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Glycoprotein Interactions in the Assembly of Hantaviruses

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ACADEMIC DISSERTATION

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“In the field of observation chance favors only the prepared mind.”

-Louis Pasteur (1822-1895)

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I Hepojoki J, Strandin T, Vaheri A, Lankinen H, 2010. Interactions and oligomerization of hantavirus glycoproteins. *Journal of Virology*, 84(1), 227-242.

II Huiskonen J, **Hepojoki J**, Laurinmäki P, Vaheri A, Lankinen H, Butcher S, Grünewald K, 2010. Electron cryotomography of Tula hantavirus suggests a unique assembly paradigm for enveloped viruses. *Journal of Virology*, 84(10), 4889–4897.

III Hepojoki J*, Strandin T*, Wang H, Vapalahti O, Vaheri A, Lankinen H, 2010. Cytoplasmic tails of hantavirus glycoproteins interact with the nucleocapsid protein. *Journal of General Virology*, 91, 2341–2350.

IV Hepojoki J, Sane J, Strandin T, Vapalahti O, Meri S, Mäkelä S, Mustonen J, Vaheri A*, Lankinen H*. 90K/Mac-2BP binds to hantavirus and its levels in plasma are elevated upon acute HFRS. A manuscript.

*Authors contributed equally

ABBREVIATIONS

3D	three-dimensional
AIDS	acquired immune deficiency syndrome
ANDV	Andes virus
BCCV	Black Creek Canal virus
BSA	bovine serum albumin
CRP	C-reactive protein
cRNA	complementary RNA
CT	cytoplasmic tail
d.p.i	days post infection
ECM	extracellular matrix
EM	electron microscopy
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
FBS, FCS	fetal bovine serum, fetal calf serum
FFU	focus-forming unit
Gc	C-terminal portion of glycoprotein precursor
Gn	N-terminal portion of glycoprotein precursor
GPC	glycoprotein precursor
HBV, HCV	hepatitis B virus, hepatitis C virus
HCPS	hantavirus cardiopulmonary syndrome
HFRS	hemorrhagic fever with renal syndrome
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HTNV	Hantaan virus
IF	immunofluorescence
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP	immunoprecipitation
kb	kilobase
kDa	kilodalton
KHF	Korean hemorrhagic fever
LSB	Laemmli sample buffer
MAb	monoclonal antibody
MALDI MS	matrix-assisted laser desorption/ionization mass spectrometry
MHC	major histocompatibility complex
MOI	multiplicity of infection
mRNA	messenger RNA
NMR	nuclear magnetic resonance
NK	natural killer
PAb	polyclonal antibody (or antiserum)
PUUV	Puumala virus
RdRp	RNA-dependent RNA-polymerase (the L segment encoded L protein)
RNP	ribonucleoprotein
RT	room temperature
RVFV	Rift Valley fever virus
SFV	Semliki Forest virus
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNV	Sin Nombre virus
SRCR	scavenger receptor cysteine-rich
TBEV	tick-borne encephalitis virus
TNF	tumor necrosis factor
TULV	Tula virus
UUKV	Uukuniemi virus
VLP	virus-like particle
vRNA	viral RNA
WB	western blot
ZF	zinc finger

ABSTRACT

Hantaviruses are one of the five genera of the vector-borne virus family *Bunyaviridae*. While other members of the family are transmitted via arthropods, hantaviruses are carried and transmitted by rodents and insectivores. Hantavirus infection in the seemingly unaffected rodent or insectivore host causes presumably a life-long persistence, throughout which the host secretes the virus in feces, urine and saliva. Occasional transmission to humans occurs via inhalation of aerosolized rodent excreta, wherein the infectious virus may survive up to two months. When transmitted to man hantaviruses cause hemorrhagic fever with renal syndrome (HFRS, in Eurasia, with mortality up to 10%) and hantavirus cardiopulmonary syndrome (HCPS, in the Americas, with mortality up to 40%).

The single-stranded, negative-sense RNA genome is in segments S, M and L that respectively encode for nucleocapsid (N), glycoproteins Gn and Gc, and RNA-dependent RNA-polymerase (RdRp or L protein). The genome segments, encapsidated by N protein to form ribonucleoprotein (RNP), are enclosed inside a lipid envelope decorated by spikes formed of Gn and Gc. The virion is approximately 70 to 210 nm in diameter and displays a round or pleomorphic morphology.

The focus of this study was to understand the mechanisms and interactions, through which the virion is formed and maintained. We started by analyzing the interactions between Gn and Gc. We observed that when extracted from virions, both Gn and Gc favor homo- over hetero-oligomerization (the Gn-Gn and Gc-Gc contacts are stronger than the Gn-Gc contact). We further showed using co-immunoprecipitation that the contacts of Gc are prone to dissociation at low pH, a feature common to class II viral fusion proteins. The minimal glycoprotein complexes extracted from virion by detergent were observed, by using ultracentrifugation and gel filtration, to be tetrameric Gn and homodimeric Gc. By applying SPOT peptide array we were able to map the regions involved in the Gn-Gc interaction at peptide level. These results led us to suggest a model where tetrameric Gn complexes are interconnected through homodimeric Gc units to form the grid-like surface architecture described for hantaviruses.

In parallel we aimed to create a three-dimensional (3D) reconstruction of the virion surface by applying cryo-electron tomography to purified virions. The study yielded a 3D-density map of the spike complex at a resolution of 3.6 nm. The spike complex formed of Gn and Gc was 10 nm high and displayed a four-fold symmetry with dimensions of 15 nm times 15 nm. This unique square-shaped complex on a roughly round virion creates a hitch for the assembly, since a sphere cannot be broken into rectangles. It is therefore probable that re-arrangements of the observed contacts between Gn and Gc are required for successful assembly.

The results of the cryo-tomographic study supported the dogma of an interaction between the cytoplasmic tail (CT) of Gn and the RNP. Using monoclonal antibodies we were able to show that, indeed, there is an interaction between the glycoproteins and RNP. Further characterization using SPOT peptide array indicated that both Gn and Gc are likely to contribute to the interaction between the spike complex and the RNP. These results led us to suggest a model, in which after the formation of the spike complex Gn-

CT becomes accessible to interaction with RNP, and this drives the budding of virions at the site of assembly.

The analysis of the efficiency of virus purification led to the discovery of a co-purifying protein. This protein, identified as galectin-3 binding protein (referred to as 90K/Mac-2BP), was shown not only to co-purify with the virions but also to interact with the virion, as confirmed by inhibition of infection and co-immunoprecipitation. Analysis of plasma samples taken from patients hospitalized for Puumala virus infection showed increased concentrations of 90K/Mac-2BP in acute phase as compared to convalescence. The increased 90K/Mac-2BP levels in patients were also found to correlate with several parameters that reflect the severity of acute HFRS.

The results of these studies confirmed, but also challenged some of the dogmas on the structure and assembly of hantaviruses. We confirmed that the Gn-CT and the RNP do interact, as long assumed. On the other hand we demonstrated that the glycoproteins Gn and Gc exist as homo-oligomers or appear in large hetero-oligomeric complexes, rather than form primarily heterodimers as was previously assumed. We concluded that while there definitely is an interaction between Gn and Gc, the interaction is not between two monomers but rather between oligomers of one or both proteins. This work provided new insight in to the structure and assembly of hantaviruses, but also raised questions to be answered by further studies.

TIIVISTELMÄ (SUMMARY IN FINNISH)

Hantavirukset kuuluvat vektorivälitteisten virusten muodostamaan *Bunyaviridae*-perheeseen ja ovat yksi perheen viidestä suvusta. Muista perheeseen kuuluvista viruksista poiketen hantavirukset ovat jyrsijä- tai hyönteissyöjävälitteisiä (Suomessa esiintyvän ja myyräkuumetta aiheuttavan Puumala-viruksen luonnollinen isäntä on metsämyyrä). Luonnollisessa isännässään hantavirus aiheuttaa pitkäikäisen infektion, joka ei kuitenkaan ilmeisesti vaikuta isäntäeläimen hyvinvointiin. Tartunnan saanut jyrsijä erittää virusta ulosteeseen, virtsaan ja sylkeen, ja ihmiseen virus siirtyy aerosolisoituneiden eritteiden mukana. Hengitysilman kautta ihmiseen siirryttyään virus voi aiheuttaa joko munuaisoireisen verenvuotokuumeen (esiintyy Eurasiassa, kuolleisuus jopa 10%), tai hantaviruksen aiheuttaman sydän-keuhko-oireyhtymän (esiintyy Amerikoissa, kuolleisuus jopa 40%).

Hantaviruksen yksijuosteinen ja negatiivisäikeinen RNA-genomi on jakautunut kolmeen osaan. Genomin osat S, M ja L koodaavat neljää rakenteellista proteiinia: nukleokapsidiproteiini (N-proteiini), glykoproteiinit Gn ja Gc sekä viruksen polymeraasientsyymi (RdRp tai L-proteiini). Viruksen genomin osat pakkautuvat N-proteiinin kanssa ribonukleoproteiiniksi (RNP), joka edelleen pakkautuu glykoproteiinien peittämän lipidivaipan sisään. Näin muodostunut viruspartikkeli on muodoltaan pyöreähkö ja halkaisijaltaan noin 70-210 nanometriä.

Tämän väitöskirjatyön tarkoituksena oli tutkia ja oppia ymmärtämään viruspartikkelin muodostumiseen sekä koossapitämiseen tarvittavia rakenneproteiinien välisiä vuorovaikutuksia. Aluksi tutkimme lipidivaipan pinnalla olevien Gn- ja Gc-proteiinien välisiä vuorovaikutuksia. Huomasimme, että viruspartikkelista eristettäessä sekä Gn- että Gc-proteiini pyrkii muodostaan yhdistelmän (engl. complex) mieluummin omankaltaisensa parin kanssa. Toisin sanoen Gn-Gn ja Gc-Gc vuorovaikutukset ovat voimakkaampia kuin Gn-Gc vuorovaikutukset. Tutkimme myös pH:n vaikutusta glykoproteiinien muodostamiin yhdistelmiin, ja havaitsimme Gc-proteiinin yhdistelmien olevan herkkiä happamalle pH:lle. Tämän tulkitsimme vahvistavan sitä oletusta, että Gc-proteiini on luokan II viraalinen fuusioproteiini. Määritimme viruksesta eristettyjen proteiiniyhdistelmien minimaalisiksi yksiköiksi yhdistelmän, jossa on neljä Gn-proteiinia ja yhdistelmän, jossa on kaksi Gc-proteiinia. Tämän perusteella loimme hypoteesin, jonka mukaan ruudukkomaiseksi kuvattu viruksen pinta muodostuu neljän Gn-proteiinin yhdistelmistä, jotka yhdistyvät toisiinsa kahden Gc-proteiinin muodostaman yksikön kautta.

Tutkimme viruksen pintarakennetta myös kryo-elektronitomografian avulla, tarkoituksenamme luoda kolmiulotteinen malli viruksen pinnasta. Tekniikan avulla loimme kolmiulotteisen mallin Gn- ja Gc-proteiinien muodostamasta ”piikistä” 3,6 nm tarkkuudella. Mallin mukaan nelisymmetrinen piikki on viruksen läpileikkauksessa 10 nm korkea ja viruksen pinnan suuntaisesti 15 nm kertaa 15 nm. Tämä ainutlaatuinen nelisymmetrisyys luo ongelman viruksen pakkautumiselle, sillä pallon pintaa ei voi rikkoa neliöiksi ja tästä johtuen pakkautumiseen tarvittaneen Gn- ja Gc-proteiinien lisäksi myös muita komponentteja.

Viruksen rakennetutkimuksen tulokset tukivat dogmaa, jonka mukaan Gn-proteiinin solunsisäinen osa (Gn-CT) ja RNP ovat vuorovaikutuksessa keskenään. Osoitimme kokeellisesti tämän vuorovaikutuksen hyödyntäen Gn-, Gc- ja N-proteiinia tunnistavia vasta-aineita. Tarkemman tutkimuksen perusteella osoittautui, että molemmat glykoproteiinit osallistuvat vuorovaikutukseen RNP:n kanssa. Tuloksen perusteella loimme mallin, jonka mukaan Gn- ja Gc-proteiinien välisten vuorovaikutusten seurauksena Gn-proteiini muuttaa rakennettaan siten, että Gn-CT kykenee sitoutumaan RNP:hen. Tämä sitoutuminen puolestaan ohjaa viruksen pakkautumista eteenpäin.

Tutkiessamme näytteitä viruspuhdistuksen eri vaiheista huomasimme, että viruksen mukana kulkeutuu tuntematon proteiini. Tunnistimme proteiinin galectin-3:a sitovaksi proteiiniksi, jota kutsumme 90K/Mac-2BP:ksi. Osoitimme, että 90K/Mac-2BP ei ainoastaan rikastu puhdistuksessa viruksen mukana, vaan myös sitoutuu viruspartikkeliin. Analysoimme sairaalahoitoa vaatineiden myyräkuumepotilaiden plasmanäytteistä 90K/Mac-2BP-pitoisuuden akuutissa vaiheessa ja vertasimme sitä toipumisen jälkeen otetun näytteen pitoisuuteen. Havaitsimme 90K/Mac-2BP:n suhteellisen pitoisuuden olevan koholla akuutissa myyräkuumeessa, ja kohonneen pitoisuuden korreloivan muiden taudin vakavuutta kuvaavien kliinisten parametrien kanssa.

Tämän väitöskirjatutkimuksen tulokset haastoivat, mutta toisaalta myös osoittivat toteen joitakin hantavirusten rakenteeseen ja pakkautumiseen liittyviä dogmia. Osoitimme kokeellisesti toteen, että Gn-CT:n ja RNP:n välillä on vuorovaikutus, kuten aiemmin oli oletettu. Toisaalta osoitimme, että glykoproteiinit muodostavat mieluummin Gn-Gn ja Gc-Gc yhdistelmiä kuin Gn-Gc yhdistelmiä. Tuloksien perusteella teimme sen johtopäätöksen, että Gn- ja Gc-proteiinien välinen vuorovaikutus vaatii useamman yksikön kumpaakin proteiinia. Väitöskirjatyön lopputuloksena ymmärrämme nyt paremmin hantavirusten rakennetta ja pakkautumista, mutta samalla nousi esiin myös uusia tutkimuskysymyksiä.

REVIEW OF THE LITERATURE

Discovery and history of hantaviruses

Descriptions of a disease similar to hemorrhagic fever with renal syndrome (HFRS) are found in Chinese records as early as 960 AD [149], however, perhaps the oldest clinical records of disease that is retrospectively linked to hantaviruses are found from Russia as early as 1913 [41]. Similar disease, termed field nephritis, was encountered by military troops during both World War I (1914-1918) and II (1939-1945) [149]. Epidemic hemorrhagic fever was reported during the Korean Conflict (1951) [149,338], when the UN troops initially encountered the causative agent of Korean Hemorrhagic Fever (KHF) [149]. The intensive attempts to isolate the causative agent of KHF resulted in isolation of the first hantavirus in 1976 [197]. The virus was named Hantaan virus (HTNV) after a small river (the Hantan River) in South Korea [195] and was eventually chosen as the prototype strain of hantaviruses [149]. Soon after identifying HTNV as the causative agent for KHF (or HFRS), nephropathia epidemica (NE) was reported to be related to KHF [190,198,358]. The causative agent of NE was identified from bank voles trapped in Puumala, Finland [34] and was given the name Puumala virus (PUUV). Clone E6 of Vero cells, derived from African green monkey kidney cells, was harnessed for the cultivation of hantaviruses [171] and, in 1985, neutralization experiments confirmed the existence of four distinct hantaviruses: HTNV, Seoul (SEOV), PUUV and Prospect Hill (PHV) [322].

In 1993 the first outbreak of the New World hantaviruses occurred in the Four Corners region (the crossing of New Mexico, Arizona, Utah and Colorado states in the USA) [149]. The causative agent was quickly identified as a hantavirus [81,259] that was given the name Sin Nombre (SNV, no name or nameless in Spanish). The disease associated was to SNV was termed hantavirus pulmonary syndrome (HPS) [133]. Due to the associated cardiopulmonary manifestations, this disease is nowadays more frequently referred to as hantavirus cardiopulmonary syndrome (HCPS) [118]. The intensive research on the hantavirus field has led to the discovery of numerous new strains, most of which are associated either with HFRS or HCPS. The changes in the climate on the global level generate a potential threat of re-emergence of hantaviral disease [39,173,363] and thus yet more hantaviruses are likely to be discovered in the future.

Classification of hantaviruses

Hantaviruses together with four other genera (*Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, *Tospovirus*) comprise the family *Bunyaviridae* [72]. While hantaviruses are carried by rodents or insectivores [72,342], the other members of the family *Bunyaviridae* viruses are arthropod-borne [73,258]. Based on the recommendations of the International Committee on Taxonomy of Viruses hantaviruses are classified as follows: I) hantavirus species are found in a unique ecological niche, i.e. in a different primary rodent reservoir species or subspecies, II) hantavirus species exhibit at least a 7% difference in aa identity on comparison of the complete GPC and N protein sequences, III) hantavirus species show at least a 4-fold difference in two-way cross-neutralization tests, IV) hantavirus species do not naturally form reassortants with other hantavirus species [72,286]. According to these criteria hantaviruses are divided into four distinct groups based on their host reservoirs [184,220].

