ERK1/2 inhibition

It has been shown that application of exogenous TNF-α augments TULV-induced apoptosis (Li *et al*., 2004). The levels of TNF-α correlate with disease severity in hantavirus-infected patients (Chen & Cosgriff, 2000; Linderholm *et al*., 1996b; Mori *et al*., 1999), and its expression has been detected in the kidneys of NE-patients (Temonen *et al*., 1996). For this reason the effects of TNF-α in respect to ERK1/2 activity in hantavirus-infected Vero E6 cells was investigated. Hantavirus-infected and mock-infected cells were cultured in the presence of low amounts of TNF-α (20 ng/ml) and after 14 d.p.i. and 25 d.p.i. there was a dramatic inhibition of ERK1/2 in response to TNF-α treatment and virus infection (I, Fig. 4). The degree of inhibition correlated with replication-efficiency of hantaviruses (I, Figs. 3 and 4) with the exception of PUUV-infected cells in which ERK1/2 inhibition was elevated with TNF-α even when virion production decreased in prolonged infection (25 days). These results corroborate the earlier findings with TNF-α in which this cytokine had detrimental effects on the viability of hantavirus-infected cells.

4.1.5 TULV regulates also EGFR and Akt (unpublished results)

ERK1/2 is involved in a ubiquitous signaling cascade, which can be activated by various stimuli (Ramos, 2008). One of the upstream activators of ERK1/2 is EGFR, a plasma membrane receptor that belongs to the family of receptor protein tyrosine kinases. Like ERK1/2, the activity of EGFR can be followed by analyzing the extent of its phosphorylation by immunoblotting. This was performed in TULV-infected Vero E6 cells and down-regulation of EGFR activity in response to TULV was detected (unpublished results, Fig. 7). The TULV-induced EGFR inactivation took place concomitantly with the inhibition of ERK1/2 suggesting that ERK1/2 activity is dependent on EGFR. In addition to the Ras/Raf/MAPK pathway, which involves ERK1/2, EGFR can also activate a phospatidylinosine 3-kinase (PI3K)/Akt pathway,

which also promotes cell survival (Lurje & Lenz, 2009). Activity of this pathway in TULV-infected cells was studied by analysing phosphorylated Akt by immunoblotting (Fig. 7). Similarly to ERK1/2 and EGFR, inhibition of Akt in TULV-infected cells was detected (4 d.p.i.). These results indicate that hantaviruses can down-regulate at least two common cell survival signaling pathways via EGFR.

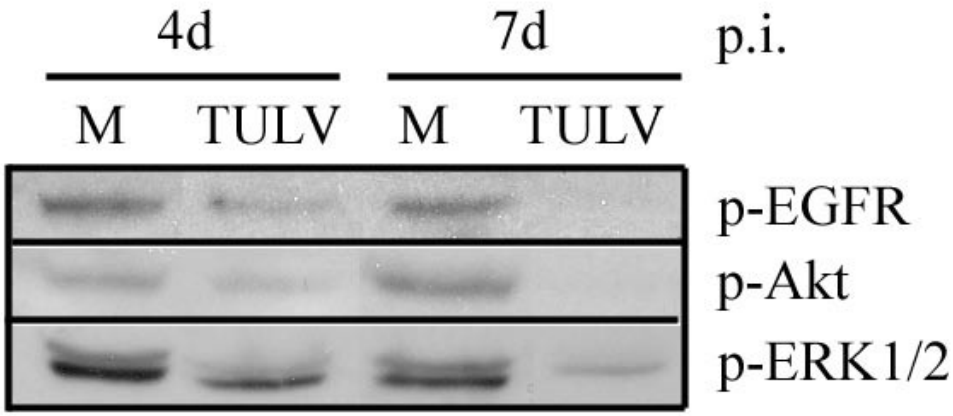


Figure 7. Regulation of EGFR and Akt by TULV. Vero E6 cells were TULV- or mock-infected (M) for 4 or 7 days. Cells were lysed and the same total amount of proteins was subjected to immunoblotting by antibodies that recognize phosphorylated (activated) forms of EGFR, Akt and ERK1/2.

4.1.6 Does anoikis play a role in TULV-induced apoptosis?

Non-transformed cells are only viable when anchored on a supportive extracellular matrix. Integrins are cell surface adhesion proteins responsible for cell attachment and play an indispensable part in cell survival through intracellular signaling in response to adhesion. Integrins form a complex with EGFR on the plasma membrane and activate EGFR-mediated downstream signaling cascades Ras/Raf/MAPK and PI3K/Akt to promote cell proliferation and survival. This type of activity has been shown for many different integrins, including hantaviral receptors $\alpha_V\beta_3$ and $\alpha_5\beta_1$ (Alexi *et al.*, 2011; Cabodi *et al.*, 2004). When the cells detach from the extracellular matrix, the pro-survival pathways are not activated and cells die due to anoikis, which is a form of apoptosis (Chiarugi & Giannoni, 2008). Hantaviruses have a preference towards inactive, bent form of integrins (Raymond *et al.*, 2005) and it is highly intriguing to speculate whether the

released virions disrupt the pre-formed integrin-extracellular matrix or integrin-EGFR complexes on the infected Vero E6 cell surface. This can lead to the observed inactivation of cell survival pathways and eventually to anoikis (Fig. 8). The involvement of anoikis in TULV-infected cells is supported by observations that up-regulation of apoptosis markers correlates with cell detachment from the supportive matrix (personal observation). Therefore, it is possible that in addition to ER stress, which has previously been shown to be involved in TULV-induced apoptosis (Li *et al*., 2005), anoikis may also play a role in this phenomenon.

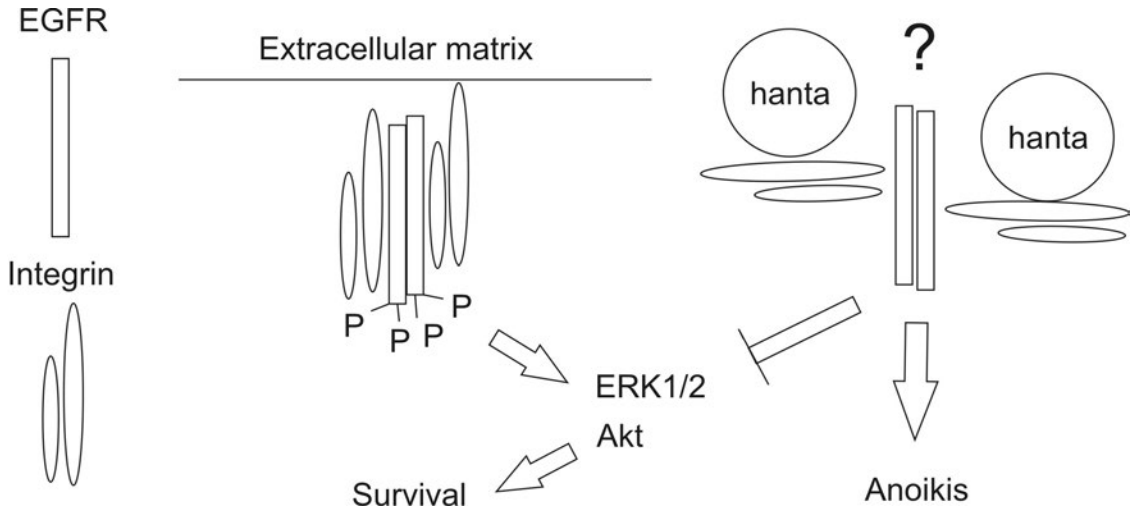


Figure 8. Anoikis in TULV-infected cells. Adherent cells survive only when they are attached to extracellular matrix through integrin receptors that exert pro-survival signals upon adhesion into the cell through EGFR activation. Hantaviruses bind to the inactive form of integrins thereby blocking the pro-survival signaling cascade resulting cell detachment and death (anoikis). P stands for phosphorylation of tyrosine residues.

4.1.7 Does hantavirus-induced apoptosis occur *in vivo*?

Monkeys infected with PUUV develop NE-like symptoms with kidney failure (Sironen *et al*., 2008). Tubular epithelial cell degeneration co-localized with PUUV antigen indicating that PUUV has a role in the formation of NE symptoms in monkey. Analysis of acute NE kidney biopsies by cleaved PARP immunohistochemistry revealed more frequent tubular cell apoptosis in NE compared to patients with other kidney diseases (Li, 2005). While the amount of samples in the latter study was low and no statistically

significant conclusions could be drawn from it, it is possible that apoptosis plays a role in the pathogenesis of NE. However, in addition to direct virus replication-caused apoptosis (enhanced by TNF-α), also cytolytic T cells could be responsible for the cell death observed *in vivo* as suggested by Sironen *et al*. (2008).

4.2 The interaction of hantavirus glycoprotein-CTs with RNP (II, IV)

4.2.1 Background

Many enveloped viruses encode a matrix protein, which acts as a bridge between the surface glycoproteins and the genomic core of the virus, thus playing an indispensable part in the proper assembly of infectious virions (Timmins *et al*., 2004). However, bunyaviruses do not have a matrix protein and, therefore, the interaction between the genomic RNA-containing ribonucleoprotein complex (RNP) and the inner surface of the viral envelope is thought to take place through the cytoplasmic tails of the surface glycoproteins Gn and Gc (see Fig. 1). In the case of hantaviruses this putative interaction is also supported by electron cryo-tomography studies (Battisti *et al*., 2010; Huiskonen *et al*., 2010). Besides that, a direct interaction between glycoprotein CTs and RNP or N protein has already been shown for many other bunyaviruses (Overby *et al*., 2007a; Piper *et al*., 2011; Ribeiro *et al*., 2009; Snippe *et al*., 2007). Furthermore, Gn-CTs of RVFV and CCHFV have been suggested to directly bind genomic RNA (Estrada & De Guzman, 2011; Piper *et al*., 2011). In papers II and IV, interactions of hantavirus glycoproteins with native RNP, recombinant N protein and nucleic acids were analyzed.

4.2.2 Native Gn interacts with RNP through its CT (II)

Conformation-dependent PUUV glycoprotein specific mAbs 5A2 (specific for Gn) and 4G2 (specific for Gc) were used to identify interactions between glycoproteins and RNP in purified virions and RNP was found to be immunoprecipitated by both mAbs (II, Fig. 1). Co-precipitation of Gn and Gc with both mAbs was also detected, which is not surprising since these proteins are known to form a complex in native virions (Hepojoki

et al., 2010). However, due to this reason, it was impossible to conclude whether RNP prefers either of the glycoproteins.

To be biologically relevant, the RNP-binding activity of Gn and Gc should be retained in their cytoplasmic tails. To study this, full-length 110 and 10 amino acid CTs of PUUV Gn and Gc, respectively, were synthesized with standard peptide synthesis chemistry. Their ability to bind isolated native RNP or recombinant *in vitro* translated N protein of PUUV and TULV was assessed in a pull-down assay where Gn-CT was coupled to thiopropyl beads through thiol-coupling and Gc-CT to avidin-beads through its biotinylated N-terminus (II, Fig. 5). Both CTs could bind RNP and recombinant N protein of PUUV and TULV origin. Furthermore, to study the involvement of CTs in the native interaction between glycoproteins and RNP in virions, the co-immunoprecipitation assay (II, Fig. 1) was performed in the presence of the CT-peptides (II, Fig. 6). Gn-CT, but not Gc-CT, was able to out-compete the native glycoprotein-RNP interaction showing that Gn-CT is absolutely required for this interaction.

4.2.3 Gn-CT binds nucleic acids (IV)

In paper II it was shown that the CTs of glycoproteins Gn and Gc can bind RNP and N protein. The RNP consists of not only N protein but also genomic RNA and it has recently been suggested that Gn-CT of RVFV and CCHFV can, in fact, directly bind genomic RNA (Estrada & De Guzman, 2011; Piper *et al*., 2011). Due to these reasons, the RNA-binding ability of Gn- and Gc-CTs was studied (paper IV). The 5A2 and 4G2 glycoprotein-specific mAbs together with pAbs against Gn and Gc were used to analyze RNA-binding potential of the PUUV glycoproteins by immunoprecipitation. The purified virus was nuclease-treated and incubated with radiolabeled *in vitro* transcribed S segment of PUUV prior to immuprecipitation. Highest amounts of precipitated RNA were obtained by Gn-specific antibodies indicating that Gn can recognize genomic RNA (IV, Fig. 3b). The RNA-binding ability of purified TULV proteins was also analyzed by using cross-reactive PUUV pAbs (IV, Fig. 3a). However, instead of using PUUV S segment RNA, an unrelated RNA was used as the substrate for TULV (IV, Fig. 3c). The TULV Gn was capable of binding to unrelated RNA indicating that the RNA-binding activity of hantavirus Gn is non-specific, i.e. it is not dependent on a specific RNA sequence or

structure. In both of these experiments N protein-specific antibodies were used as positive control for RNA binding, but surprisingly there was no significant precipitation of RNA with these antibodies. This is probably due to the nuclease-treatment of the sample, which could render N protein unrecognizable by antibodies. To assess the RNA-binding activity of hantaviral proteins in a more direct way, the applied radiolabeled RNAs were cross-linked to virus proteins and proteins separated in SDS-PAGE. The RNA-bound proteins were identified by autoradiography and immunoblotting (IV, Figs 1 and 2). Radioactive bands overlapped with Gn and N protein, but not with Gc, in immunoblots, thus confirming the ability of Gn and N to bind RNA.