The viruses in the first group are harbored by hosts belonging to the *Murinae* subfamily of *Muridae* [263] and contain HFRS-causing viruses such as HTNV (the type species), SEOV and Dobrava virus (DOBV) [184]. The host of the second group of hantaviruses belong to the *Arvicolinae* subfamily of *Cricetidae* [263] and while PUUV causes HFRS, the others viruses of the group like Tula virus (TULV), PHV, Khabarovsk virus (KBRV) and Topografov virus (TOPV) are considered apathogenic [184]. The third group of hantaviruses are associated with hosts belonging to the *Neotominae* and *Sigmodontinae* subfamilies of *Cricetidae* and the associated viruses: SNV, Andes virus (ANDV), New York virus (NYV), Black Creek Canal virus (BCCV) and Laguna Negra virus (LNV) cause HCPS when transmitted to man [184,220]. Even though the prototype, Thottapalayam virus (TPMV), of the fourth group of hantaviruses was isolated in 1964 [40], this group is the most recent addition to the list of hantaviruses [342]. The members of the fourth group of hantaviruses are carried by hosts belonging to the family *Soricidae* [220,263,342]. In addition to TPMV also the other members such as Tanganya virus (TGNV), Seewis virus (SWSV), Camp Ripley virus (RPLV), Cao Bang virus (CBNV), Ash River virus (ARRV), Jemez Springs virus (JMSV) and Imjin virus (MJNV) of this group are considered apathogenic to man [15,16,124,156,342-345].

Transmission of hantaviruses

The carrier rodents of hantaviruses become chronically infected and secrete the virus in saliva, urine and feces that are considered to be the route for vertical transmission [127,150,263]. Inhalation of aerosolized rodent excreta is considered to be the main route for transmission to man [125,150,164,375], however, transmissions via rodent bites have also been reported [66,350,367]. While humans are generally considered dead-end hosts for hantaviruses [372,387], there are some reports of HCPS caused by person-to-person transmission of ANDV [227,273].

Pathogenesis of hantaviruses

Hantavirus infection in man causes either HFRS (Old World hantaviruses in Eurasia) or HCPS (New World hantaviruses in the Americas) with respective mortalities of up to 15% and 40% [150,164].

Clinical manifestations of HFRS

After a relatively long incubation time, ranging from 10 days to 6 weeks [150], the course of HFRS can be divided into five phases: I) febrile, II) hypotensive, III) oliguric, IV) polyuric and V) convalescent phase [205]. The febrile stage typically lasts 3 to 6 days and is generally followed by a severe decline in blood pressure (hypotensive stage), that occasionally leads to a severe or even a fatal shock [205,281]. The first four phases are manifested by symptoms such as thirst, headache, abdominal pain, backache, nausea, dizziness, myalgia, flushing of the face (and V-area) and blurred vision [150,205,281]. Thrombocytopenia appears during the hypotensive phase, and may contribute to the occasionally appearing bleeding manifestations [281]. Renal involvement is indicated by hematuria and proteinuria during the hypotensive phase [150]. The following oliguric phase lasts from 1 to 16 days and is manifested by weight gain, risk for hypertension, pulmonary edema and renal insufficiency [150,205,281]. The gradual recovery of renal functions begins by the polyuric stage, during which the electrolyte balance may be

disturbed by the extensive urine secretion [205,281]. Full recovery of renal functions during convalescent phase may take up to several weeks [205].

Clinical manifestations of HCPS

Similarly to HFRS, the incubation period for HCPS is relatively long ranging from 7 days to 5 weeks [78,150]. The clinical course of HCPS is divided into four phases: I) febrile, II) cardiopulmonary, III) diuretic and IV) convalescent phase [78]. The febrile phase of typically 3 to 5 days may be accompanied by symptoms such as headache, dizziness, anorexia, nausea, vomiting, diarrhea and/or abdominal pain [78,150]. Dyspnea, tachypnea and non-productive cough follow the febrile phase and are indications of a pulmonary edema [78,150,281]. During the pulmonary edema, the disease proceeds fast and death due to hypoxia, circulatory compromise or a combination of both may occur within 24 to 48 h [78]. The impairment of the myocardial functions are manifested by a low cardiac output, high systemic vascular resistance and hypotension [78,281]. The clearance of the pulmonary edema occurs during the diuretic phase [78]. Symptoms, such as thrombocytopenia, oliguria and renal failure, commonly encountered in HFRS; have also been described in some HCPS cases [150]. Comparably, HFRS cases have been described to involve pathological findings similar to HCPS cases, and thus it has been suggested that hantaviruses should be considered a cause of acute respiratory distress irrespective of the endemic area [297].

Innate and cellular immune response to hantaviruses

The initial response to hantaviruses is the induction of inflammatory cytokine production [296]. During the acute stage of hantavirus infection the levels of at least the following cytokines and/or acute-phase proteins have been reported to be elevated: TNF-α, interleukin (IL)-1, IL-5, IL-6, IL-10, IL-15, interferon (IFN)-α, IFN-β, IFN-γ, RANTES/CCL5 and C-reactive protein (CRP) [26-28,31,112,119,181,204,252,291,296,353]. Even though some cytokines such as IL-6, IL-10 and TNF-α have been associated with a poor prognosis of hantavirus infection, there seems to be a great variation in the cytokine responses between individuals [29,150]. The activation of the complement system has been reported in acute hantavirus infection [272] and the complement activation is suggested to contribute to the disease severity [317]. Recently hantavirus infection was reported to induce a rapid increase in the amount of natural killer (NK) cells and furthermore the high NK cell level was found to be sustained over a period of more than 60 days [26]. The existence of hantavirus-specific $CD8^+$ T cells with CTL (cytotoxic T lymphocyte) epitopes in N and Gc proteins has also been documented during hantavirus infection, and they are suggested to contribute to the pathogenesis [77,168,368,373,378].

Humoral immune response to hantaviruses

Hantavirus infection in man causes an extensive immune response towards the protein (N, Gn and Gc) components of the virion [34,376,378]. Already upon the onset of symptoms the patients are positive for N protein specific IgM and IgG [150,372]. Additionally, the presence of virus-specific IgA and IgE antibodies has been reported during the acute phase of illness [2,58,219]. The appearance of N-protein-specific antibodies during the acute phase of illness is the basis of hantavirus serology

[34,154,214,337,376], and thus recombinant N protein is commonly utilized in the serodiagnosis of hantavirus infection [69,377,384,403]. Even though some early epitopes in Gn have been reported [145], the glycoprotein-specific antibody response generally appears in the later stage of the infection [145,213] and neutralizing antibodies (epitopes are in the glycoproteins) can be detected during the convalescent phase [378]. The neutralizing antibodies prevail decades after hantavirus infection [213] and thus infection with one hantavirus is thought to provide a life-long immunity [378].

Genome and replication of hantaviruses

Characteristically, as members of the family *Bunyaviridae*, hantaviruses are negative-stranded RNA viruses with a tripartite genome segmented into S (small, 1.8-2.1 kb), M (medium, 3.7-3.8 kb) and L (large 6.5-6.6 kb) segments [74,288]. The viral RNA (vRNA) of each segment comprises of an open reading frame (ORF) and non-coding regions (NCR, or NTR for non-translated region) located at the 3'- and 5'-ends of the segment (Figure 1) [74]. While the lengths of the ORFs in the segments are rather conserved between genotypes, the length of especially the 3' NCR of S segment is rather variable [288].

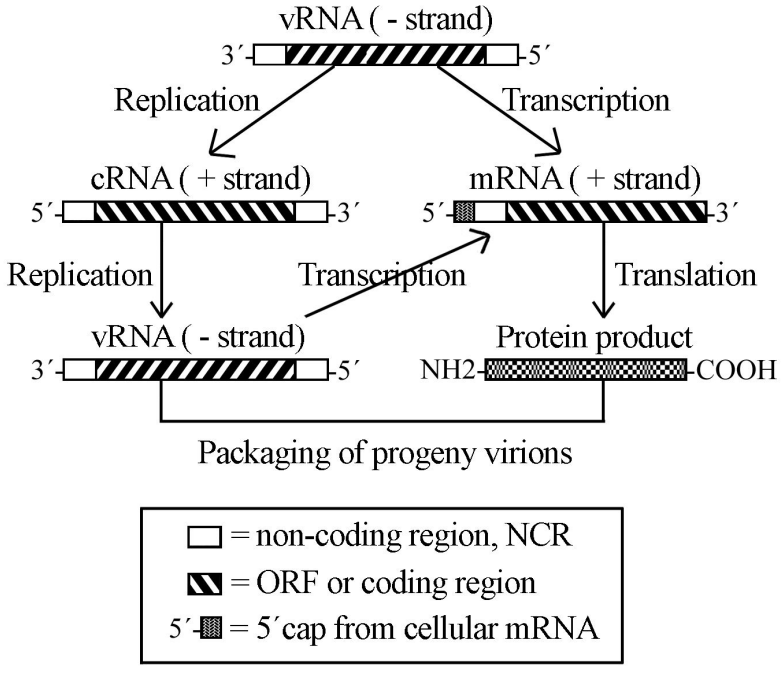

Figure 1. *Coding strategy of hantaviruses (replication, transcription and translation), according to Jonsson and Schmaljohn* [152].

A distinguishing feature of the hantavirus genome as compared to the other *Bunyaviridae* members is the conservation of the very terminal nucleotides of S, M and L segments (see Figure 2) first shown for HTNV [321], and later confirmed with SNV and PUUV [45,282]. The conserved and complementary nucleotides at the 3'- and 5'- termini of each segment are predicted to form a panhandle structure, which is thought to function as the viral promoter and to play a crucial role in the replication [74,152,186,238]. Indeed, terminal deletions in each segment have been observed to correlate with a decreased replication level in persistently infected cell cultures [186,238,239].

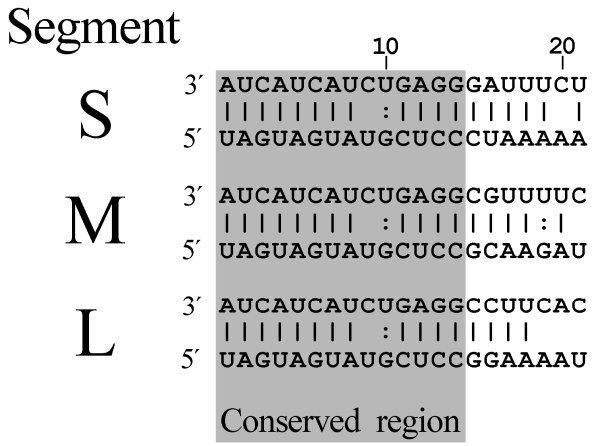

Figure 2. *3'and 5' nucleotides of vRNA segments* [288].

RNA replication

The initial process towards the production of progeny virions begins, as described later in detail, soon after uncoating of the entering virions [152]. The RNA synthesis can be divided into transcription (the production of mRNA) and replication (the production cRNA and vRNA) [152], as depicted in Figure 1. The minimal components required for replication are the RNA-dependent RNA-polymerase (RdRp), nucleocapsid (N) protein and the vRNA [321]. Thus the crucial protein components for replication are the RdRp and N protein [84,85]. Based on the localization of N protein and RdRp in infected cells, the RNA synthesis is assumed to take place in the perinuclear region [158,187,298]. Since both N protein and RdRp are shown to be membrane associated, RNA synthesis presumably requires the presence of cellular membranes [187,298]. The transcription and translation of mRNA is believed to precede the initiation of replication, because N protein is required for replication [276].

mRNA synthesis

The binding of the mRNA 5'-cap to eukaryotic initiation factor 4F (eIF4F) is a prerequisite for efficient translation in eukaryotic cells [237]. The transcription initiation of hantaviruses and other family *Bunyaviridae* viruses requires a continuous supply of suitable primers carrying the 5'-cap [152]. These primers are derived from the 5'-caps of cellular mRNAs by RdRp-mediated endonucleolytic cleavage of the cellular mRNA 7-18 nucleotides downstream from the 5'-cap [96]. However, recent evidence suggests that the primers are sequestered from cellular processing (P) bodies by N protein, which also acts as a surrogate for the eIF4F complex [135]. The “cap-snatched” primers align with the vRNA template a few nucleotides from the 3'-end, presumably via pairing to only a single nucleotide [96,152]. The primer is then extended by a few nucleotides, after which the nascent RNA slips back a few nucleotides on the repeated terminal sequence, and continuum of this “prime-and-realign” produces an exact copy of the 3'-end of vRNA [94-96,146]. In addition to the 5'-cap, mRNA synthesis is distinguished from cRNA synthesis by the fact that the mRNA is terminated after the ORF [136,238,239,327]. This is suggested to be mediated by a secondary structure present only in the naked RNA, devoid of N protein [152]. This would be reasonable, since trimers of the N protein are described to recognize the conserved panhandle structure present in cRNA and vRNA, resulting in the initiation of encapsidation [135,163].

vRNA and cRNA synthesis

The switch from mRNA synthesis towards replication is thought to be mediated by the concentration of free N protein [152]. As depicted in Figure 1, vRNA is used as the template for the synthesis of cRNA, which then in turn is used as the template for new vRNA [152,188]. The initiation of cRNA and vRNA replication is suggested to be similar to the initiation of mRNA transcription with the exception that, instead of the 5'-cap, a triphosphorylated guanosine (pppG) serves as the primer [94,96]. After production of the repeated terminal sequence (see conserved region in Figure 2) by consecutive "prime-and-realign" cycles, the RdRp is suggested to cleave the pppG, thus leaving a monophosphorylated U to the 5'-end of the nascent RNA [152].

Structure of hantavirus particle

Hantavirus particle, the virion, consists of a lipid envelope, N protein-encapsidated vRNA, glycoproteins (Gn and Gc) and the RdRp [74,322,323]. The viral lipid envelope encloses three helical RNPs created by encapsidation of the vRNA segments around N protein [135]. The hantavirus particle has been described as being round or pleiomorphic, with a broad size range from 70 to 210 nm, when purified viruses are studied in EM [226,359]. In EM sections of infected cells the virions appear mainly as round or roundish particles [105,226,359,390], suggesting that the virions are sensitive to mechanical stress, and that the observed pleomorphism is at least partially artefactual. The lipid envelope of the virion is 5 nm thick and the spikes formed of Gn and Gc protrude approximately 10 nm from the membrane [20]. The virions, consisting of >50% protein, 20-30% lipid, 7% of carbohydrate and 2% RNA, have a buoyant density of 1.16-1.18 g/cm^3 in sucrose [135,323]. A schematic presentation of a hantavirus particle is shown in Figure 3A.

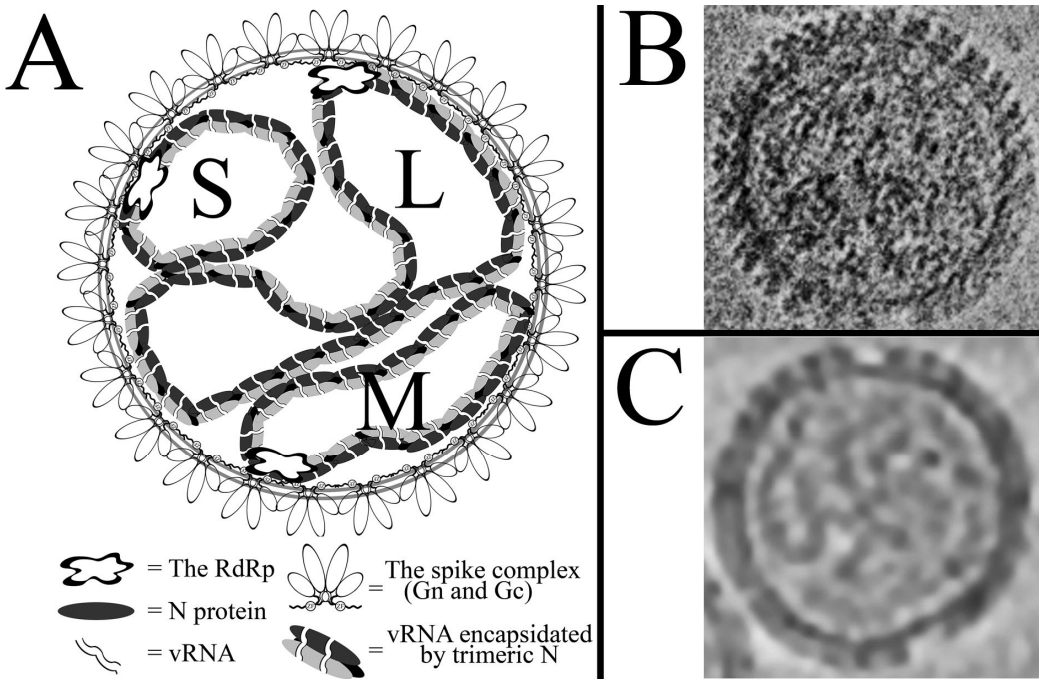

Figure 3. *Hantavirus particle. A) A schematic representation of the virion, B) A hantavirus particle viewed by cryo-EM, C) A slice (= 5nm) from an electron cryo-tomogram of hantavirus particle. B is courtesy of Pasi Laurinmäki and Prof. Sarah Butcher, and C is courtesy of Dr. Juha Huiskonen.*

Protein components of hantavirus

The S, M and L genome segments respectively encode the N protein, glycoprotein precursor (GPC) and RdRp [135], as depicted in Figure 4. The S segment of the *Cricetidae* rodent, but not the *Muridae* rodent viruses, contains an alternate ORF that encodes for a non-structural protein, NSs [286]. The genome-encoded protein components, comprising the virion as discussed above, are described in more detail in the following sections.

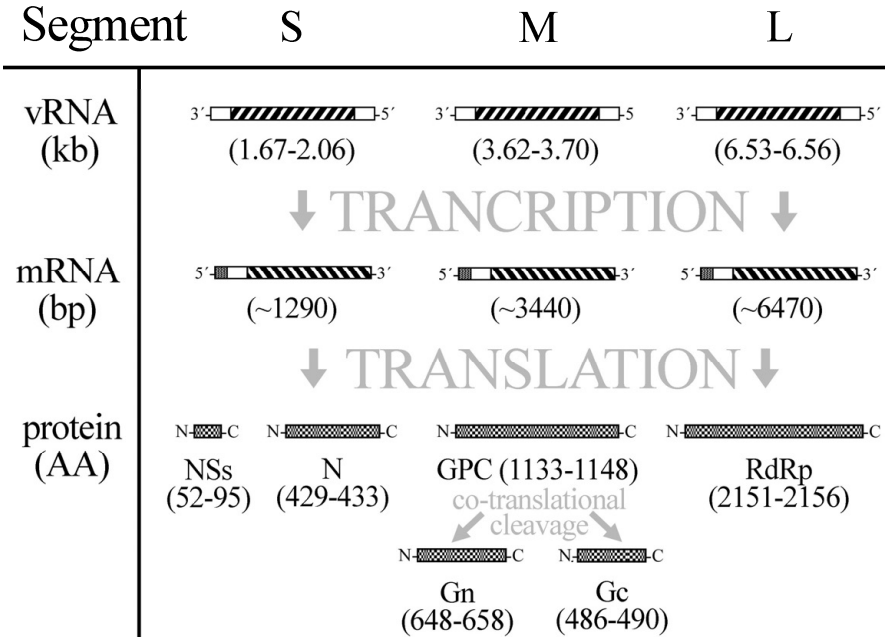

Figure 4. *Proteins encoded by the genome segments, numbers according to Plyusnin* [286].