Having established that full-length Gn can bind RNA, the next step was to analyze the RNA-binding activity of Gn-CTs. GST-fused recombinant Gn-CTs of PUUV and TULV were expressed in *E. coli* and studied for their interaction with the radiolabeled PUUV S segment RNA and unrelated RNA by GST pull-down. Also, an IRdye800-conjugated single stranded 42-mer DNA was included for comparison (IV, Fig. 4). All nucleic acids bound to both CTs but not to GST alone confirming the RNA-binding activity of Gn-CT. In addition, these results showed that Gn-CT can also bind relatively short DNA that alleviates the nonspecific nature of its interaction with nucleic acids.

4.2.4 Mapping the binding sites of Gn-CT towards RNP, N protein and nucleic acids (II and IV)

As discussed in section 1.2.4 hantaviral Gn-CT harbours some conserved regions, i.e. the ZF domain and endocytosis motif YxxL, that might exert functions important for virion assembly. Therefore it was of interest to determine the binding sites of Gn- and Gc-CT to RNP, N protein and nucleic acids. The binding sites for RNP and recombinant baculovirus-expressed PUUV N protein (bacN) were analyzed by SPOT peptide arrays. The arrays consisted of 16- to 18-mer peptides covering the whole sequence of Gn-CT with a 3-residue overlap. The Gn-CTs of PUUV, TULV, PHV and NY-1V together with Gc-CTs of multiple hantaviruses were synthesized on a cellulose membrane and probed either with PUUV lysate or bacN. The binding of N protein in both cases was detected by N protein antibodies and enhanced chemiluminescence. Four different binding domains in all Gn-CT sequences were initially detected towards PUUV RNP and bacN. These

domains were located on either side adjacently to the ZF domain, in the middle of the ZF and in the C-terminus of Gn-CT (II, Fig. 2 and 5). The ability of Gc-CTs to bind N protein was also confirmed. The residues important for the RNP interaction in each binding domain of PUUV CTs, except for the one that resides in ZF domain, was further analyzed by peptide mutagenesis and deletions. The binding site in ZF was not included since the whole ZF domain was analyzed separately. Firstly, each individual residue of a chosen parent peptide corresponding to one binding domain was mutated to alanine. Secondly, one by one amino acid deletions from either side of the peptide were applied for all except the C-terminal binding peptide Gn-CT. The peptides were synthesized as SPOT peptide arrays, probed with PUUV lysate and RNP binding detected as previously (II, Fig. 3). Individual residues important for the RNP interaction could be identified and which are highlighted in the Gn-CT alignment (II, Fig. 4). Positively charged residues were often important for RNP binding indicating a possible involvement of the negatively charged nucleic acid backbone in the interaction. The same peptides, which bound RNP, were also shown to bind bacN. However, it is not known whether the bacN preparation is absolutely pure of nucleic acids and whether the N protein exists as a complex with non-viral RNA, which may have co-purified with the protein during its extraction from cells.

To identify the binding sites of PUUV and TULV Gn-CT towards nucleic acids, a CelluSpot peptide array with the same general principle as in the SPOT peptide arrays was employed but where the peptides were first synthesized in soluble form and later printed on glass slides in array format. The advantages of this methodology over the conventional SPOT array technique are the possibility to get multiple identical arrays from one round of peptide synthesis and miniaturization of the assay platform. The IRdye800-conjugated nucleic acid previously detected to bind Gn-CTs was used as probe, and in the case of PUUV Gn-CT three different binding sites that localized similarly to the previously determined binding sites for RNP were identified (IV, Fig. 5a). In the case of TULV, however, only C-terminal peptides were found to have affinity towards nucleic acids. Furthermore this binding region extended beyond the C-terminal RNP binding site, which was identified for PUUV Gn-CT, and towards the N-terminus of the protein (IV, Fig. 6). In all, it seems that the C-terminal part of hantaviral Gn-CT harbours a conserved nucleic acid binding site, which overlaps with the RNP binding site.

4.2.5 The binding of Gn-CT to RNP, N protein and nucleic acids is primarily mediated by its C-terminal part (II and IV)

Peptide arrays are sensitive for false positive results due to high concentration of peptides in a single SPOT and lack of conformational and positional stringency asserted by the natural biological context of a peptide. Therefore, the aim was to verify the mapping data obtained by peptide arrays with soluble peptides and recombinant proteins. Three peptides of PUUV Gn-CT identified as RNP binding peptides were synthesized in soluble form (designated Gn_N, Gn_M and Gn_C; see Table 2) together with the ZF domain. Surface plasmon resonance assay in which the peptides were immobilized to a Biacore sensor chip and bacN was used as the analyte in liquid phase was performed (unpublished results, Fig. 9). A concentration-dependent interaction of PUUV to all peptides except the ZF was observed. First of all this result confirmed that the peptides, which were identified by SPOT arrays as N-binding peptides, were functional also in this type of assay where peptide concentration on the chip surface was probably much lower than in the individual SPOTs. This result also showed that the ZF domain is unable to bind N protein. This was not due to inability of the peptide to bind zinc-ions (see section 4.3). This result contradicts the study which suggested that the interaction between Gn-CT and N proteins was mediated through an intact ZF domain (Wang *et al*., 2010). However, our peptide mapping data showed that the N protein can interact with Gn-CT through binding sites adjacent to the ZF and which were not present in ZF peptide (Table 2). It is, therefore, possible that the folding of the ZF domain is important for the binding of N protein possibly by bringing the binding sites in Gn-CT closer to each other.

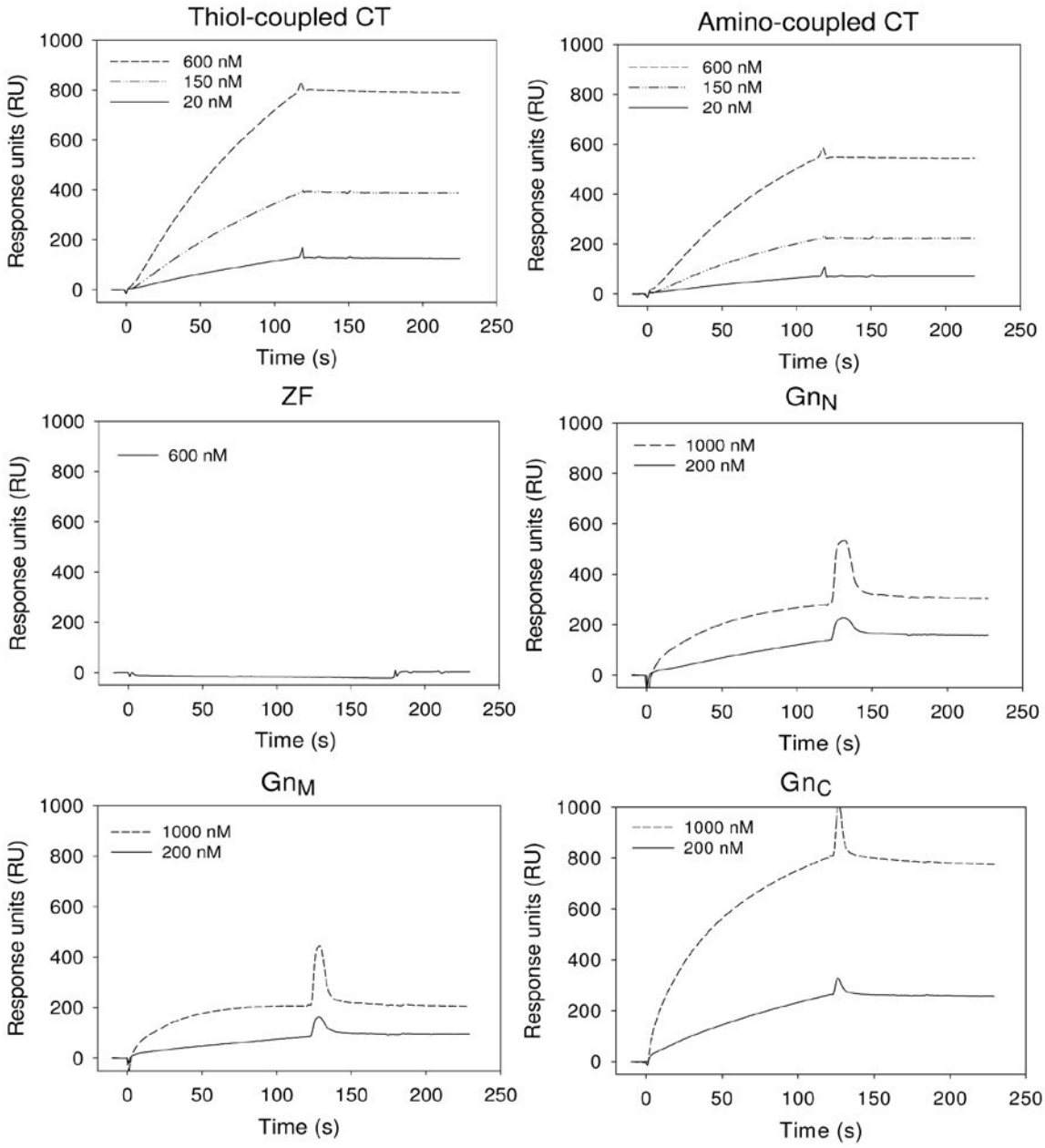


Figure 9**.** Binding of Gn-derived peptides to N protein as measured by surface plasmon resonance assay. The Gn-CT was coupled to a sensor chip either through free thiols or free amines. The biotin-conjugated Gn_N, Gn_M, Gn_C and ZF peptides were coupled to a streptavidin-coated sensor chip. Baculovirus-expressed N protein was injected over the sensor chips at indicated concentrations. The sensograms presented here were obtained by subtracting their signal from an empty reference flow cell.

To analyze the binding capabilities of Gn_N, Gn_M and Gn_C peptides further, they were coupled to avidin-beads through their biotinylated N-terminus prior to performing pull-down assays with isolated RNP, *in vitro* translated N protein and nucleic acids. All

peptides bound the recombinant N protein of PUUV and TULV, and Gn_C showed the highest affinity followed by Gn_M and Gn_N (II, Fig. 5). The peptides were found to bind nucleic acids with similar relative affinity as observed for N protein (IV, Fig. 5b). However, in the case of RNP binding, Gn_M was the weakest binder of PUUV RNP and could not bind TULV RNP at all. In contrast, Gn_N showed RNP binding levels comparable to Gn_C. The reason for the differences between the binding of N protein and RNP to Gn_M and Gn_N is unknown but the results suggest distinct mechanisms of how Gn-CT recognizes N and RNP. It is possible that genomic RNA mediates the binding of RNP to Gn-CT either directly or by inducing conformational changes in the native N protein.

For TULV Gn-CT, a GST-tagged recombinant protein lacking the C-terminal part of Gn-CT including the nucleic acid binding region and the putative endocytosis motif YxxL was expressed (IV, Figs 6 and 7b). In pull-down assay, the RNA-binding of this deletion construct was observed to be completely abolished; whereas approximately 50% remained of its DNA-binding activity as compared to the full-length Gn-CT (IV, Fig. 7c). The differences in RNA and DNA binding may be explained by the different sizes of the applied nucleic acids. It is possible that the internal binding sites of Gn-CT, located adjacent to the ZF, are inaccessible for the larger RNA molecules. These binding sites were only detected for PUUV but they could also be involved in the Gn-nucleic acid interaction of TULV. Taken together, these results suggest that the C-terminal part of Gn-CT has the highest affinity towards native RNP, N protein and nucleic acids.

4.2.6 Significance of the interaction of Gn-CT with RNP and nucleic acids in virus life cycle

The peptide mapping results indicated that Gn-CT of hantaviruses can bind nucleic acids independently of N protein but through the same binding sites. Since the recombinant N protein preparations used in these assays may also contain nucleic acids associated with the N protein, it is possible that the observed interaction of Gn-CT with N protein and possibly also RNP is mediated solely by nucleic acids. Therefore, the involvement of nonspecific nucleic acid-mediated binding event in the native Gn-RNP interaction was analyzed using the previously described co-immunoprecipitation assay. The virus was treated with nucleases prior to immunoprecipitation or alternatively the

immunoprecipitation was performed in the presence of excess DNA (unpublished results, Fig. 10). In both cases the RNP binding activity of Gn was preserved indicating that although Gn binds nucleic acids nonspecifically, this is not solely responsible for its interaction with RNP.