N protein

The N protein, encoded by the S segment ORF, is a non-glycosylated protein of 429-433 amino acids with a molecular weight of approximately 50 kDa [135,163]. Even though N protein is devoid of a transmembrane sequence, it is membrane associated and localizes in infected cells to the perinnuclear region [158,298]. The N protein is expressed in large quantities, and hantavirus-infected cells stained with N protein antibodies are characterized by a granular staining pattern in immunofluorescence microscopy [196-198,393]. This staining pattern is presumably caused by aggregation of N protein to inclusion bodies that appear during infection [105,360], or by accumulation of N protein to cytoplasmic processing bodies (P bodies) [241]. The primary structure of this highly abundant protein is very conserved between all rodent-borne hantaviruses [163,286] and thus N protein contains a number of cross-reactive epitopes [69,70,107,211,214,215,217,247,322,325,376, 377,392,396]. In addition to the protection of vRNA and cRNA, the functions of N protein (described in more detail below) include interactions with the other structural proteins of the virion and with various cellular components [135].

Oligomerization of N protein

The oligomerization of N protein has been intensively studied, and the current understanding is that the oligomerization is mediated via both N- and C-terminal residues

[3,4,7,8,159-161]. A structural study of the N-terminal residues from 1 to 80, predicted to form a coiled-coil domain [4], revealed this region to form antiparallel coiled-coils when expressed alone [30,385]. Mutagenesis studies have pin-pointed the critical residues of the interaction between monomers, thus demonstrating the role of this domain in oligomerization of N protein [7,8]. Both intra- [7,8,30,385] and intermolecular [4] coiled-coiling via the N-terminal domain has been suggested. The crystal structure of this domain showed intramolecular coiled-coils [30], but intermolecular coiled-coils at high concentrations of this domain were observed by another group [4]. Regardles of the exact oligomerization mechanism of the N-terminal domain, the N protein trimer is most likely formed by head-to-head and tail-to-tail interactions [3,4,7,8,162]. The interaction with nucleic acids may contribute to the oligomerization of N protein, since its conformation has been observed to change as a result of binding to either mRNA caps or vRNA [243,246].

Interaction with RNA and formation of the RNP complex

The N protein has been shown to bind various different RNAs: unpecific RNA, tRNA, vRNA, cRNA and mRNA [106]. Even though N protein has the ability to associate with RNAs rather unspecifically, it binds to the 5'-terminus of vRNA with the highest affinity [151,243,329]. N protein has been shown to act as an RNA chaperone that mediates the dissociation and re-folding of the nascent RNA chains, thus presumably promoting the biological functions of hantavirus RNA [243]. The region that mediates the binding of HTNV N protein to RNA has been mapped to residues 175-217 [391]. Additionally, the C-terminal region has been shown to bind RNA [106], and also several positively charged residues in the N-terminal half of the molecule have been shown to contribute to the RNA binding [330].

The packaging of vRNA to form the RNP complex begins by formation of a trimeric N protein complex [3,7,159-161,163]. This complex then specifically recognizes the vRNA panhandle [242]. Curiously, the N protein trimer is also capable of genus-specific recognition of the viral panhandles [240], suggesting that also the panhandle structure plays a key role in the recognition. The binding of the panhandle results in conformational changes that presumably drive the further oligomerization of N protein [135,243]. Mature RNPs either extracted from virions or expressed in recombinant form appear as long helical filaments, when studied in EM [23,105]. The vRNA in RNPs is partially susceptible to RNase treatment, but the exact mechanism of RNA binding of hantavirus N protein is unclear [321]. For influenza A and vesicular stomatis virus (VSV) the sugar-phosphate backbone of vRNA is protected by interactions with N protein [21,142,178]. Similarly, the studies on Rift Valley fever virus (RVFV) N protein support the hypothesis that the vRNA would be wrapped around N protein in the RNP [302].

In addition to packaging, N protein has also a role in the transcription and replication via interactions to vRNA and cRNA [135,244]. It has also been shown to mediate the binding and sequestering of the cellular mRNA caps [241,246]. N protein localizes to the P bodies, where the cellular mRNAs are decapped and deadenylated [241]. N protein rescues the 5'-caps of cellular mRNAs from the P bodies in order to be used in viral transcription and translation processes [241,246,274]. After binding to the mRNA cap the conformation of N protein is altered, resulting in an increased affinity towards vRNA

[246]. As a result of binding to N protein the cap and vRNA anneal, and the mRNA cap is used as primer for the synthesis of the nascent viral mRNA [246].

Interactions with other viral proteins

The RNP is assumed either to contain or to be associated with the RdRp [321]. Reverse genetics studies of various bunyaviruses have indicated that both N protein and the RdRp are required for successful replication of vRNA [25,32,67,86,87], and indeed also hantaviruses obey this rule [84,287]. Even though the N protein and RdRp co-localize to the perinuclear and/or Golgi region [187], and increasing evidence suggests interplay between these proteins [43,244,246], no direct interaction between these proteins has been reported. A direct interaction would perhaps not be a necessity for packaging, since the cytoplasmic tails (CTs) of glycoproteins Gn and Gc could bind both components independently.

N protein or the RNP has for a long time been speculated to interact with the CT of one or both glycoproteins. This interaction would be crucial for the packaging of virions, since hantaviruses are devoid of a matrix protein [347]. The interaction between Gn-CT and N protein was demonstrated for UUKV [269,271] and very recently also recombinant Gn-CT of hantavirus was shown to interact with recombinant N protein [381]. At the beginning of this study this interaction had not been demonstrated for hantaviruses with full-length proteins.

Interactions with cellular proteins

In addition to the direct functions in the virus life cycle, discussed earlier in detail, the N protein interacts with various cellular proteins. IFNs, the key components of innate immune system, are considered as the first line of defense against invading viruses [314]. Type I and III IFNs induce the production of Mx proteins, antiviral effectors, which inhibit viral replication [116,117]. In humans MxA provides resistance towards RNA viruses of the families *Orthomyxoviridae, Paramyxoviridae, Rhabdoviridae, Togaviridae and Bunyaviridae* [116]. The induction of MxA production and subsequent inhibition of replication has been described for several hantaviruses [5,93,155,165,169,296,361]. The mechanism of MxA antiviral activity is not understood in detail, but presumably the main targets are viral nucleocapsid-like structures [314]. The antiviral effect of MxA in hantavirus infection is suggested to be mediated by reduction of the amount of free N protein required in the replication of vRNA [163]. Both pathogenic and apathogenic hantaviruses have been shown to induce MxA, but the induction is delayed in the case of pathogenic hantaviruses [102,296]. This delay is thought to be caused by either Gn-CT or NSs protein [5,6,143,144,169,231].

Viruses are known to utilize cytoskeletal components, such as the actin microfilaments and the microtubules, at various stages during their life cycle [54,132]. Actin may be deployed either during the entry (to transport the genetic material to the site of replication) or during the assembly and egress of progeny virions [340]. Microtubules have generally been associated with the entry of viruses, but they have also been hypothesized to be used by viruses in the egress [340]. The N protein of BCCV interacts with both monomeric and filamentous actin [300]. Furthermore, depolymerization of filamentous actin was demonstrated to reduce virus production, suggesting that actin

plays a role in the assembly or egress of Black Creek Canal virus (BCCV) [300]. In the case of HTNV the localization of N protein to the endoplasmic reticulum (ER) is driven by dynamin-mediated microtubule transport [294]. Additionally, the authors observed vimentin to re-organize into cage-like structures around the HTNV N protein, a phenomenon that they speculated to be essential for replication. In summary, both actin and microtubule networks are essential for the entry and post-entry steps of hantaviruses, and these interactions are mediated via N protein [295].

The screening of cDNA libraries either in yeast or mammalian two-hybrid system has revealed a multiplicity of interaction partners for N protein [162,194,202,218]. The first ligand that was identified using these methods was Daxx [202]. Daxx was initially identified as a Fas-mediated activator of Jun N-terminal kinase (JNK), thus being directly involved in apoptosis signaling [55,394]. Somewhat conflicting reports of hantavirus-induced apoptosis have been published, some showing that hantaviruses induce apoptosis and some not [1,121,157,175,201,224,264,293,354]. In addition to the apoptosis-related functions in the cytoplasm, Daxx functions as a transcriptional regulator in the nucleus [335]. The targeting of Daxx functions is mediated by small ubiquitin-related modifier-1 (SUMO-1) either via covalent modification or via non-covalent interactions [203,335]. Interestingly, the other reported interactions of N protein are linked to SUMO-1. Initially the N protein of PUUV [202], and soon after also the N protein of TULV was observed to interact with SUMO-1 [162]. Later reports described also the N proteins of other hantaviruses to interact with SUMO-1 related molecules [194,218]. The interactions of HTNV and SEOV N proteins with SUMO-1 conjugating enzyme (Ubc9) and with a SUMO-1 ligase, the protein inhibitor of activated STAT called PIAS, suggested that the hantaviral N protein might be modified by SUMO-1 [179,194]. Since Daxx and SUMO-1 have been shown to interact, it is tempting to speculate that there would be some sort of interplay between N protein and Daxx through SUMO-1. It remains to be studied, what would be the role of this suggested interaction and how it relates to the suggested hantavirus-induced apoptosis [202].

Quite recently the extensive studies by Mir *et al.* have indicated that the N protein of hantaviruses is directly involved in the initiation of translation at ribosomes [43,135]. This adds yet another crucial role to the broad repertoire of functionalities associated to N protein. First, N protein was shown to replace the multisubunit eI4F, indicating that N protein can directly mediate the initiation of translation [245]. The eIF4F complex consists of the cap-binding protein eIF4E and the RNA helicase eIF4A that are assembled on the scaffolding protein eIF4G [380]. No significant homology exists between N protein and the eIF4F complex, even though both are binders of the mRNA caps [245]. Additionally, N protein acts as a translation initiation factor also for non-viral proteins, suggesting that N protein bridges between the mRNA and 43S pre-initiation complex [274]. Indeed, N protein was found to interact with the small ribosomal unit 40S (a component of the 43S pre-initiation complex) via binding to its sub-components, ribosomal protein S19 (RPS19) and 18S rRNA [120]. A simultaneous binding of N protein to RPS19 and to the conserved triplet repeat sequence at the 5'-end of vRNA has led to the conclusion that N protein mainly promotes the translation of viral mRNAs [43].

Non-structural protein (NSs)

The N-protein-encoding ORF in the S segment of hantaviruses carried by *Arvicolinae* and *Sigmodontinae* rodents contains also an overlapping (+1 nucleotide) ORF that encodes for a putative NSs [286,349]. In the case of PUUV and TULV the NSs has been detected from infected cells and it is shown to localize to the perinuclear area [143,379]. The NSs of hantaviruses inhibits the activities of IFN-β, nuclear factor κB and IFN regulatory factor 3 (IRF-3) and thus presumably functions to prevent the innate immune response [143]. This hypothesis is supported by the observation that TULV with ORF for NSs survives longer than TULV without the ORF in an IFN-competent cell culture [144].

Glycoprotein precursor, GPC (Gn and Gc glycoproteins)

The M segment of hantaviruses codes for 1133-1158 amino acid residue GPC, depicted in Figure 5A [279,326,347]. The GPC is cleaved during translation, and full-length GPC has neither been observed during infection, in recombinant expression nor in virions [71,278,348]. Even though not shown for hantaviruses, the cleavage is presumably mediated by cellular enzymes since the GPC of UUKV phlebovirus is not cleaved during *in vitro* translation [370]. Furthermore, the cleavage efficiency of UUKV GPC differs depending on the cell type [10]. In the case of hantaviruses the cleavage occurs C-terminally to a completely conserved WAASA amino acid sequence, presumably by the cellular signal peptidase complex [207]. The WAASA sequence is located at the very C-terminus of Gn and it is a part of the transmembrane sequence (V in figure 5) that also acts as a signal sequence for Gc [207,278]. Successful cleavage of UUKV GPC also requires the presence of more than 50 (but less than 98) amino acid residues of the Gc [10]. In the case of UUKV only one processing site has been reported, and presumably this is the case also for hantaviruses, since the C-terminus of HTNV Gn has been reported to cover at least residues 588-614 and the cleavage occurs after residue 648 [10,327]. Also, there is no experimental evidence, such as identification of the resulting peptide, to support a secondary cleavage [207,327]. After co-translational processing of the GPC, the resulting proteins fold and mature together (as described in detail below); the N-terminal portion matures to Gn and the C-terminal portion to Gc [278,279,326].

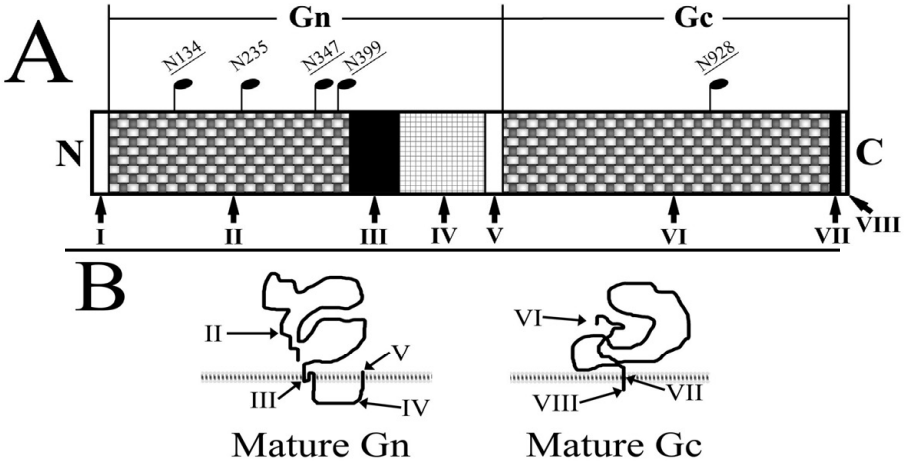

Figure 5. *The GPC encoded by the ORF in M segment. A) Organization of Gn and Gc in the GPC. The N-glycosylation sites are numbered based on HTNV GPC, underlined label indicates a conserved N-glycosylation site among hantaviruses. The other labels are: I signal sequence, II Gn ectodomain, III transmembrane helix, IV Gn-CT, V transmembrane helix and the signal sequence for Gc, VI Gc ectodomain, VII transmembrane helix, VIII Gc-CT. B) A schematic representation of the membrane topology of mature Gn and Gc.*

Folding and maturation of Gn and Gc

Besides the lipid envelope of the virion, the only structural components “visible” and exposed to the surrounds are the lipid-embedded glycoproteins [135]. Due to this very nature of the virion, virus-neutralizing antibodies target the glycoproteins Gn and Gc [56,130,131,320]. Neutralizing antibodies are an ideal tool for the study of glycoprotein folding, since they are able to bind the native and mature glycoproteins of the virion. Both intracellular and virion-associated glycoproteins have been shown to be endoglycosidase H (endo H) sensitive, indicating a high-mannose type of glycosylation [324]. Thus studying the folding and maturation by analyzing the trimming of sugar moieties is inapplicable in the case of hantaviruses.

Both Gn and Gc of hantaviruses localize to the Golgi complex during infection; however, conflicting results have been reported when Gn and Gc are expressed independently [278,279,312]. Ruusala *et al.* reported that the expression of both proteins is required for localization to the Golgi complex, and that they remain in the ER when expressed individually [312]. In contrast to this Pensiero *et al.* reported Gn to be able to localize to the Golgi complex without Gc, but co-expression was shown to be required for the transport of Gc from ER to the Golgi complex [278]. The Gn of viruses from all other genera of the family *Bunyaviridae* have been shown to localize to the Golgi complex independent of the expression of Gc [35,115,167,192,228,229,308,334]. Interestingly phleboviruses, for example, have been shown to possess a Golgi retention/targeting motif at the N-terminus of the Gn-CT [9,11,103]. The finding that a recombinant hantavirus Gn containing the 20 N-terminal residues of the CT was found to localize at Golgi complex would support the existence of such a signal also in hantaviruses [278]. However, other reports have shown Gn to reside at the ER when expressed independently [332,348], supporting the observations of Ruusala *et al*. Curiously, it was shown that the co-expression of SNV Gn and ANDV Gc resulted in localization of both proteins to the Golgi complex [60]. These authors also saw individually expressed Gn to at least partially localize to the Golgi complex. These reports provide clear-cut evidence for the fact that when expressed together both Gn and Gc localize to the Golgi complex. Whether hantaviruses are the only genus of the family *Bunyaviridae*, whose Gn is incapable of Golgi complex localization, or whether the observed differences are due to the expression system or the cell line used, remains to be studied further. If true, it could reflect the different surface structure of hantaviruses as compared to the other viruses of the family [226].

Curiously, some reports have demonstrated that the glycoproteins may, depending on cell type, be also transported to the plasma membrane [261,348]. Spiropoulou *et al*. suggested this phenomenon to reflect the difference between Old and New World hantaviruses, whereas Ogino *et al*. explained this phenomenon by the use of different cell lines [261,348]. It remains to be studied further, whether this is due to different budding sites utilized in different cell types or true differences between virus strains. It would, however, seem reasonable that hantaviruses would bud into the *cis*-Golgi similarly to other members of the family *Bunyaviridae*.