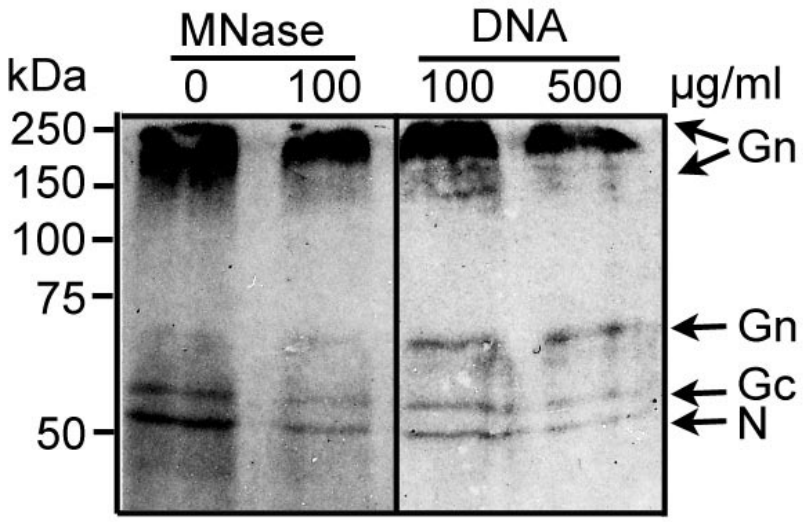


Figure 10. Involvement of nucleic acids in Gn-RNP interaction. Purified radiolabeled PUUV was either treated with micrococcal nuclease (MNase) prior to immunoprecipitation with 5A2 or alternatively immunoprecipitation was performed in the presence of extensive amounts of a 42-mer single-stranded DNA. The precipitated proteins were separated in SDS-PAGE and detected by autoradiography according to their typical migration pattern.

Matrix proteins of NSRVs have been shown to inhibit viral RNA synthesis. In the case of influenza M1 matrix protein, this is mediated through its RNA binding motifs (Perez & Donis, 1998; Watanabe *et al*., 1996; Ye *et al*., 1989). Like discussed in section 1.2.4, bunyaviruses do not encode matrix protein, and Gn-CT (at least in the case of hantaviruses) is thought to substitute for its activity. Therefore, it is intriguing to speculate whether the hantaviral Gn-CT actually regulates viral transcription or replication. Inhibition of viral RNA synthesis would act as a signal for the viral polymerase or the RNP complex to engage in packaging and assembly of progeny virions rather than producing more viral proteins or genomes (Fig. 11). The ZF domain of Gn-CT may play the major role in this putative function, similarly to influenza M1 and arenavirus Z proteins (Cornu & de la Torre, 2001; Nasser *et al*., 1996). In the case of Z

protein, no RNA-binding motifs have been identified in the protein but instead its ZF have been shown to bind the viral polymerase (Jacamo *et al.*, 2003).

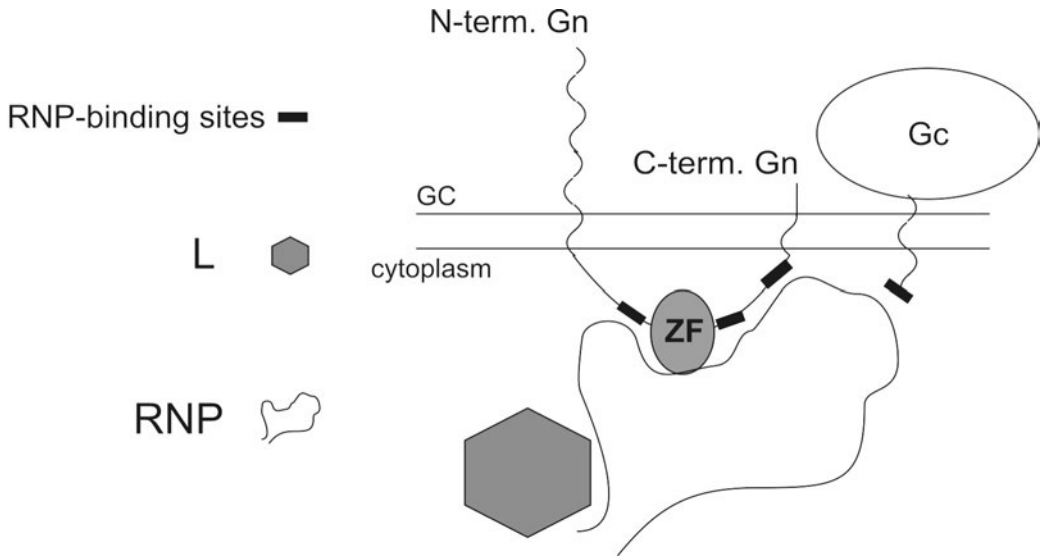


Figure 11. Hypothetical scheme of the packaging of hantavirus RNP into progeny viruses. The CTs of Gn and Gc form a matrix-type proteinaceous layer underneath the viral envelope in the virions or at Golgi membranes (GC) in the infected cells to which viral RNPs bind. The L protein is associated with RNPs but is inactive due to RNP-CT interaction.

In order to produce infectious viruses, hantaviruses need to pack at least one copy of each of the three differently-sized RNPs into a single virion. The Gn-CT of TULV seemed to be interchangeable to PUUV Gn-CT in its binding ability towards RNP, N protein and nucleic acids of PUUV origin. In addition, according to SPOT peptide arrays, even Gn-CTs of HCPS-causing viruses (e.g. NY-1V) can bind PUUV RNP and bacN. This cross-species binding suggests that the interaction between Gn-CT and RNP is rather nonspecific; i.e. there is no specific packaging signal in the individual RNPs and that the CTs could possibly interact with all the three viral RNPs independently. The robustness of Gn-RNP interaction unorthodoxically implies that complementary RNPs (cRNPs) encapsidating positive-stranded viral RNAs are being packaged together with the viral RNPs (vRNPs) into progeny virions. However, it is possible that the vRNPs are expressed in high excess over cRNPs in infected cells and, therefore, no differentiation of these complexes by CTs is needed in order to produce highly infectious virions. The nonspecific nature of the CT-RNP interaction can result in virions with multiple copies of

individual segments but evidence for this is lacking. However, the high variability in size and shape of purified TULV (Huiskonen *et al*., 2010) is indicative of inconsistent numbers of RNPs in the virion.