Glycosylation of Gn and Gc

Viral glycoproteins typically contain N-linked glycans, the function of which is linked not only to folding but also to receptor binding, membrane fusion and viral morphogenesis [63]. The GPC of HTNV contains six potential N-glycosylation sites, five on Gn (N residues 134, 235, 347, 399 and 609) and one on Gc (N928) [327]. Of these sites N134, N347, N399 and N928 are conserved among all hantaviruses [333] and are depicted in Figure 5A. At least in the case of HTNV and PUUV these sites have experimentally been shown to be occupied [148,333]. In addition to these sites, studies suggest that there is also variation in the glycosylation pattern between hantaviruses. HTNV Gn contains an additional N-glycosylation site at N235 and PUUV Gc contains an O-glycosylation site at threonine residue 985 [148,333]. The glycans of Gn and Gc are endo H sensitive, indicating them to be of high-mannose type [14,324]. N-glycosylation has further been shown, in addition to folding and intracellular transport, to also play a key role in the formation of glycoprotein epitopes [333]. Mutations in N-glycosylation sites were also observed to affect the complex formation and thus also the intracellular localization of Gn and Gc [333]. The composition of the N-glycans is indicative of the cellular localization of the target protein, since further processing of high-mannose type glycans occurs at medial- and *trans*-Golgi compartments [192]. This indicates that the Gn of hantaviruses is only able to transit to the *cis*-Golgi, as suggested earlier by co-localization with a *cis*-Golgi matrix protein GM130 [332,333].

Fusogenic activity of the GPC

Hantaviruses possess a fusogenic activity, as indicated by induction of infected cells to form syncytia under acidic conditions [18]. The formation of syncytia was demonstrated to occur at pH values below 6.3 [18]. The ability to induce syncytia varies between hantavirus strains, since acid treatment of PUUV, HTNV and SEOV infected cells respectively yielded small, medium-sized and large syncytia [232]. The size difference of the induced syncytia might indicate differences in the pH required for induction, the surface localization of glycoproteins or the amount of virus produced. The expression of full-length GPC transforms cells prone to syncytia formation under acidic pH and glycoprotein antibodies have been demonstrated to prevent is phenomenon [261]. Two of these fusion-preventing antibodies have their epitopes in Gc, but also one Gn specific antibody (3D5) was reported [261]. This antibody has been suggested to recognize a heterodimeric glycoprotein complex [333,383], and thus it can be concluded that the fusogenicity is more likely to reside in Gc. The fusogenicity of the GPC was further confirmed by demonstrating that also the GPC of different hantavirus strains transforms cells prone to low pH induced syncytium formation [401].

Mutational analysis of the N-glycosylation sites in the GPC have shown that only the N-glycosylation of Gc is crucial for fusogenicity, thus favoring the hypothesis that Gc is the fusion protein of hantaviruses [400]. Sequence comparison and bioinformatics studies have further suggested that the Gc of hantaviruses (and other *Bunyaviridae* members) is a class II viral fusion protein [97,366], as described later in more detail. Tischler *et al*. demonstrated that the putative fusion loop of Gc is able to bind artificial membranes [366]. The same group continued their studies using Gc expressed from a lentiviral vector system [48] and confirmed the presence of a functional fusion loop in the intact Gc

[47]. Additionally, they showed by mutagenesis that the aromatic and the polar residues in the fusion loop are crucial, not only for cell fusion, but also for infectivity [47].

Formation of spike complex

It is obvious that the spike consists of the glycoproteins Gn and Gc; however, the number of Gn and Gc units in the spike, in other words the exact composition of the spike is not known. The fact that Gn and Gc are expressed from a common precursor, and that co-expression is needed for their localization to the Golgi complex [279,312,327], suggests that equal amounts of both proteins would be present in the virion. The folding studies indicating that Gn and Gc co-expressed from two separate vectors localize similarly to the glycoproteins expressed from a common precursor [60,278,279,312,332,348] suggest that also unequal expression of Gn and Gc would be possible. This would enable the incorporation of unequal amounts of Gn and Gc to the virion.

The surface of hantaviruses has been described as grid-like [226], suggesting at least a two-fold, most likely a four-fold, symmetry to exist on the surface of the virion. This geometry could be explained by the oligomerization of heterodimeric Gn-Gc as suggested by Antic *et al*. [14]. The Gn of hantaviruses has been described to form SDS-stable complexes [348], suggesting that Gn has a tendency to form homo-oligomeric complexes. Both Gn and Gc of UUKV exist as homodimers when extracted from virions [307], even though, similarly to hantaviruses the proper folding of UUKV Gc requires co-expression with Gn [280]. The surface structure of phleboviruses UUKV and RVFV displays an icosahedral symmetry [92,134,268] that is presumably maintained by contacts between heterodimeric Gn-Gc units [134]. Assuming that the Gn-Gc heterodimer is the basic unit on the surface of the virion, it remains unclear how homodimeric Gn and Gc units are formed when UUKV glycoproteins are extracted from virions [307]. Since the Gn and Gc of hantaviruses have been reported to resemble in cellular localization the Gn and Gc of other family *Bunyaviridae* viruses, the surface differences between hantaviruses and other viruses are enigmatic. Based on the co-localization of glycoproteins to the Golgi complex, the spike complex of the family *Bunyaviridae* viruses is presumably assembled at either ER or *cis*-Golgi [35,115,167,192,228,229,308,334].

Cytoplasmic tails (CTs) of Gn and Gc

Both Gn and Gc of hantaviruses contain a CT (or a cytoplasmic domain) with respective sizes of ~110 and ~10 amino acid residues [347]. The CT of Gn has been studied more extensively, since it has been proposed to act as a surrogate matrix protein of hantaviruses [135]. By sequence comparison Gn-CT is also more conserved than Gc-CT, where only the transmembrane flanking cysteine and proline are conserved, as indicated in Figure 6B. The conserved cysteine residue right at the cytoplasmic face of the transmembrane sequence of Gc could mediate palmitoylation, since both Gn and Gc of UUKV are palmitylated [10]; however, this has not been studied in the case of hantaviruses.

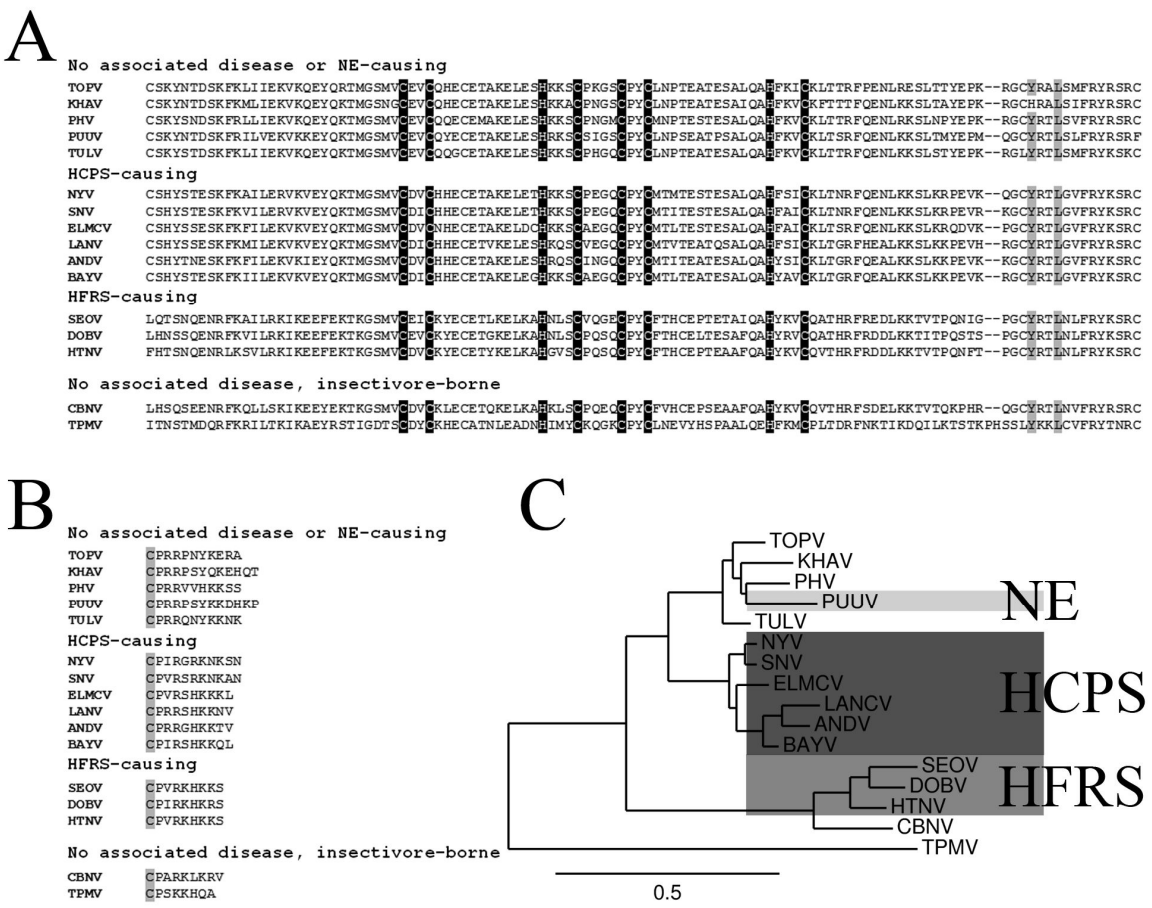

***Figure 6**. Cytoplasmic residues of Gn- and Gc-CT. A) Alignments of the Gn-CTs. The residues involved in the zinc finger (ZF) domain are highlighted by black boxes. The highly conserved YxxL motif is indicated by grey boxes. B) The Gc-CTs of hantaviruses, the grey box indicates cys-residue that by CSS-Palm 2.0 prediction (available at: http://csspalm.biocuckoo.org/,* [304]*) is palmitoylated. C) A phylogenic tree based on the Gn-CT sequences (created by Phylogeny.fr, available at http://www.phylogeny.fr/,* [59]*). The non-highlighted viruses are considered apathogenic. Alignments in A and B were done using ClustalW2 (available at: http://www.ebi.ac.uk/Tools/msa/clustalw2/,* [44]*).*

The Gn-CT of hantaviruses contains an approximately 50-residue domain that by prediction forms a zinc finger (ZF) [100], highlighted in Figure 6A. This region of Gn-CT was suggested to be a RING finger domain [100], but NMR analysis of the domain revealed it to fold as a tandem CCHC ZF [79]. It remains to be seen whether the regions flanking the ZF domain, present in intact Gn-CT, would affect the folding of the ZF domain [79]. The function of this domain remains to be solved, since it did not bind nucleic acids [79] and presumably it also does not bind N protein.

The C-terminus of Gn-CT contains a degradation signal or degron (V in Figure 5) that has been suggested to direct Gn-CT to proteolytic degradation [100,328,382]. Expression of this region resulted in the degradation Gn-CT of pathogenic but not of apathogenic hantaviruses [328]. Using a similar approach also the Gn-CT of TULV was shown to be degraded, arguing against this hypothesis [382]. The degron is in fact the signal sequence for Gc and thus also a transmembrane sequence. Therefore the most likely explanation for the observed proteolytic degradation is the aggregation of Gn-CT expressed without proper targeting to the ER [382]. Unfortunately only in the initial study describing the degradation of Gn-CT was the full length Gn-CT including both

transmembrane helices expressed [100], and by using a single inhibitor (ALLN, less specific than other inhibitors of the proteasome) the authors concluded Gn-CT to be proteasomally degraded. The degradation was shown to be mediated by tyrosine residues locating to the C-terminus of the Gn-CT, where immunoreceptor tyrosine-based activation motifs (ITAMs) have been found for HCPS-causing hantaviruses [100,101,249]. However, by prediction only the first YxxL of the ITAM motif should be in the cytosol (Figure 6A shows only cytosolic residues and thus lacks the second YxxL), while the second would be buried in the membrane. The first YxxL is conserved among practically all hantaviruses (Figure 6A.), and typically such a motif acts as a targeting signal for endosomal-lysomal pathway [24,374]. Therefore the initially observed degradation of Gn-CT [100] could also be mediated by endosome-lysosome route, supported by the observation that a population of Gn co-localizes with LAMP-1 (a marker for late endosome-lysosome) during expression [348]. In order to mimic the natural localization of Gn, the functions of Gn-CT should be studied by including both N- and C-terminal transmembrane sequences in a construct with a proper signal sequence. This has unfortunately not been done.

The pathogenic and apathogenic hantaviruses have been shown to regulate differently the cellular responses to infection [102]. This difference is suggested to reside in the Gn-CT, which in the case of pathogenic hantaviruses has been reported to interfere with retinoic acid inducible gene I (RIG-I)- and TANK-binding kinase 1 (TBK1)-directed IFN responses [5]. This interference has further been shown to be mediated upstream of IRF-3, at the level of complex formed by TBK1 and TNF receptor associated factor 3 (TRAF-3) [6]. Gn-CT inhibits the formation of TBK1–TRAF-3 complex by binding to TRAF-3 [6]. Also the C-terminal residues of TULV Gn-CT have been shown to inhibit early IFN responses, but do so by interacting with TBK1-complexes rather than TRAF-3 [231]. Curiously, also replication deficient SNV particles induce a strong IFN response independent of IRF-3 activation [292].

RNA-dependent RNA-polymerase (RdRp, L Protein)

The RdRp encoded by the L segment of the genome consists of approximately 2150 amino acids, and is the largest protein encoded by hantaviruses [12,13,74,186,282,352]. The molecular weight of the RdRp has been estimated to be approximately 250 kDa by mobility in SDS-PAGE, and the protein is shown to localize into the perinuclear area [187]. The RdRp of phleboviruses functions as oligomers [398] and likely this is also the case for the RdRp of hantaviruses. Membrane association of the RdRp and N protein of hantaviruses has suggested that the transcription and replication events take place on cellular membranes [187,298]. An interesting feature of the hantavirus RdRp is its ability to induce recombination of homologous RNA sequences, thus enabling virus evolution via superinfection [287]. The number of complete hantavirus RdRp sequences is limited, and among the known sequences the amount of identical amino acids varies between 69% and 98% [257]. It has been shown that the RdRp of hantaviruses, like the RdRps of other viruses, requires a divalent cation (either Mg^{2+} of Mn^{2+}) for activity [321]. There is a preference for Mn^{2+} but whether it is essential for *de novo* initiation of RNA synthesis, as is the case for hepatis C virus (HCV) RdRp, is not known [22,208,402]. Even though the primary sequences of the RdRps from different viruses are variable, some conserved

motifs do exist [33,38]. These motifs, consisting of conserved amino acid side chain functionalities, appear among different RdRps in a specific sequence with similar distances between the motifs [33,250,289]. Apart from the sequence and homology studies [13,186,257,282] little is known of this, perhaps the most important protein of hantaviruses.

Entry of viruses

In order to be internalized into (mammalian) host cell, a virus needs an effective disguise to complete a number of steps such as to hide from the immune system, to attach to target cells, to find and to bind to the receptor [339]. There are several variations on a theme, that is, the internalization of viruses can be mediated via a wide variety of mechanisms such as clathrin-mediated endocytosis, macropinocytosis, caveolar/lipid raft mediated endocytosis and phagocytosis [235]. Being internalized to the host cell is not enough per se, since the virus may still need to escape to the cytosol and to be transported to the site of replication [225]. The release of the genetic material in the case of enveloped viruses occurs via fusion of the viral membrane with the cellular target membrane [339]. The fusion can be triggered by binding to a receptor, by exposure to low pH or to reducing environment, by enzyme-induced modification, and by combinations of the above [225,339].

Entry of hantaviruses

Unlike for the other members of family *Bunyaviridae* viruses, several putative receptors have been described for hantaviruses. The receptors include decay-accelerating factor (DAF), complement receptor gC1qR, an unknown 70 kDa protein, as well as β1- and β3-integrins [36,46,98,99,170,182,193,248,303,346]. Additionally, it has been shown that lectins promote the entry of hantaviruses [262], suggesting that the glycosylation of viral proteins plays a role in the attachment to host cells. The entry of hantaviruses has been demonstrated to occur preferably from the apical side of polarized epithelial cells [299], however, also entry from the basolateral side has been reported [310].

After successful attachment to the cell surface, the entry of HTNV continues in clathrin-coated vesicles [147,295]. However, contrary to HTNV, ANDV was shown not to use clathrin-mediated endocytosis, indicating that different routes may be utilized among hantaviruses [295]. This agrees with the observations made with UUKV, whose entry is partially clathrin-mediated but involves also other routes for internalization [210]. Most probably the internalization routes of bunyaviruses overlap after the viruses have entered the early endosome. The entry of other family *Bunyaviridae* viruses requires low pH (from 5.4 to 6.3) [114,210,284,285,307] and for hantaviruses the required pH ranges from 5.8 to 6.3 [18,47,232,261,301,401]. This suggests that the bunyaviruses fuse either with early or late endosomes [210,225]. It is, however, not known whether there would be differences regarding the site of fusion between genera or even strains. The N protein of internalized HTNV has been demonstrated to co-localize with LAMP-1, a marker for late endosomes and lysosomes, suggesting that the fusion does not occur at the early endosome [147]. This observation could also reflect a different route used by hantaviruses as compared to other family *Bunyaviridae* viruses or “overloading” of the cells with the virus. The mechanism how hantaviruses manage to transport their RNPs to

the site of replication is not known; however, the interactions of N protein with microtubules and actin suggests that these cellular machineries might be utilized [294,295,300].

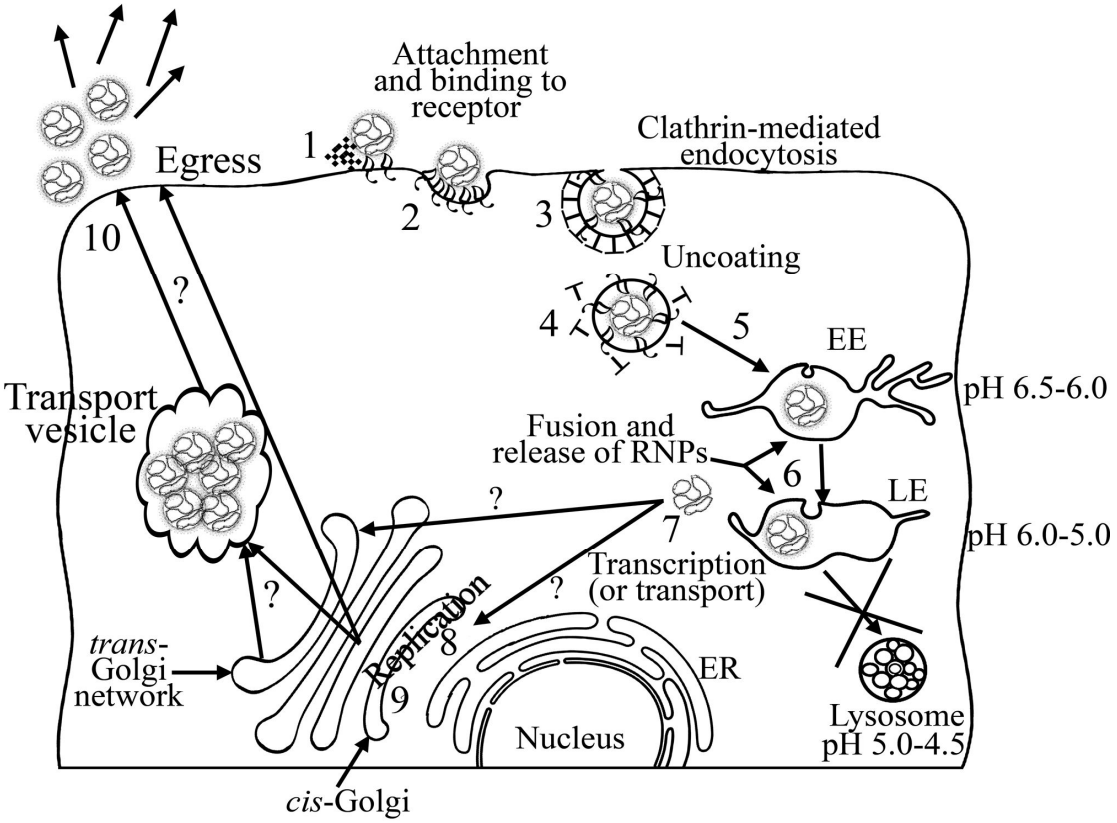

Figure 7. *Hantavirus life cycle. (1) The virion attaches to the cell surface by binding to the receptor either directly or via a viral attachment factor. (2) The binding of virion to the receptor induces endocytosis signaling, for instance via clustering of the receptors. (3) The virion enters the cell in clathrin-coated vesicles, (4) the clatrin-coat of the vesicle is disassembled and (5) the virion-harboring vesicle enters the early endosome (EE), which (6) matures to late endosome (LE). (7) Fusion of the viral membrane and the membrane of EE or LE is driven by acid-induced conformation changes in the Gc, resulting in the release of the RNPs. The initial transcription takes place following fusion either at the site of release or the RNPs may be transported to the ER-Golgi intermediate compartment (ERGIC) via dynein-dependent transport of N protein* [294]*, where the initial transcription would alternatively occur. It is also possible that the virus could be directly transported to the Golgi complex from LE either after or before the fusion. (8) The replication occurs presumably in virus factories located either at the ERGIC or the cis-Golgi, as shown for Bunyamwera virus* [89]*. (9) The viruses bud into the cis-Golgi, from where they are transported to the plasma membrane for release, presumably via recycling endosomes. (10) The egress of progeny virions takes place at the plasma membrane. Alternatively, budding directly at the plasma membrane has been suggested for New World hantaviruses.*

Viral fusion proteins

The successful entry of enveloped viruses requires at some stage the release of the genetic material from the virion [88]. This process is mediated by the membrane-embedded viral fusion proteins [61,126]. There are ten characteristic features that apply to most viral fusion proteins, which are (1) the fusion protein is a type I membrane protein, (2) the majority of fusion protein locates outside the viral membrane, (3) the protein(s) contains an N-glycosylation(s), (4) the protein(s) has the ability to form oligomeric complexes, (5) the protein is abundant in the viral membrane, (6) the protein contains a sequence responsible for the fusion, (7) the protein exists in the virion as a

tight homo- or hetero-oligomeric complex, (8) the protein either mediates the binding to cell directly or is in complex with a protein that serves this function, (9) the protein is typically expressed as a large precursor, and (10) the protein requires proteolytic activation [68,126]. The fusion proteins have been classified depending on factors such as the mechanism of activation, the complexes formed by the fusion protein, the orientation in respect to the viral membrane and the location of the fusion peptide [61]. The initial classification contained two classes [61,341]; however, recently a third class has been included [388]. Common to all classes of viral fusion proteins is that after a successful fusion the protein exists in a homotrimeric complex, where the N- and C-terminal regions are brought into close proximity to one another [388].

Class I fusion proteins

The characteristic feature of class I fusion proteins is that they exist as homotrimeric complexes both on the virion surface (in the pre-fusion state) and after the fusion [388]. The homotrimeric complex is maintained by contacts between a central trimeric α-helical coiled-coil structure that is complemented with C-terminal helices, forming a bundle of six helices [68,388]. The fusion peptide is located to the N-terminus of the protein, and the proteins are typically primed for fusion by a proteolytic cleavage [61]. The fusion of a class I fusion protein is triggered either by binding to a receptor or by exposure to acidic pH [61,341,388]. Class I fusion proteins are irreversibly altered in conformation after the fusion reaction [61].

Class II fusion proteins

The class II fusion proteins exist in virion as either homo- or heterodimers [341]. The fusion peptide or actually a loop (since it is not N- or C-terminal) is in the pre-fusion conformation hidden by a tight interaction between two glycoproteins [341,388]. The fusion of class II fusion proteins is triggered by acidic pH that initially induces the dissociation of the proteins and exposes the fusion loop [68,341]. The resulting changes in conformation of the fusion protein lead to the attachment of the fusion loop to the target membrane, which is followed by further changes in fusion proteins conformation, eventually leading to the formation of a tight homotrimeric complex [61,68,341]. The changes in protein conformation finalize the fusion between viral and cellular target membrane [341]. The conformational changes upon exposure to low pH are considered to be irreversible in the case of class II fusion proteins [61].

Class III fusion proteins

The “new-comers” to the viral fusion proteins are the group of class III fusion proteins [388]. This class shares features from both class I and II, being trimers with central α-helical coiled coils in the pre-fusion state, but having fusion domains/loops that resemble those of class II fusion proteins [388]. A distinguishing feature is that the conformation changes of class III fusion protein(s) are described to be reversible [61,388].

Assembly of hantavirus particle

The assembly of hantaviruses presumably takes place at the *cis*-Golgi, where the mature Gn and Gc along with N protein locate during infection and expression [298,311,332,333]. For Bunyamwera virus the assembly has been shown occur in a viral

factory situated in the ERGIC or the Golgi complex [260,315]. Even though not shown for hantaviruses, the evidence of N protein associating with microtubules and actin along with localization to the ER-Golgi suggests a similar system [294,295,300], as indicated in Figure 7. The required contacts for successful packaging of infectious progeny viruses consist of the following: the encapsidation of vRNA by N protein (described above), the interaction between RdRp and RNP, the binding of RNP to the spike complex and the interactions between spikes. From these key interactions only the encapsidation of vRNA has been studied in great detail [3,4,7,8,106,159-161,240,242,243,329]. Even though the formation of the spike complex is poorly understood (as discussed above), Gn-CT has been shown to bind N protein using recombinant proteins [381]. Furthermore it has been shown that VLPs can be created by co-expression of the S and M segment [23,200]. This indicates that the assembly would require N, Gn and Gc protein but not necessarily the RdRp, as shown also for RVFV [283]. Currently it is not known how the RdRp gets incorporated into the progeny virions but most probably interaction to either vRNA, to N protein or to the spike complex is required. The assembled virions are thought to bud into the Golgi, from where they are presumably transported to the plasma membrane via recycling endosomes [311]. The release of virions may depend on the cell type since both apical and basolateral egress has been reported [299,310].

AIMS OF THE STUDY

Hantaviruses were initially isolated at the end of 1970s. The studies on hantavirus structure have been hampered by the fact that hantaviruses generally grow to low titers, thus limiting their EM studies. Prior to this study the surface of hantavirus particle had been described to display a grid-like pattern in negatively stained EM preparations. It was not, however, known how this grid-like appearance would be generated by the glycoproteins Gn and Gc. The glycoproteins were previously assumed to form a heterodimeric Gn-Gc complex, mainly because the proper folding and localization requires co-expression. The assembly of virions was assumed to be mediated by an interaction between Gn and the RNP. This study was initially designed to experimentally demonstrate these prevailing dogmas of the hantavirus research field.

The specific aims were:

I To establish an efficient but gentle purification protocol for hantaviruses.

II To study the interactions between the structural components of the virion.

III To determine the surface structure of hantavirus in detail.

IV To study the interactions of structural proteins with cellular proteins.

V To study the assembly and/or packaging of virions.

MATERIALS AND METHODS

The materials and methods used are briefly described below. A more detailed description for each method is provided in the respective original publication.

Cells and virus cultivation (I-IV)

Vero E6 green monkey kidney epithelial cells (ATCC 94 CRL-1586) were used in all studies for virus cultivation. The cells were grown and propagated in minimal essential medium (MEM) supplemented with 2 to 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

The viruses used were PUUV (strain Sotkamo) and TULV (strain Moravia 5302). Virus inoculations were in all studies done in 75-cm^2 flasks to 75-90% confluent Vero E6 monolayers by allowing the 1:50-1:100 (multiplicity of infection, MOI, 0.01-0.1) diluted virus-containing supernatant to adsorb for 1 h at 37 °C. The virus-containing supernatants were collected at 5 to 14 d.p.i. with TULV and at 7 to 21 d.p.i. with PUUV. The titers, determined according to Kallio *et al*. [153], for virus batches varied between 10^6-10^8 focus-forming unit (FFU)/ml for TULV and 10^4-10^6 FFU/ml for PUUV.

Purification of viruses (I-IV)

Depending on the following experiments the viruses were either purified by pelleting through a sucrose cushion or by sedimentation in a density gradient. For a *Bunyaviridae* virion the M_r and the S_{20w} values are 3-4*10^8 and 350-500 S, respectively [221]. The *k*-factor for SW28 rotor (Beckman) at 27.000 RPM is 264.0 (http://www.beckmancoulter.com/resourcecenter/labresources/centrifuges/rotorcalc.asp). Thus using the S_{20w} of *Bunyaviruses* and the known relation between *k*-factor and sedimentation coefficient, T=K/S [306], the pelleting of hantaviruses would require roughly 30 to 45 min ultracentrifugation at this speed.

Rough purification and approximately 250- to 1000-fold concentration of viruses was done by pelleting the pre-cleared (passed through a 0.22 μm-pore-size filter) growth medium through a 3-ml 30% (wt/vol) sucrose cushion (Beckman SW28 rotor; 27,000 rpm for 2-4 h at 4 °C) in a suitable buffer. Commonly 10-25 mM HEPES with 100-150 mM NaCl, pH 7.4 was used as the buffer for sucrose solutions.

More delicate purification of viruses was done by sedimentation in a preformed density gradient. Viruses, grown in MEM supplemented with 2-3% of FCS prefiltered through a 100-kDa cut-off centrifugal filter device (Millipore), were preconcentrated approximately 250- to 500-fold using similar device, applied on top of a preformed density gradient of either sucrose (0-70%) or OptiPrep™ (0-50%) in SW41 tubes (Beckman) and centrifuged for 14-17 h at 25,000-28,000 RPM at 4 °C.

Antibodies (I-IV)

The antibodies used in the original articles are described in table 1.

Table 1. *Antibodies used in articles* ***I-IV****. The abbreviations used are IP=immunoprecipitation, IF=immunofluorescence and WB=western blot.*

Hantavirus specific antibodies

MAbs	Origin	Specific antigen	Cross-reactive with	Applicable in	Reference
MAb 5A2	mouse	PUUV Gn	-	IP, IF	[216]
MAb 1C9	mouse	PUUV Gc	-	IP, IF	[212]
MAb 4G2	mouse	PUUV Gc	-	IP, IF	[216]
MAb 5E1	mouse	PUUV N	TULV N	IP, IF, WB	[216]
MAb 3C11	mouse	TULV N	PUUV N	IP, IF, WB	[217]
PAbs					
Anti-Gn	rabbit	GST - PUUV Gn	TULV Gn	IP, IF, WB	[376]
Anti-Gc	rabbit	GST - PUUV Gc	TULV Gc	IP, IF, WB	[376]
Anti-2/3N	rabbit	GST - PUUV 2/3N	TULV N	IP, IF, WB	[376]
Patient sera	human	PUUV	TULV	N.D	

Other antibodies

MAb	Origin	Specific antigen	Supplier	Applicable in	Reference
SP-2	mouse	90K/Mac-2BP	Bender MedSystems	IP, WB	[137]
PAb					
Anti-Mac-2BP	rabbit	Mac-2BP (84-383)	Santa Cruz Biotech	WB	-

Secondary antibodies / Conjugates

Specificity	Origin	Label	Supplier
Anti-mouse IgG	rabbit	HRP	Dako
Anti-mouse IgG	goat	Alexa Fluor 680	Invitrogen
Anti-mouse IgG	donkey	IRDye 800CW	LI-COR Biosciences
Anti-mouse IgG	sheep	IRDye 800	Rockland
Anti-rabbit IgG	swine	HRP	Dako
Anti-rabbit IgG	goat	Alexa Fluor 680	Invitrogen
Anti-rabbit IgG	sheep	IRDye 700	Rockland
Anti-rabbit IgG	donkey	IRDye 800CW	LI-COR Biosciences

Immunoprecipitation and pull-down (I,III,IV)

Immunoprecipitation (IP) and co-IP experiments were done using either protein G-Sepharose (GE Healthcare, Amersham Biosciences) or Gammabind G Sepharose (GE Healthcare, Amersham Biosciences). The amount of primary/specific antibody in IP or co-IP varied from 5 to 10 μg and the amount of Sepharose beads from 20-30 μl between the experiments. Two different approaches for the IP and/or co-IP experiments were used; the Sepharose beads pre-loaded with the primary antibody were incubated with protein solution, or the antibody was allowed to form immunocomplexes with the protein

solution prior to precipitation. The precipitated proteins were typically eluted using Laemmli sample buffer (LSB).

In pull-down experiments thiopropyl Sepharose 6B (GE Healthcare, Amersham Biosciences) and monomeric avidin (Pierce Biotechnology) beads were used to immobilize peptides via cysteine residues or via N-terminal D-biotin, respectively. Peptide coupled beads were used to pull down proteins from solutions similarly as described for the IP and co-IP experiments. Wheat Germ Agglutinin Sepharose (WGA, Amersham/GE Healthcare) beads were used to pull down glycoproteins via carbohydrate moieties. Elution in pull-down experiments was done by boiling the beads in LSB.

SDS-PAGE and immunoblotting (I,III,IV)

Protein separations on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, [189]) were carried out using routine protocols on gels with acrylamide concentrations varying from 5 to 15%. The separations were done either under reducing or non-reducing conditions, respectively using LSB with or without mercaptoethanol. The proteins separated in SDS-PAGE were transferred onto nitrocellulose using wet-blotting when performing immunoblots. The nitrocellulose membranes were incubated 15 to 60 min at RT in buffer containing 1-3% of either skim milk or BSA (blocking buffer) to block unspecific binding to the membrane. The primary antibodies at 1:1000 to 1:100 dilutions in blocking buffer were incubated with membrane either 1 to 2 h at RT or overnight at +4 °C. Horseradish peroxidase (HRP) labeled secondary antibodies were used at 1:1000 dilutions in blocking buffer and IRDye conjugated secondary antibodies at 1:10000 to 1:5000 dilutions.

Peptide synthesis (I,III,IV)

Soluble peptide syntheses were carried out either using Applied Biosystems peptide synthesizer 433A or Intavis MultiPep via Fmoc chemistry. Coupling of each amino acid residue was done using 2.5- to 10-fold excess of an activated Fmoc-derivative. Activation was done by mixing amino acid, HCTU (2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate) and NMM (4-methylmorpholine) at respective 1:1:2 ratio. The couplings, generally done in duplicate, were performed at RT with 15 to 45 min reaction time. The side chain deprotection of peptides was done simultaneously to the resin cleavage. Peptide purifications were done using reversed-phase HPLC (RP-HPLC) and peptide quality was checked using mass spectrometry.

The synthesis of SPOT and CelluSpot peptide arrays (described below) was carried out using Intavis MultiPep or by Abimed Autospot Robot ASP222. Synthesis on modified cellulose was done utilizing Fmoc chemistry via DIC (*N,N'*-Diisopropylcarbodiimide)/HOBt (1-Hydroxybenzotriazole) activation. Capping with acetic anhydride was done after each coupling to limit the sequence mutations to N-terminal deletions of the parent sequence.

Interaction site mapping (I,III)

The protein-protein interaction site mappings were done mainly by utilizing SPOT peptide array (reviewed in [91,128,389]). A set of overlapping peptides covering the whole primary structure of one of the interaction partners was synthesized on a cellulose

membrane. The membrane-bound peptides were probed with the interaction partner, whose binding was detected by using a specific antibody. The probing and detection of the SPOT membranes was done analogously to immunoblotting. A schematic illustration of the interaction site mapping using SPOT peptide assay is shown in Figure 8.