4.3 Hantavirus infectivity is dependent on free thiols (III)

4.3.1 Background

The biological functions of the ZF domain located in hantavirus Gn-CT remains unknown. In addition to the possible role of the ZF in virus assembly its role in hantavirus infectivity was addressed (paper III). This is because retroviruses possess a similar dual CCHC-type ZF in their nucleocapsid protein, and alkylation of the thiols that coordinate zinc-ions results in complete retrovirus inactivation (Arthur *et al*., 1998; Chertova *et al*., 2003; Jenkins *et al*., 2005; Morcock *et al*., 2005; Morcock *et al*., 2008; Musah, 2004; Rein *et al*., 1996; Rice *et al*., 1993; Rice *et al*., 1995; Rossio *et al*., 1998). In addition, a similar observation has been made for arenaviruses in which the RING finger domain of its Z protein is likely to be required for infectivity (Garcia *et al*., 2000; Garcia *et al*., 2002; Garcia *et al*., 2006; Garcia *et al*., 2009). In these two cases the studied thiols are located inside the virus envelope but, on the other hand, thiols located on the surface of viral glycoproteins can also be exploited in virus entry. They can be substrates for virally- or cell-encoded protein disulfide isomerases (PDIs) that act to re-organize disulfide linkages in viral glycoproteins in order to facilitate viral entry (Sanders, 2000). The active site of PDIs resides in the CxxC motif, which is also found in hantavirus Gc adjacent to its proposed fusion peptide (Cifuentes-Munoz *et al*., 2011; Garry & Garry, 2004; Tischler *et al*., 2005).

4.3.2 Hantaviruses are inactivated by thiol-blocking reagents

N-ethylmaleimide (NEM) and 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) were used to probe the role of thiols in hantavirus infectivity. Of these chemicals, NEM is membrane-permeable and irreversible whereas DTNB is membrane-impermeable and reduction-labile. PUUV- or TULV-infected Vero E6 cell culture supernatants were treated with increasing amounts of the thiol-reagents and the remaining infectivity was analyzed in the supernatants by FFU assay (III, Fig. 1 and Table 1). Both reagents could inhibit the infectivity of PUUV and TULV but NEM was by far the more virucidal. By using NEM at concentrations between 10-100 μM close to 100% inactivation of the virus stocks was achieved. The potency of NEM was increased when purified TULV was used, indicating a protective effect of serum in virus-containing cell culture supernatants (III, Fig. 1c). These results suggested that membrane-permeability of thiol-alkylating reagent, as is the case with NEM, may be important for high virucidal efficiency. However since also DTNB had significant inhibitory activity the possibility of at least some level of thiol-disulfide shuffling in hantavirus glycoproteins, required for infectivity, could not be excluded. We tested the effect of these chemicals also on UUKV phlebovirus infectivity since these viruses do not contain a ZF domain (personal observation). This was achieved by infecting Vero E6 cell monolayers with UUKV and TULV (III, Fig. 2). In the case of NEM, excess reagent reactivity was quenched prior to virus adhesion but the membrane-impermeable DTNB was allowed to react with the cellular PDIs upon infection. Results showed that NEM is clearly more virucidal towards hantaviruses than phleboviruses, thus supporting a role for the ZF in hantavirus infectivity. In addition, DTNB had no measurable inhibitory effect on TULV or UUKV in this assay suggesting that no cellular PDI activity is involved in hantavirus or phlebovirus entry.

4.3.3 Inactivation of hantaviruses by NEM retains virion integrity

The mechanism of how NEM inactivates hantaviruses was then investigated by studying structural and functional integrity of the virus after inactivation. Firstly, the structural integrity of the inactivated virions was studied by sucrose density gradient ultracentrifugation (III, Fig. 3). Secondly, the conformational stability of the NEM-treated

viral glycoproteins was assessed by immunoprecipitation with conformation-sensitive antibodies (III, Fig. 4). Thirdly, cell-binding capacity of the inactivated virus was analyzed by binding of radiolabeled virus to Vero E6 cell monolayers (III, Fig. 5). All these experiments suggested that virus structure and surface functionality was unaltered due to NEM treatment supporting the idea that the inactivating potential of NEM probably lies in its ability to traverse the viral membrane. Although it was not the main intention in this study; these results also suggested that NEM-inactivated hantaviruses can be used to provoke a neutralizing antibody response in affected individuals and they could, therefore, be used as vaccines. For retroviruses, where chemical inactivation by thiol-blocking reagents is widely studied, the use of thiol-inactivated viruses as vaccines has also been investigated (Lifson *et al*., 2002; Lifson *et al*., 2004; Lu *et al*., 2004).

4.3.4 The possible role of the ZF in hantavirus entry

The results obtained by using NEM as hantavirus-inactivating agent supported the idea that ZF could in fact be involved in hantavirus infectivity. To obtain some idea of which of the viral proteins are subjected to alkylation with NEM, biotinylated maleimide (B-mal), a mechanistic analogue for NEM, was used. First of all, it was observed that this reagent is not as virucidal as NEM (III, Fig. 6a) probably reflecting its bulkier molecular composition. Intact and detergent–disrupted sucrose-gradient purified TULV was then treated with this chemical and immunoblotting using streptavidin as probe was performed to detect the B-mal reacted proteins (III, Fig. 6b). B-mal reacted with Gn, Gc and N proteins even in intact viruses indicating that all these proteins have free thiols. N protein was clearly more heavily labelled in disrupted virions as compared to intact ones showing that B-mal does not cross membranes readily, which could also be the reason for its lower virucidal efficiency compared to NEM. In addition to Gn, labelling of Gc and N protein was also detected, and, therefore, it was impossible to deduce which thiols were critical for virus infectivity. Despite of this, the effect of NEM on the zinc binding capacity of previously synthesized PUUV ZF peptide was analyzed (Table 2) in order to determine whether NEM is actually capable of destroying the ZF fold in hantaviruses. This was done by exploiting the spectroscopic activity of Co(II)-ions, which can functionally substitute for Zn(II)-ions in ZF-folds. The Co(II)-ion treated peptide gave a peak at 650 nm, which was not visible in the presence of EDTA, indicating ion-binding to the peptide

(unpublished results, Fig. 12). Treatment with NEM, but not with iodoacetamide (IAA), clearly ejected the Co(II)-ion from the peptide. IAA is also a thiol-blocking reagent, which in concordance with its inability to destroy the PUUV ZF fold has low inactivation potential of hantaviruses in FFU assay (data not shown).