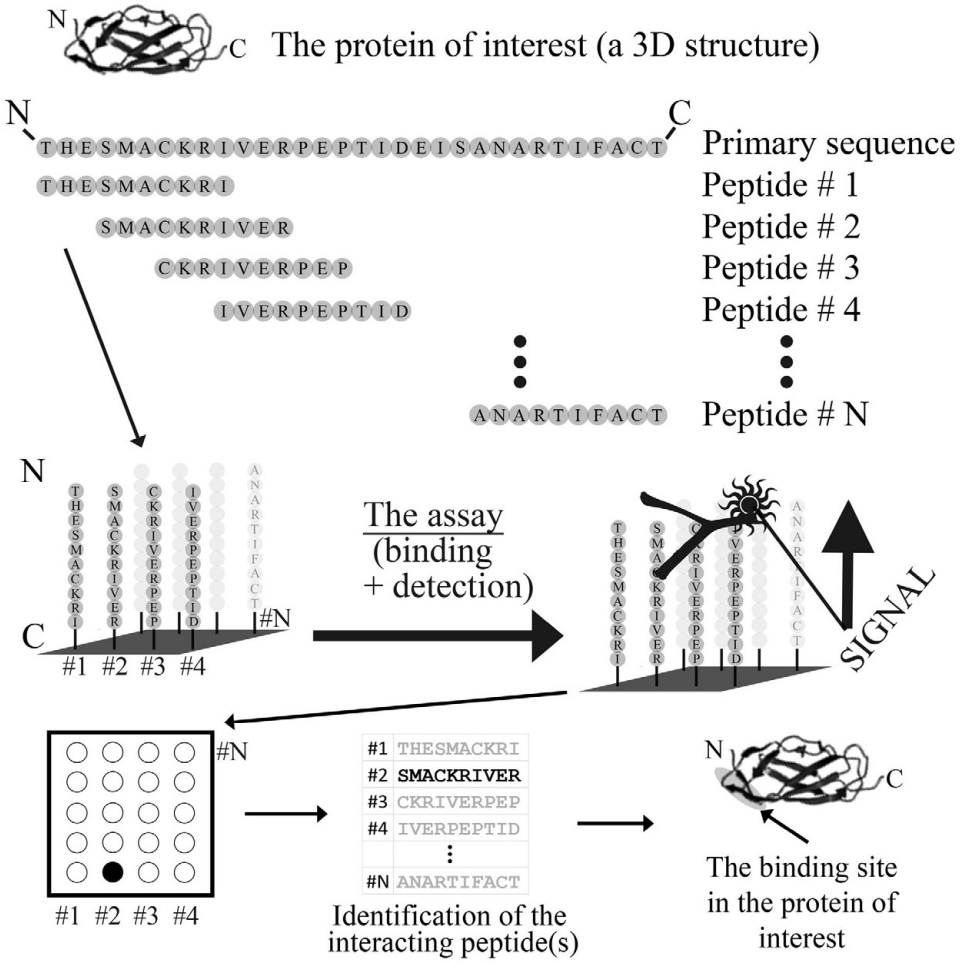

***Figure 8**. Schematic illustration of the interaction site mapping using SPOT peptide array. A set of overlapping peptides covering the primary sequence of the protein of interest is synthesized on a modified cellulose membrane. The membrane is probed with the interaction partner, followed by detection of the binding peptides (enhanced chemiluminescence (ECL), autoradiography, fluorescence, etc.). Each positive "spot" corresponds to a known peptide sequence, and thus the region involved in the interaction can be mapped to peptide level. If the structure of the protein of interest is known, the binding site can be studied in the structure.*

Cryo electron microscopy (I,II)

Fresh preparations of TULV purified by density gradient ultracentrifugation were pipetted onto 400-mesh copper grids covered with carbon film and vitrified in liquid ethane. Standard cryo-EM micrographs were recorded at -180 °C under low electron dose conditions using Tecnai F20 field emission gun (FEI) transmission EM operated at 200 kV with a nominal magnification of 50,000x. Samples for electron cryotomography were prepared similarly, but with addition of BSA-coupled 10-nm colloidal gold particles. For electron cryotomography the micrographs were recorded at -196 °C with 300-keV Polara FEI equipped with a GIF 2002 energy filter. The tilt series covering an angular range of -60 to 60° were collected at -5 to -6 μm defocus under low-dose conditions (less than 100 electrons/Å^2 per series) with a magnification of 67,300x.

Electron cryotomography (II)

The 3D reconstructions (tomograms) of virions were calculated in IMOD utilizing colloidal gold particles as markers. A total of forty-five spherical and five tubular virions were used in the analysis of surface structure. The structure of the spike complex was solved using iterative template matching, alignment and averaging of aligned subvolumes by a program, Jsubtomo (available at http://www.opic.ox.ac.uk/mediawiki/index.php/Downloads), written to carry these tasks. Alignment with 2-fold symmetry was based on cross-correlation between the template volume for the spike and the tomographic volume of a virion. The initial template volume was generated by manually averaging 10 subvolumes, and in further rounds these subvolumes provided a template for the next round. The final average for the spike volume was calculated using 2353 subvolumes and the resolution was assessed by Fourier shell correlation.

Sedimentation in sucrose gradient (I)

Estimations of the molecular weights of protein complexes were done by sedimentation in sucrose density gradient. Molecular weight determinations were based on both comparisons to the mobility of marker proteins of known molecular weights and on calculations based on sucrose concentrations of the density gradient fractions. The calculations were done based on pre-calculated tables [233] assuming of 1.4 g/cm^3 [83] to be the density of proteins.

Gel-filtration chromatography (I,III)

Gel filtration chromatography was applied for the separation of viral components and for the estimation of their molecular weight, based on comparisons to the retention times of marker proteins. The separations were done in Sephacryl S-200HR (GE Healthcare) column operated by BioCAD Vision Workstation (Perceptive BioSystems, Applied Biosystems). Fractionation of the structural components of virion was done by applying a lysate of viruses, pelleted through sucrose cushion, to gel filtration column and by collecting equal-sized fractions.

Mass spectrometry, MS (I, III, IV)

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS was used to confirm the quality of the synthetic peptides. The RP-HPLC fractions containing the peptide of interest were mixed with α-cyano-4-hydroxycinnamic acid and dried on a massive steel plate. The MS (Auto-Flex III TOF/TOF, Bruker Daltonics) analysis was typically done in negative mode. When analyzing the trypsin digests of the unknown protein (IV), two peptides were *de novo* sequenced by laser induced fragmentation. The peak lists of the fragmented peptides were sent to Mascot MS/MS ion search (http://www.matrixscience.com/) for the identification of the unknown protein.

RESULTS

Purification of viruses (I-IV)

Crude purification and concentration of hantaviruses has classically been done by pelleting viruses through a sucrose cushion. The separation is based on the sedimentation velocity; the larger the particle, the faster it reaches the buoyant density. In study II, we designed a protocol where the viruses are grown in MEM supplemented with 2-3% FCS that has been filtered through a 100 kDa membrane. This enabled 200- to 500-fold concentration of the virus containing growth medium prior to ultracentrifugation in a density gradient (II). The results (Table 1 in II and Figure 1A in IV) indicated that purification using this protocol produced virions largely free of contaminating proteins and roughly 20% of the original infectivity was recovered. However, a major contaminant of approximately 90 to 100 kDa in SDS-PAGE mobility was observed in Optiprep™ (used as synonym for iodixanol) virus-containing density gradient fractions (Figure 1A in IV). Ultracentrifugation of similar virus preparation in a sucrose gradient of approximately the same density range resulted in more pure preparation of viruses (Figure 3 in IV). This purification scheme was mainly utilized in study II, where high purity of virions was essential. In studies I, III and IV mainly viruses concentrated by pelleting through sucrose cushion were used, since the contaminating proteins (from FCS, mainly BSA) were considered insignificant for the interaction studies (I and III).

Interactions between the structural components of virion (I-III)

The previous reports of interactions between the structural proteins of hantaviruses have mainly been focused on the interactions between Gn and Gc. Most of these studies have been carried out using recombinant expression of the M segment encoded GPC. Since the recombinant expression and especially proper folding of Gn and Gc is complex, we chose to study mature proteins and complexes thereof extracted from intact virions. The rationale behind this was that once the virion is formed it contains all components at optimal ratios in respect to one another. Thus extracting the structural components from virions could help to understand how the virion is constructed.

Gn-Gc interaction (I and II)

We initially confirmed the interaction between Gn and Gc by co-IP experiments with previously characterized neutralizing glycoprotein specific MAbs. Both Gn (5A2) and Gc (1C9 and 4G2) specific MAbs were found to co-IP Gn-Gc complex (Figure 1A and D in I). Additionally, we observed that the Gn-Gc interaction is pH dependent and that the dissociation occurs at around pH 6.2 (Figure 1A in I). In addition, we observed that Gn and Gc could re-associate to form Gn-Gc if the pH was returned to 8.0 prior to co-IP. However, the co-IP of Gn and Gc after low pH treatment was obvious only using MAb 1C9, and using MAb 4G2 mainly Gc was immunoprecipitated (Figure 1D in I). This suggested to us that the epitope of MAb 1C9 is actually formed of both Gn and Gc, and that the epitope of MAb 4G2 would be present only in Gc homodimer. We also exposed intact virions to low pH and observed a reduction in infectivity (Figure 2 in II). Curiously, low pH treatment did not result in complete inactivation of viruses, as would be expected

in the case of class II viral fusion proteins. We rather suggested the observed reduction in virus titer to be caused by aggregation of virions. This observation indicated to us that other factors than merely acidic pH would be required for the fusion of hantaviruses.

To further characterize the minimal interacting complex, we used membrane impermeable cross-linker to fix the glycoprotein complexes on the surface of intact virions. The complexes analyzed by SDS-PAGE under reducing and non-reducing conditions were found to be mainly homo-oligomeric rather than hetero-oligomeric (Figure 3A and B in I). Using cross-linking we were unable to clearly define the hetero-oligomeric complexes.

Since cross-linking did not help us to resolve the glycoprotein complexes, we decided to study the effect of reductants TCEP [tris(2-carboxyethyl)phosphine] and DTT (1,4-Dithio-DL-threitol) to the mobility of Gn and Gc in SDS-PAGE. By western blotting we observed that complexes positive for both Gn and Gc were present in non-reduced samples (Figure 4B in I). The sizes of complexes Gn-Gc, $(Gn\text{-}Gc)_2$ and $(Gn\text{-}Gc)_x$ were 110, 220 and >250 kDa, respectively. These complexes disappeared under reducing conditions (Figure 4C in I), suggesting thiol bridges to play a role in the Gn-Gc interaction. Apparently the thiol bridges exist between Gn and Gc molecules, since TCEP (at the concentration applied) was found not to break intramolecular thiol bridges (see Gc mobility, Figure 4A in I). Finally, to avoid possible complex formation during SDS-PAGE, we analyzed the glycoprotein complexes extracted from virions by density gradient ultracentrifugation. Using precalculated tables for linear sucrose gradients and by comparing to migration of known marker proteins, we were able to create an estimate for the sizes of Gn-Gc complexes (Figure 5 in I). Based on the estimated molecular weights 376 and 470 kDa we concluded that these complexes respectively represent $Gn_4\text{-}Gc_2$ and $Gn_4\text{-}Gc_4$ (Figure 5 in I).

Next we decided to map the regions involved in the Gn-Gc interaction by using an array of overlapping peptides covering the primary sequence of GPC. The binding of Gn to peptides was detected using a Gn specific MAb. In total we identified five distinct binding sites in the primary sequence of Gc (Figure 8A in I). To visualize the results of the interaction site mapping, we created a 3D structure model of PUUV Gc using the structure of SFV E1 protein as a template (Figure 7 in I). Three of the interaction sites (Gc_I, Gc_{II} and Gc_{IV}) were found to locate into domain I of the Gc structure model (Figure 8A in I). The interaction site Gc_{III} covered the putative fusion loop of Gc; however, this region was also found to bind Gc (Figure 8A in I). Thus we could not conclude whether it is Gn-Gc or Gc-Gc interaction that is responsible for preventing premature fusion.

The summary (Figure 9 in I) of our results pointed out that more than one unit of each glycoprotein is required for the formation of Gn-Gc complex and thus the complex is not a heterodimer. Based on the complexes extracted from virions, we created a hypothesis on how the surface of virion is created. The hypothesis where four Gn units are connected by two Gc units (Figure 9C in I) was later found to correlate with the surface arrangement of TULV spike complex (Figure 5A and C in II). However, even after obtaining the cryo-EM density data we were unable to decide what would be the minimal hetero-oligomeric complex of Gn and Gc.

Gn-Gn interaction (I and II)

The characterization of Gn-Gc complex indicated that Gn is prone to homo-oligomerization and tends to form large complexes (Figure 1A and D in I). In fact Gn has previously been described to form SDS-stable complexes [348]. We decided to study if we could determine the composition of the Gn oligomers. Cross-linking of intact virions with membrane-impermeable reagent revealed Gn to be extremely prone to react (Figure 3 in I). This we interpreted to indicate that Gn is more exposed in the virion than Gc.

Next we compared the mobility of Gn in SDS-PAGE under reducing and non-reducing conditions. While Gc mainly existed as monomer independent of addition of reductant, monomeric Gn was hardly visible under non-reducing conditions (Figure 4A in I). Additionally, we observed that reduction with TCEP (at the concentration applied) enhances the migration of Gn as compared to reduction with DTT (Figure 4A in I). The treatment of virions with free thiol-blocking NEM (*N*-ethylmaleimide) prior to SDS-PAGE enabled a small amount of Gn to migrate as monomer also under non-reducing conditions (Figure 4B in I). NEM treatment also reduced the amount of >250 kDa complexes containing both Gn and Gc seen in non-reducing SDS-PAGE separation (Figure 4B in I). Together these results indicated that some amount of thiol bridge formation occurs during the SDS-PAGE separation. When separated in SDS-PAGE under reducing conditions the most prevalent forms of Gn were monomeric and dimeric (Figure 4C in I). Curiously, addition of non-ionic detergent (Triton X-100) to virions prior to SDS-PAGE separation increased the overall amount of Gn by producing trimeric and tetrameric Gn complexes in addition to the monomeric and dimeric complexes (Figure 4C in I). Based on these results, the strongest interactions of Gn are in favor for homo- rather than hetero-oligomerization. When analyzing the detergent solubilized complexes of Gn in density gradient, we found Gn only in complexes that were also positive for Gc (Figure 5A in I). The majority of Gn had migrated through the density gradient, suggesting Gn to aggregate when extracted from virions.

Interaction site mapping using SPOT peptide array revealed five binding sites for Gn in the primary sequence of Gn, likely representing the regions involved in Gn-Gn interaction (Figure 8A in I). Due to the experimental setup (the membrane was probed with virus lysate where Gn-Gc complexes are present) it is also possible that binding to some of these peptides was through Gc from a Gn-Gc complex. The Gn in such complexes would not have been observed using the Gc specific MAb 4G2, since the IP experiments with this MAb had indicated that its epitope is present only in Gc-Gc homodimer, as discussed below.

The density map of TULV surface solved by EM cryotomography showed an obvious tetrameric assembly of globular heads (Figure 5 in II). This observation together with the tetrameric Gn complexes (SDS-PAGE, Figure 4A-C in I) and the high molecular weight Gn complexes (density gradient ultracentrifugation, Figure 5A in I) suggests that the four-fold symmetry is maintained via Gn-Gn contacts. The central stalk below the globular heads of the tetrameric assembly (Figure 5B, D and E in II) suggests that the region involved in the Gn-Gn oligomerization would locate just before the first transmembrane helix of Gn. Peptides from this region of Gn were not; however, found to bind Gn in the SPOT peptide array (Figure 8A in I). This could simply be because monomeric (or di-

and trimeric) Gn available for such interaction is not present in complexes extracted from virions using non-ionic detergent. This would reflect the observations that even SDS cannot break the glycoprotein complexes to yield Gn monomers (Figure 4A-C in I).

Gc-Gc interaction (I and II)

After we had observed that Gn-Gc complex dissociated at low pH, we performed a set of IP experiments with Gc specific MAb 4G2. The results showed that MAb 4G2 is unable to immunoprecipitate Gc at pH below 6.2 (Figure 1B in I) but if Gc is bound to MAb 4G2 prior to low pH treatment, it does not dissociate at pH 6.0 (Figure 1C in I). Our interpretations of these results were that Gc exists as oligomers and that the epitope of MAb 4G2 is most likely formed of two Gc molecules (Figure 8B in I).

To gain more insight into the complexes formed by Gc, we performed cross-linking experiments, studied its mobility in SDS-PAGE and sedimented Gc complexes in density gradient as described for Gn complexes. We observed Gc to be less prone to cross-linking than Gn, as judged by the reduction in the amount of monomeric Gc after adding the cross-linker (Figure 3B in I). This we interpreted to indicate that Gc is somehow buried or otherwise “invisible” to the cross-linker in the intact virion. Thus Gc would likely lie parallel to the viral membrane, as would be assumed according to the definition of class II fusion proteins.

When studying the effect of reductants TCEP and DTT to Gc, we observed that TCEP was unable to break the intramolecular thiol bridges of Gc at the concentration applied. We came to this conclusion since there was approximately 10 kDa mobility shift between the Gc reduced with TCEP as compared to DTT reduced Gc (TCEP reduced migrated similarly to non-reduced, see figure 4A in I). We utilized this observation in the following experiments to determine whether there actually are covalent, thiol bridge-mediated, dimers of Gc. Indeed, we found Gc to form covalent homodimers (Figure 4B and C in I). However, most of Gc migrated as monomers, regardless of the treatments prior to SDS-PAGE separation.

The results of the analyses of Gc complexes extracted from intact virions using non-ionic detergent were very clear as compared to the analysis results of Gn complexes. Gc was found mainly devoid of other protein components and in complexes that were approximately 90 to 110 kDa in size and thus represented Gc homodimers. The molecular weight estimations were based on sedimentation velocity in density gradient (Figure 5A in I) and on retention time in gel filtration (Figure 6 in I) as compared to respective values of known marker proteins.

Interaction site mapping using SPOT peptide array showed only one region in the primary sequence of GPC to interact with Gc. This region, Gc_{III}, that represents the putative fusion loop of Gc and was found to also interact with Gn (Figure 8A in I). The earlier results (Figure 1B to D in I) had suggested that the epitope of MAb 4G2 is present only in Gc homodimer. It is thus possible that Gc_{III} together with intact Gc molecule forms the epitope of MAb 4G2 and therefore only this binding site was detected for Gc. This would be supported by epitope mapping studies for MAb 4G2 [122]. We highlighted the peptides characterized to form the epitope of this MAb to the 3D structure model of PUUV Gc. Then we docked two such Gc molecules as a dimer, so that the epitopes in

the two molecules would form a unanimous surface (Figure 8B in I). This was achieved quite easily and the docking produced a Gc dimer where the epitope would locate towards solution. This indicated to us that our 3D model could, in fact, reflect reality to some extent. Additionally, this led us to believe that the mechanism of neutralization by this MAb is via prevention of Gc-Gc complex dissociation, since this event is crucial to the fusion.

The EM cryotomography structure of TULV surface was found to have both two-fold (between spikes) and four-fold (at the center of spike) planes of symmetries (Figure 5A in II). Since Gc was found to be solubilized as homodimers, we interpreted Gc to maintain the connections between the neighboring spikes. A density corresponding to these connections is visible in the slices through the tomogram (Figure 1D and in II), indicating that Gc most likely lies in between the spikes. Additionally, the density between spikes (Figure 1B in II) lies parallel to the membrane and is less exposed to the surrounding environment. This agrees both with the cross-linking data (Figure 3B in I) and the hypothetical docking of Gc homodimer (Figure 8B in I). The density on the virion surface could not accommodate the heterodimer as such, and thus the 3D model would not exactly fit the observed structure. Slight modifications on the angles between the domains of the model would, however, enable fairly successful docking as discussed below.

The spike complex (I and II)

The interaction data of the complexes formed by Gn and Gc led us to suggest a model to account for the previously reported grid-like surface of virion [226]. Since the virion presumably contains equal number of both Gn and Gc molecules, we were left with practically only one plausible model for the glycoprotein organization on the virion surface (hypothesis 3 of Figure 9C in I). The electron cryotomography data of the TULV surface structure was found to support this hypothesis surprisingly accurately (Figure 5 in II). The spike complex was determined to be 15 nm by 15 nm and 12 nm in height (Figure 5A and B in III). The four-fold symmetry of the spike is obviously mediated via connections between four Gn molecules and two such structures are connected to one another via two Gc molecules. Whether Gc also contributes to the globular heads of the spikes (Figure 5A and B in II) is not known. Should Gc contribute to this part of the spike, one would have expected to observe Gn-Gc heterodimers in the cross-linking experiments. Since such complexes were not abundant, we assumed that Gc does not contribute to the globular head of the spike. Whichever the case, there still are obvious contacts between the spike and the density in between spikes, and these contacts very likely are maintained by Gn-Gc interaction. Thus to describe a single spike, one could say that it is a complex of four Gn and four Gc molecules, but not necessarily a tetramer of Gn-Gc heterodimers. Thus the Gn-Gc positive large complex observed in the density gradient ultracentifugation (Figure 5A in I) probably represents the spike complex.

Spike-RNP interaction (II and III)

Computational slicing through the tomogram of TULV indicated that the RNP makes occasional contacts to the viral membrane (Figure 5 in II). Additionally, we observed that in tubular virions (such as depicted in Figure 1B in II) the spikes on the surface of the

virion are arranged directly above the RNP (an unpublished observation with Dr. Juha Huiskonen). The density map created by averaging the spike volumes collected mainly from round virions did not show any detectable density under the spike complex (Figure 5B in II). This indicates that not all spikes are connected to RNP in intact virions.

We decided to confirm the interaction between the spike complex and RNP using co-IP. Previous results showed that both Gn (MAb 5A2) and Gc (MAb 4G2) specific neutralizing antibodies co-immunoprecipitate the spike complex (Figure 1A and D in I), and thus we chose to use these MAbs in this study. Co-IP of N protein was observed with both MAbs (Figure 1 in III), thus confirming that either Gn or Gc (or both) mediates the binding to N protein.

Next we synthesized the Gn-CTs of PUUV, TULV, PHV and NYV on SPOT peptide array as a set of overlapping peptides and included also the Gc-CT of several hantaviruses. We probed the membrane with both RNP of PUUV and recombinant PUUV N protein, and detected the binding of N protein using an N protein specific MAb. We saw that PUUV N protein had three distinct binding sites in the primary sequence of Gn-CT (Figure 2A in III). Curiously, similar binding pattern was observed with all Gn-CTs studied (Figure 2A-D in III). Additionally, all the Gc-CTs included in the study were found to bind N protein (Figure 2E in III). This we interpreted to enable recombination of hantavirus segments in the case of co-infection of the rodent host.

We used N- and C-terminal deletion analysis and alanine scanning of the interacting peptides to locate and identify the critical residues for the binding of N protein (Figure 3A-E in III). As a result of these analyses we were able to verify that there are three distinct binding sites for N protein in the primary sequence of Gn-CT (Figure 4 in III). Furthermore, two of these binding sites actually contained two sub-binding sites. The analysis did not clearly resolve the Gc-CT residues that are critical to the interaction, probably because Gc-CT is short (at max 12 residues inside the virion) and thus most residues contribute to binding. The Gc-CT contains several positively charged residues, and thus it is likely that the binding of N protein is mediated via electrostatic interaction (see sequences in Figure 2E in III).

To further confirm the ability of peptides to interact with RNPs extracted from virions, we performed peptide pull-downs with purified TULV and PUUV RNPs. The peptides Gn_N, Gn_M and Gn_C, representing the three binding sites in Gn-CT of PUUV, successfully pulled down the RNP of PUUV (Figure 5 in III). However, only Gn_N and Gn_C pulled down the RNP of TULV. Additionally, all peptides were capable of pulling down *in vitro* translated N protein of both PUUV and TULV (Figure 5 in III). Also the whole Gn-CT of PUUV, synthesized via solid phase peptide synthesis, was able to pull down both TULV and PUUV N protein independent of the source (Figure 5 in III). Finally, we attempted to use the peptides to outcompete the natural RNP-spike interaction in co-IP. We found out that only the whole Gn-CT was enough to prevent the co-IP of RNP and the spike complex (Figure 6 in III). This indicated that while several regions in the primary sequence of Gn-CT are able to bind N protein, also the folding of the intact Gn-CT is essential for the interaction.

The morphology and structure of virion (I and II)

Hantaviruses have previously been described as pleomorphic or round particles with a diameter ranging from 70 to 210 nm and the surface of which displays a grid-like pattern [226]. We observed mainly round particles in the cryo-EM samples; however, depending on the purification protocol also tubular particles were occasionally present (Figure 9B in I and Figure 1 in II). The diameter of round virions was between 120 to 160 nm and the size of the tubular particles varied between 80 to 350 nm in diameter/length.

The surfaces of the virions were found to be nearly entirely covered by glycoprotein spikes measuring 15 nm by 15 nm and projecting 12 nm from the 5 nm thick viral membrane (Figure 5B and C in II). The RNP inside the virions appeared as threads of 8 nm in diameter, and was more clearly resolved in the tubular virions (Figure 1B in II). There were patches devoid of spikes on the virion surface (Figures 1B and 3C in II), and also the glycoprotein spikes were found to form regularly organized patches on the surface of the virion (Figure 3B in II). The four-fold symmetry of the spike indirectly explains this type of organization and especially the regions devoid of spikes, since the surface of a ball cannot be broken into squares. In the ordered lattices the spikes were found to regularly make contacts to the neighboring spikes from two opposing faces of the square-shaped spike. In other words the spikes tended to make highly organized rows of spikes. The contacts from the two other faces of the square-shaped assembly were found to be less regular, as seen in the averaged structure of the spike complex (Figure 5A in II, compare the contacts left and right to the contacts above and below).

The contacts between the spikes were further analyzed by determining the orientation of each spike to its neighboring spike. The results showed that the minimum spike-to-spike distance was 15 nm, representing spikes aligned side-by-side (Figure 4A in II). The out-of-plane angle between two neighboring spikes was determined to be 15º (Figure 4B in II), indicating that the glycoprotein contacts induce a specific curvature to the viral membrane. Thus the great circle of a virion would on average accommodate 24 spikes (360º/15º=24). The preferred in-plane angle between spike was 0º (Figure 4C in II), reflecting the tendency of spikes to create organized rows. In conclusion, the regular spike-to-spike distances are in favor for specific contacts between the glycoproteins.

Interaction between galectin-3 binding protein and virion (IV)

When analyzing the fractions collected at different steps through the purification of TULV for cryo-EM studies (II), we observed a co-purifying protein (Figure 1A in IV). The band of the unknown protein was cut out from the gel, in-gel trypsinized and analyzed by MALDI-MS/MS. The protein was identified as galectin-3 binding protein. We further verified the identity of this protein by western blotting using a commercial galectin-3 binding protein specific MAb (Figure 1B in IV). This protein has earlier been identified from several sources and originally as a tumor-associated antigen 90K [137-139,369]. Due to the fact that this protein is also able to bind galectins-1 and -7 [108,110], we prefer to call it 90K/Mac-2BP (galectin-3 was previously known as Mac-2, [141]).

Up-regulation of 90K/Mac-2BP at mRNA level was observed in hantavirus infection of cultured cells as analyzed by gene microarray [102]. We thus wanted to check whether the production of 90K/Mac-2BP is induced by hantavirus infection in Vero E6 cells. We

collected cell culture supernatants from infected and mock-infected cells, and separated the preconcentrated proteins by density gradient ultracentrifugation. The result indicated that also mock-infected cells secrete 90K/Mac-2BP to the cell culture medium (Figure 2 in IV). We were unable to determine whether the production of 90K/Mac-2BP is increased upon infection in Vero E6 cells; however, up-regulation of 90K/Mac-2BP production has been reported in endothelial cells as a result of hantavirus infection [102].

Next we compared the co-purification of 90K/Mac-2BP with TULV in sucrose and OptiPrep™ (we use OptiPrep™ as a synonym for iodixanol) density gradients. We observed that the co-migration was more pronounced when OptiPrep™ was used as the density gradient medium (Figure 3 in IV). However, 90K/Mac-2BP also co-migrated with TULV in sucrose density gradient as confirmed by western blotting (Figure 4 in IV). The observed co-migration in both gradients suggested an interaction between 90K/Mac-2BP and TULV (buoyant density 1.16-1.18 g/cm^3 in sucrose and 1.10-1.12 g/cm^3 in OptiPrep™).

To study whether 90K/Mac-2BP interacts with TULV we performed a co-IP experiment using anti-human 90K MAb and added different amounts of 90K/Mac-2BP containing fraction to the co-IP. The 90K/Mac-2BP MAb was able to co-IP TULV when small amount of 90K/Mac-2BP containing fraction was added (Figure 5 in IV). When an excess of this fraction was added the co-IP of TULV was abolished (Figure 5 in IV). We further confirmed the interaction using wheat germ agglutinin pull-down of 90K/Mac-2BP-TULV complex (Figure 6 in IV). When the pull-down was done from a buffer containing non-ionic detergent, mainly Gn was seen to co-precipitate with 90K/Mac-2BP (Figure 6 in IV). This suggested that 90K/Mac-2BP would bind to Gn. In addition, we observed that MAb against 90K/Mac-2BP was able to inhibit TULV infection of Vero E6 cells (Figure 7 in IV). This we concluded to confirm an interaction between 90K/Mac-2BP and TULV. Using these methods we could not rule out the possibility that there might be additional players involved in this interaction.

Up-regulation of 90K/Mac-2BP has been reported in pathological conditions such as cancers and chronic viral infections [19,42,53,139,140,172,256,275]. We thus decided to analyze the level of 90K/Mac-2BP in the plasma of patients hospitalized due to acute PUUV infection. We analyzed the 90K/Mac-2BP level in plasma samples collected from 61 individuals during hospitalization, and compared these values to the values in samples taken roughly 1 month after hospitalization. We observed that the level of 90K/Mac-2BP was significantly higher during the acute phase as compared to convalescence (Figure 8 in IV). The same samples had previously been analyzed for a multiplicity of clinical parameters and cytokines such as complement activation, C-reactive protein, IL-6 and indoleamine 2,3-dioxygenase [191,222,266,267,317]. We thus compared the 90K/Mac-2BP levels to the previously measured parameters. We observed that the level of 90K/Mac-2BP had the strongest correlation with complement activation, but it was also found to correlate with several other clinical parameters reflecting the severity of HFRS.

This was to our knowledge the first report to indicate interaction between 90K/Mac-2BP and a micro-organism, and also the first report to describe up-regulation of 90K/Mac-2BP during an acute or transient viral infection.

DISCUSSION

Interactions between glycoproteins

We started to work with hantaviruses by aiming to purify the viral proteins from a concentrated batch of viruses. When analyzing the purity of virus preparations, we begun asking how many bands should a pure preparation of hantavirus particles produce in SDS-PAGE separation? The literature describes the Gn of hantaviruses to form SDS-stable [348] complexes, thus yielding several bands in SDS-PAGE [14,130,278,348]. Also the glycoproteins of La Crosse virus and UUKV have been characterized as homo-oligomers either when expressed as recombinant proteins or when extracted from virions [277,307]. These results somewhat challenge the dogma of a purely heterodimeric glycoprotein complex in the case of *Bunyaviridae* viruses.

While there are interactions between Gn and Gc [14,278,312], our results demonstrated that the strongest interactions between the glycoproteins are in favor of homo-oligomerization. We studied mainly glycoproteins extracted from virions, and thus we cannot exclude the possibility that the viral membrane would alter the preference for the interaction partner. In fact, in the case of the virion it is rather obvious that Gn and Gc do interact with each other. However, even in this case, the minimal unit for heteromeric complex formation does not need to be a heterodimer. Our results led us to suggest a model where tetrameric Gn complexes are interconnected by homodimeric Gc, as shown in Figure 9. This model was later found to agree with the structure of the spike complex (Figures 10 and 11).

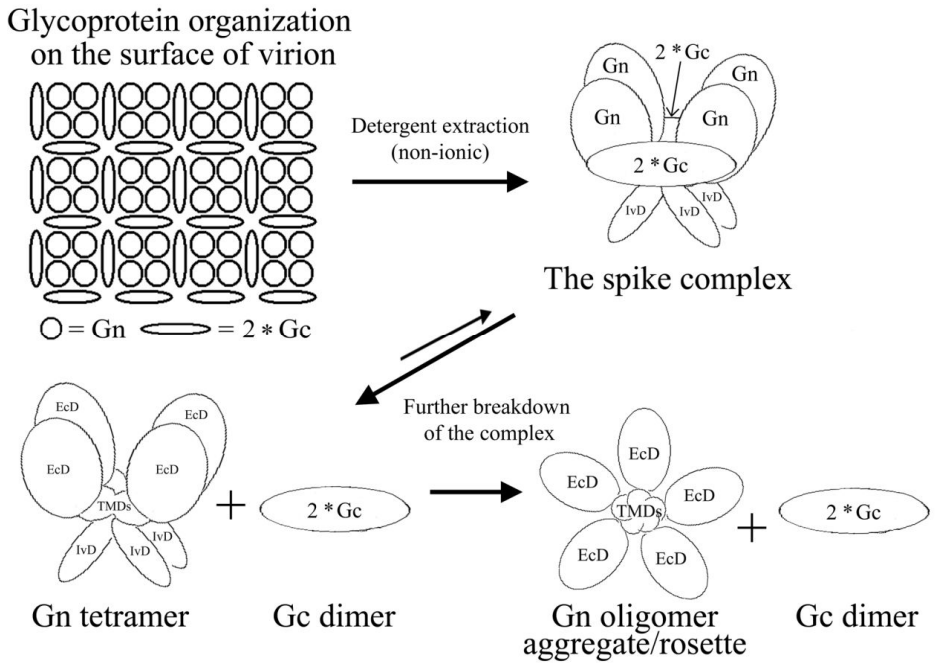

Figure 9. *Hypothesis on the glycoprotein organization in the virion, and the glycoprotein complexes formed when the membrane is disrupted. Initially, when the virion is immersed to a non-ionic detergent, the complete spike complex (four units of both Gn and Gc) is extracted. Further breakdown of the spike complex results first in dissociation of Gn (homotetramer) and Gc (homodimer), and at later stages large Gn homo-oligomers are formed presumably by hydrophobic interactions. EcD=ectodomain,TMD=transmembrane domain, IvD=intraviral domain.*

The question that should be addressed when characterizing the biochemical properties of the glycoproteins is: how is the heterodimer defined? The term heterodimer is defined by biology online dictionary (http://www.biology-online.org/dictionary/Heterodimer) as a dimer made up of two, but not identical subunits. Thus due to the fact that both Gn and Gc were found to preferably exist as multimers of themselves, and not even necessarily dimers, we conclude that both Gn and Gc are rather homo-oligomers (Gn a homotetramer and Gc a homodimer). We did also observe Gn and Gc to co-immunoprecipitate, most likely as the complete spike complex (as illustrated in Figure 9). This concurs with other reports of the glycoproteins [17], where even Gn and Gc from two different hantavirus species have been shown to co- immunoprecipitate [60]. We saw efficient co-IP of Gn and Gc only when using fresh virus lysates, thus favoring the hypothesis presented in Figure 9 and agreeing with the results obtained during the characterization of the MAbs used [216]. It seems obvious that the interaction between Gn and Gc requires homo-oligomers of either one or both glycoproteins. The virion surface is maintained by contacts between Gn and Gc, however, based on our results the interacting units are rather tetrameric Gn complexes that are interconnected through homodimeric Gc units than heterodimers.