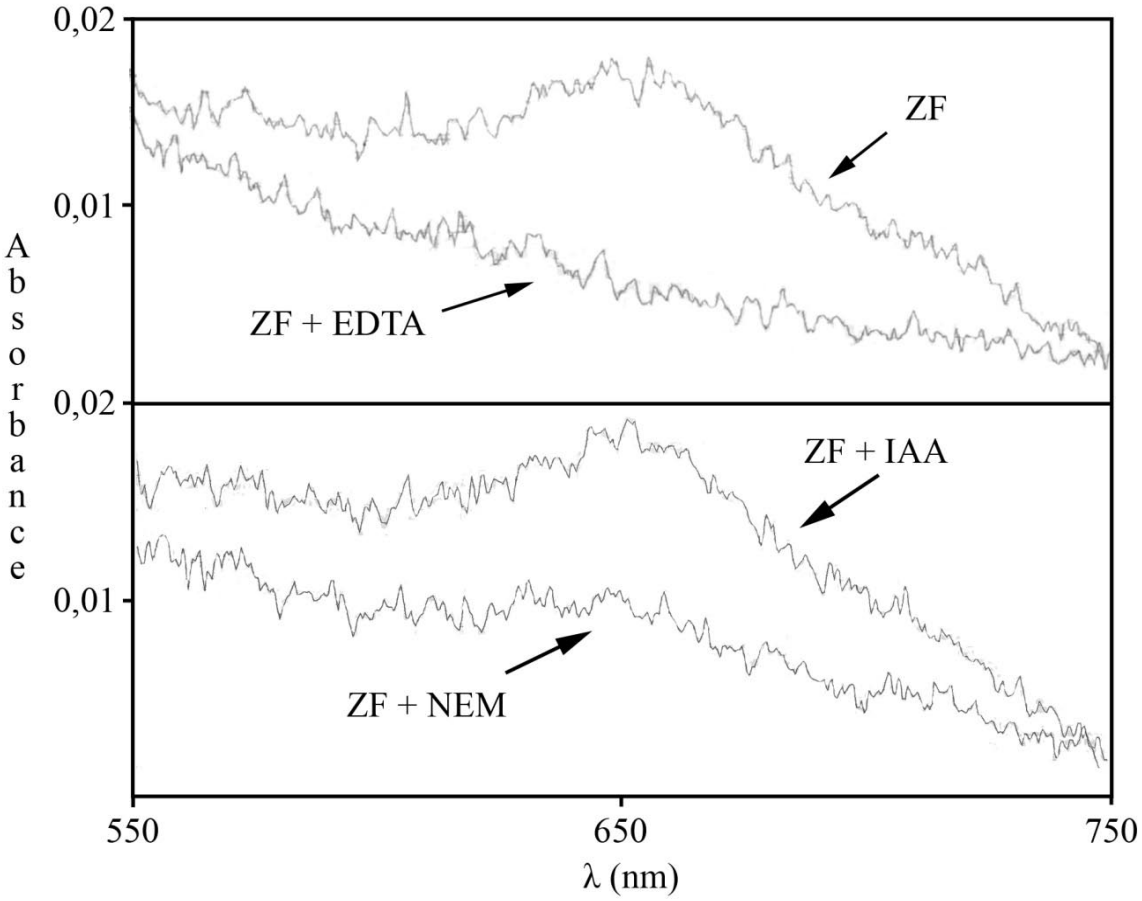


Figure 12. PUUV zinc finger peptide metal binding capacity is destroyed by NEM but not by IAA. The ZF peptide was folded in the presence of spectroscopically active Co(II)-ion as a substitute for the spectroscopically inactive Zn(II)-ion as the metal ligand. Absorption spectrum was recorded from 550 to 750 nm and a local absorption maximum detected at 650 nm, indicating Co(II) chelation to the peptide. To abolish the metal binding capacity of the zinc finger, two equivalents of NEM or IAA in respect to cysteine residues and 2 equivalents of EDTA in respect to Co(II)-ion were added.

In the case of NEM the result in Fig. 12 was expected due to the known ability of NEM to displace metal-ions from ZFs (Chertova *et al.*, 1998; Morcock *et al.*, 2005). However, whether the efficiency of NEM as a hantavirus-inactivating agent lies in its ability to

destroy ZFs still remains elusive since this reagent was also shown to react with Gc and N proteins. In addition, possible L protein-reactivity of NEM could not be excluded in these experiments. It is also unclear whether NEM can actually react with hantavirus ZF in its biological context inside the virion. Furthermore, the possibility that NEM reacts with free thiols that are hidden from the more bulky B-mal, IAA or DTNB on the surface of the virion (in Gn or Gc) cannot be ruled out. Nevertheless, if it is assumed that ZF is required for hantavirus infectivity, it is interesting to speculate about its mode of action. There is a single report showing that immunodepletion of arenavirus RING finger-harboring Z protein from infected cells blocks RNA synthesis (Kolakofsky & Hacker, 1991). This contradicts the role of Z protein in inhibition of RNA synthesis but suggests that the role of the matrix protein in the case of arenaviral RNA synthesis is more diverse than expected. The fact that ZF-reactive chemicals abolish arenavirus infectivity certainly supports a role for the RING finger in a post-entry RNA synthesis initiation step. Since hantaviruses are also inactivated with ZF-reactive chemicals, it is possible that the ZF of NSRV possess a more conserved role e.g. in the initiation of viral RNA synthesis. Another possibility is that intact ZF of arenaviruses or hantaviruses is required for the fusion event of the virus in endosomal membranes. Intact hantavirus ZF was also suggested to be required for N protein binding (Wang *et al*., 2010). However, in the immunoprecipitation experiments of native and NEM-inactivated virions with glycoprotein-specific mAbs, the interaction between glycoproteins and RNP in response to NEM was preserved (III, Fig. 4). This result showed that hantavirus inactivation by NEM is not caused by disruption of this interaction. In conclusion, while the possible role of the ZF in hantavirus infectivity remains elusive, these results clearly indicate that free thiols are necessary for hantavirus infectivity.

5 Concluding remarks

This thesis project can be divided into three sub-projects: Hantavirus-induced apoptosis, hantavirus packaging and hantavirus entry. In addition, attempts were made to identify functions for the Gn zinc finger in hantavirus entry and assembly.

Hantaviruses do not generally cause apoptosis in cell cultures. Therefore, it was a bit of a surprise when it was demonstrated that TULV causes apoptosis in Vero E6 cell line in which the virus is propagated (Li *et al.*, 2004). One of the aims of this thesis was to study the regulation of cellular signaling pathways in response to TULV infection in order to elucidate the mechanisms of TULV-induced apoptosis. Results showed that ERK1/2, which belongs to a ubiquitous cell survival pathway, was down-regulated prior to induction of apoptosis in TULV-infected cells. Also a coincidental down-regulation of a cell surface protein EGFR, which functions upstream of ERK1/2, was observed. Since EGFR is known to form a complex with integrins, the cellular receptors of hantaviruses, it was hypothesized that apoptosis is linked to regulation of integrins and malfunctioning adhesion properties of the infected cells. In our laboratory, apoptosis was previously observed only in the case of TULV infection but in this study it was also shown that other hantaviruses can cause apoptosis, albeit at a low level. We also determined that the level of induced apoptosis is probably related to different growth properties of the individual viruses. For unknown reasons TULV seemed to grow faster and to higher titers than other hantaviruses studied. In these studies a particular strain of TULV called Moravia was used. However, there are other strains of TULV that do not induce apoptosis as readily as the Moravia strain (Dr. Alex Plyusnin, personal communication), which suggests that these strains have differences in their growth properties. It is probable that the special growth properties of TULV Moravia, as revealed in these studies, are obtained by spontaneous mutation and is not an inherent difference between hantavirus species. Finally, the biological significance of the hantavirus-induced apoptosis could relate to kidney dysfunction and proteinuria, which are associated with HFRS. Hopefully, future studies will address this question and possibly demonstrate a direct link between hantavirus-induced apoptosis and pathogenesis. The mechanistic understanding of TULV-induced apoptosis could potentially provide means to combat HFRS.

For viral RNPs to be efficiently packaged into progeny virions during hantavirus budding, it has been proposed that RNPs need to interact with the envelope glycoproteins. This is thought to be achieved by a direct interaction between N protein and cytoplasmic tail of Gn. Initial studies suggested that this hypothesis may, indeed, be correct (Koistinen and Li *et al.*, personal communication). However, while the initial studies were conducted solely by recombinant proteins, in the present study it was shown that there is interaction between native Gn and N protein in purified virions. In addition, it was observed that Gn-CT also interacted with nucleic acids, thereby suggesting that the recognition of RNP by Gn-CT is not entirely mediated by protein-protein interactions. These results showed that the Gn-CT of hantaviruses is a surrogate matrix protein, which, in the case of other NSRVs, have functions outside its role in acting as a link between the viral envelope and the virus core. One such function is the regulation of viral RNA synthesis. Thus, it may be hypothesized that this could also be the case in hantaviruses where Gn-CT might interfere with RNA synthesis, perhaps through its nucleic-acid binding activity. The initial hypothesis was that the Gn-CT could interact with RNP through its ZF domain but in contradictory to this assumption the mapping results revealed binding sites on both sides adjacent to ZF fold. Therefore, as suggested by Wang *et al*. (2010), an intact ZF is likely to play a regulatory role in the recognition of the RNP but probably cannot directly bind N protein. In addition, the C-terminal part of Gn-CT was found to have the strongest affinity towards RNP, N protein and nucleic acids. This domain includes a putative endocytosis motif YxxL, which has been suggested to be important for Gn-CT interaction with N protein (Koistinen and Li, personal communication). The interactions that are described in this study are probably fundamental to hantavirus assembly and are, therefore, a suitable target for antivirals. The mapping data of these interactions revealed possible peptide targets which could be used as lead compounds in the development of chemicals that inhibit virus assembly and thus infectivity.

The third sub-project of this thesis focused on possible role of hantaviral ZF in virus entry. This was because in the case of retro- and arenaviruses thiol-blocking compounds can block virus infectivity by destroying the ZF-fold of these viruses. Therefore, the possible virus-inactivating effects of this type of compounds were tested also for hantaviruses. Indeed, it was found that a thiol-alkylating compound, NEM, blocked hantavirus infectivity. This reagent was able to react with multiple proteins of the virus and unfortunately the observed loss of infectivity could not be pin-pointed to the

destruction of the ZF fold. Further studies are still needed to fully elucidate the actual mechanism of how NEM can inactivate hantaviruses and whether it involves the functions of the ZF. Nevertheless, it was also determined that the inactivated hantavirus retained its surface structure and functions of its glycoproteins. This suggested that NEM-inactivated hantaviruses could provoke effective neutralizing antibody responses in individuals and could, therefore, be considered as candidates for vaccine development.

6 Acknowledgements

This work was performed at the Peptide and Protein laboratory, Department of Virology, Haartman Institute, University of Helsinki during 2004-2011. In addition to performing hantavirus research, our laboratory also acts as core facility of the Institute by providing services in peptide synthesis and interaction kinetics. I wish to acknowledge the head of the Institute, Professor Seppo Meri, and head of the Department, Professor Kalle Saksela, for the support to our Core Facility laboratory over these years. I also want to thank Academy of Finland, Finnish Cultural Foundation, Magnus Ehrnrooth Foundation, Paulo Foundation, Maud Kuistila Memorial Foundation, Otto Malm Foundation, Orion-Farmos Research Foundation and Finnish Kidney Foundation for making this thesis financially possible.

This thesis was co-supervised by Docent Hilkka Lankinen, head of the Peptide and Protein laboratory, and Professor emeritus Antti Vaheri, former head of the Department. Mostly, I would like to thank Hilkka for providing me the opportunity to conduct research in her lab. She encouraged me to do independent research and to follow my own interests and intuition that I think has been highly valuable for me in developing as a scientist. Her ability to find the essential points from at times complicated data has been very fruitful to me. Antti is equally acknowledged first of all for providing the facilities to do research but also for introducing novel ideas in the field of hantavirus pathogenesis and especially for his clear-sightedness in performing the art of publication. I'd like to thank also Docents Maija Vihinen-Ranta and Petri Susi for reviewing this thesis and providing very helpful ideas to improve it.

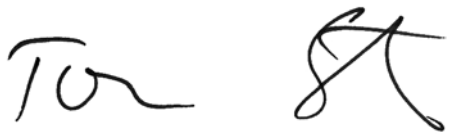
I feel very much indebed to my colleague Jussi Hepojoki for being a close friend to me during all these years. He has made the research much more fun and I do not know how I could have managed without him! In addition to being good company his profound understanding of biochemistry has helped me a lot in my own work. I am grateful for our scientific discussions. I'd like to also specially acknowledge Hao Wang with whom I have shared good scientific discussions and publications. Of course I'd like to thank also other former (Agne, Kirill, Pasi, Paula, Sami, Tuomas H., Xiao), long-standing (Anna, Anne, Anu, Eili, Liina, Maria, Niina, Satu K., Tarja) and a bit newer (Elina, Erika, Essi, Jussi S., Lev, Satu S., Suvi, Tuomas R.) colleagues at the Zoonosis lab with whom I have

shared very nice tick-gathering trips, other trips and also sparkling. I'd like to thank Docent Alex Plyusnin and Professor Olli Vapalahti as group leaders in the Zoonosis Unit for their scientific and social contributions. I will not forget to mention members of our "sähly"-team Marko, Oskari, Pekka, Perttu and Markus among others. I am grateful for the technical assistance of the members of the Zoonosis Lab; Irina, Kirsi, Leena, Pirjo and Tytti.

Of course, I would not be here without my parents Mirja and Göran. They have always supported me even when I have occasionally given them some rough time. My deepest thanks belong to Kati who does not let me dwell in science but bring so many other things to think (and occasionally stress) about. After Kati and I had our daughter Essi, I realized there are other even more important things to life than science. Kati and Essi, you are the most important things in my life.

This thesis is written in memory of my brother Jesse.

Helsinki, August 2011



Tomas Strandin

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