Structure of the glycoprotein spike complex

Initial EM studies on the surface structure of hantaviruses revealed that the surface is formed of cross-like subunits, creating a grid-like pattern [226,359,360]. At the time of discovery this was a unique finding among the animal-infecting enveloped RNA viruses, and perhaps still is (II and [20]). The only other viruses of the family *Bunyaviridae* with a clearly ordered and characterized surface structure, RVFV and UUKV, are members of the genus *Phlebovirus*. The surface of the phleboviruses has been reported to show an icosahedral symmetry [92,113,134,206,268,270]. Additionally, the surface of Bunyamwera virus of the genus *Orthobunyavirus* has been reported to contain substructures (penta- and hexagonal profiles) related to icosahedral symmetry [260]. Currently no ordered surface structure has been assigned to the viruses of the genera *Nairovirus* and *Tospovirus*; however, their structures have not been studied as intensively.

Clearly the spike complex of phleboviruses (see Figure 10) is different from its hantaviral counterpart. The contacts between the glycoproteins of RVFV are not obvious from Figure 10, and thus no conclusions on the nature of contacts (homo- or hetero-oligomeric?) between the glycoproteins can be drawn based on the structure. The apparent six-fold symmetry of the RVFV spike suggests the involvement of at least six glycoprotein units in the spike. Respectively for hantaviruses the four-fold symmetry of the spike suggests the involvement of four glycoprotein units. In both cases the unit could either be hetero- or homodimer (or even oligomer).

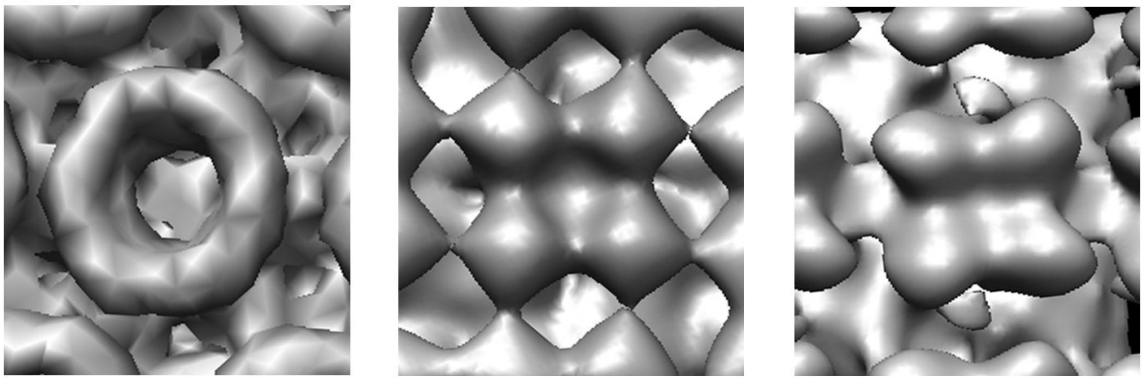

Figure 10. *Spike complexes of phlebovirus (RVFV) and hantavirus (HTNV and TULV). The RVFV spike was solved at 2.2 nm resolution* [134]*, the HTNV spike at 2.5 nm resolution* [20] *and the TULV spike at 3.6 nm resolution (II). The HTNV density map was kindly provided by Prof. Michael Rossmann and Anthony Battisti.*

The spike complexes of TULV and HTNV [20] are compared in Figure 11. The spike complexes share same features such as the obvious four-fold symmetry; however, there are obvious differences between the two structures. As depicted in Figure 11 (panels 4-6 in A and panels 4 in C), the stalk below the central tetrameric complex is missing from the structure of HTNV. This most likely is an artifact created by the different sampling parameters applied for the averaging of the volumes. In both density maps there is a volume directly below the central tetrameric complex (Figure 11 panels 8 in A and panels 4 in C), most likely representing the Gn-CT. Because the density lies directly below the spike, it is fairly safe to assume that the tetrameric complex would possess the stalk seen in the TULV structure. Another difference between the structures lies in the space between spikes (the interspike density). This interspike region seems to be more clearly resolved in the HTNV structure, or at least the faces of the rectangular spike complex seem identical. In the case of TULV two-fold symmetry was assumed when creating the tomogram, and this partially explains why the two faces of the spike complex differ in structure. The spikes in the TULV tomograms were observed to form ordered rows having strong contacts to the neighboring spikes on both sides. The contacts between such rows were less evident and this explains the different appearance of the two faces in the TULV spike. The averaging on the study of Battisti *et al*. [20] was mostly based on edge-on views of spikes, and thus the selection of spikes in the two studies differed markedly. This combined with the observations on TULV (faces of the spike complex may differ) would suggest that neither of the structures explains the whole surface of the virion. Thus neither of the maps completely fits to the biochemical data of glycoprotein complexes extracted from virions. It is very likely that actually the surfaces of TULV and HTNV are identical, and perhaps the features of these maps could be combined to form a universal map for hantaviruses.

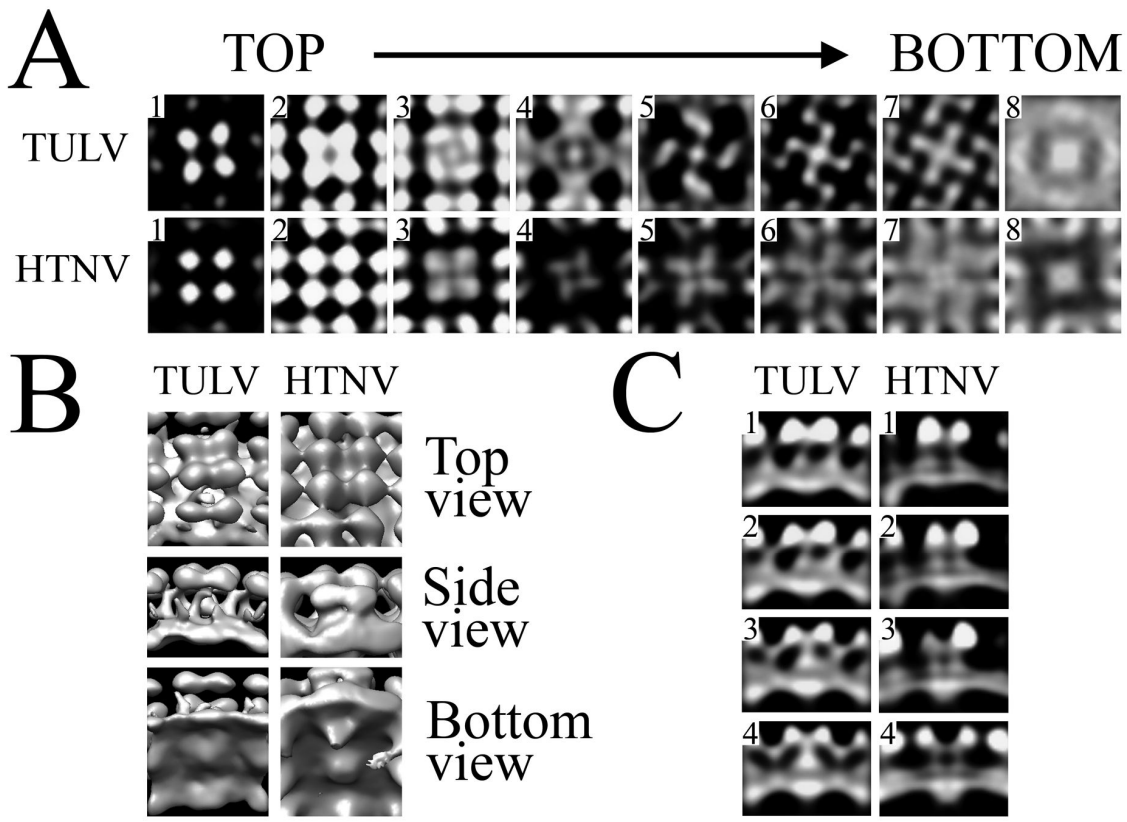

Figure 11. *Comparison between the HTNV* [20] *and the TULV (II) spike structures. A) Slices through the spike density from above. B) The spike complex viewed from different angles. C) Slices through the spike density from side. The HTNV density map was kindly provided by Prof. Michael Rossmann and Anthony Battisti.*

Based on the structural data of TULV and the extraordinary stability of Gn oligomers, one could build a hypothesis that Gn proteins would be connected to each other via coiled-coil-type of contacts. The α-helical coiled-coil is a very widespread fold in proteins [50], and it is considered to be the most frequent oligomerization motif between proteins or between subunits of proteins [234]. Coiled-coils are oligomers of 2 to 7 α-helices that wind around one another to form a superhelical twist, which can be either left-handed, straight or right-handed [234]. The left-handed coiled-coils are characterized by heptad (7 units) repeats but hendecad (11), pentadecad (15) and heptadecad (17) repeats have been described for the straight and right-handed superhelices [37,234,351]. The coiled-coil interactions of some proteins have proven to provide extreme thermostability to protein complexes of enzymes that are required to function under extreme conditions [37]. This type of an interaction could explain the observed SDS-stability of the Gn oligomers. Such a structure in the density map of TULV spike complex would exist in the stalk below the globular heads (see Figure 11 and 12). To clarify this hypothesis a structure of a tetrameric coiled coil from a protein called tetrabrachion was docked into the stalk of the TULV density map (shown in Figure 12). The docked crystal structure shows a right-handed coiled-coil formed of four 52-residue chains with hendecad repeats [351]. The docking (Figure 12) indicates that such a coiled-coil tetramer could be accommodated by the stalk density of the hantavirus spike complex.

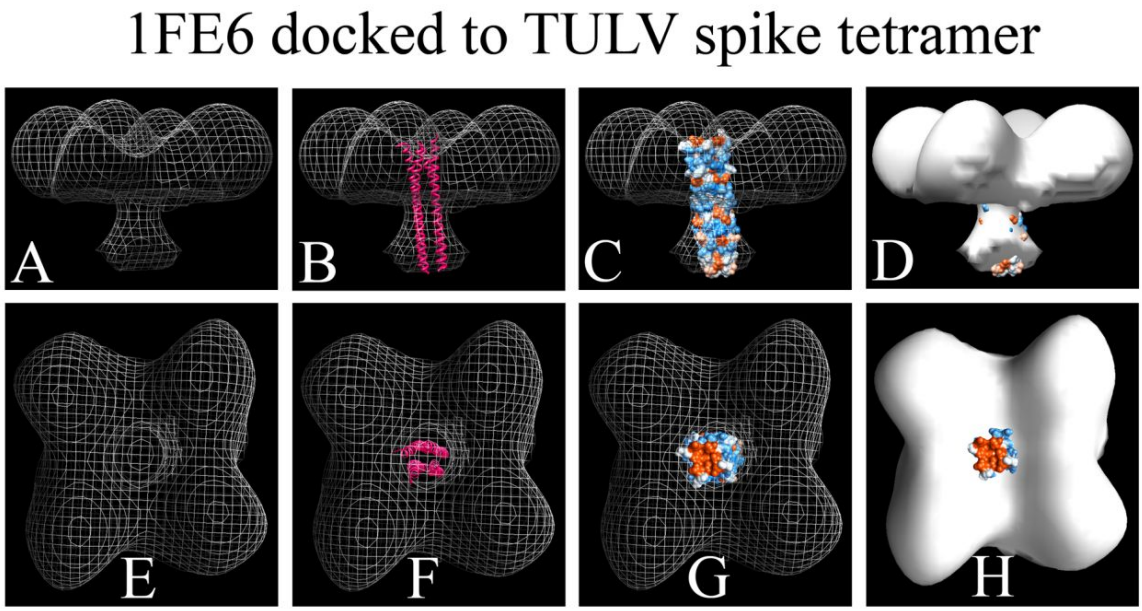

Figure 12. *Hypothesis on the interaction mechanism underlying the tetrameric spike complex. In panels A-D a side-view of the tetrameric spike complex is displayed (extracted from the density map of TULV) and in panels E-H the same complex is viewed from above. In A and E only the spike density is shown, in B and F a ribbon presentation of a tetrameric coiled-coil (1FE6,* [351]*) is docked into the structure, in C and G 1FE6 is represented as hydrophobic surface, and in D and H the spike density is shown as a solvent accessible surface to indicate that the stalk density can accommodate the volume of the tetrameric coiled-coil.*

Assuming that four-fold contacts inside the spike are mediated by coiled-coiling between four Gn units, the Gc should mediate the interspike contacts. The evidence strongly suggests that Gc is a class II fusion protein, and the known class II fusion proteins have common structural features [166]. The class II fusion proteins are formed of three domains: I (a β-barrel domain), II (the fusion domain) and III (a β-barrel domain with immunoglobulin-like fold) [166], see Figure 7 in I for the structure of class II viral fusion proteins. We created a 3D model for PUUV Gc structure (Figure 7 in I) based on the known structure of SFV E1 protein [199,309] and previously Tischler *et al*. [366] had used the structure of TBEV E protein [305] as the template to create a 3D model of the Gc of ANDV. Since our results had suggested Gc to form homodimers when extracted from virions, we suggested a hypothesis where the interspike connections are mediated by Gc. In Figure 13 we tested this hypothesis by docking the known structures of SFV E1 (2ALA, [199]) and TBEV E (1SVB, [305]), and our model of PUUV Gc (Figure 7 in I) into the density map of the TULV spike complex. The docking is hypothetical; however, the observed Gn binding sites in Gc (Figure 8 in I) would favor this type of docking. In the docking presented in Figure 13 also Gc contributes to the globular heads of the spike complex and is oriented slightly perpendicular to the viral membrane. Such an orientation would be atypical for a class II fusion protein, since the known examples of class II fusion proteins have been described to lie more or less parallel to the viral membrane [68,341].

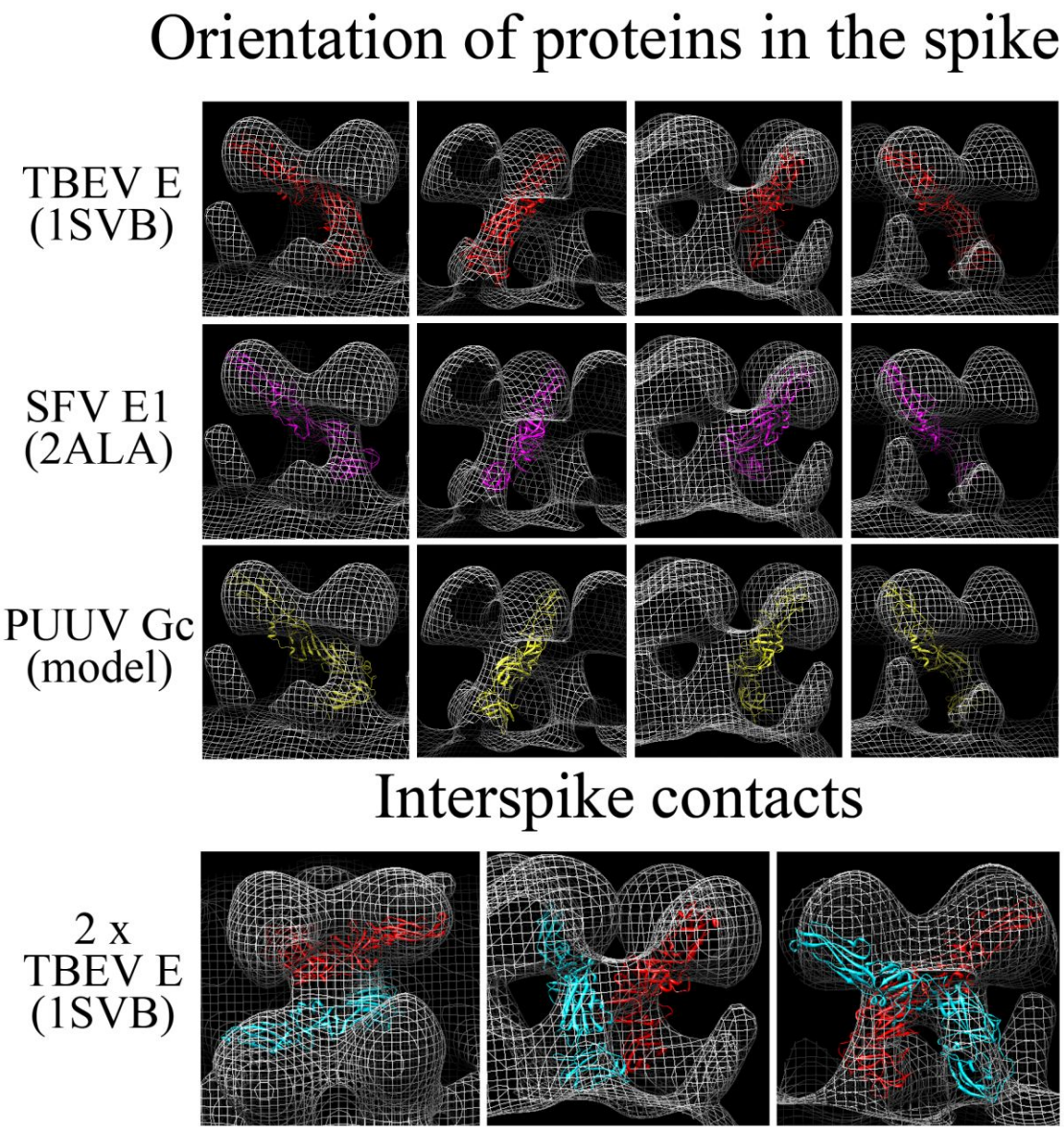

Figure 13. *Docking of fusion protein structures into the density map of TULV. Dockings of a single molecule of TBEV E (1SVB,* [305]*), SFV E1 (2ALA,* [199]*) and PUUV Gc (a model) are shown in the upper panels with respective colors red, purple and yellow. The panels at the bottom represent the interspike connection. In the interspike connections two TBEV E protein molecules (colored red and cyan) are docked in a similar fashion as in the panels above. An alternate docking could be done so that the tip of the TBEV E protein (harbors the fusion loop) would make contact to the globular head of the neighboring spike (not shown).*

Even though class II fusion proteins share the same domain organization and overall fold, there are differences between SFV E1 and TBEV E protein. For instance, the angles between the domain I and III are different in the two structures. In Figure 13 the domain III lies parallel to the membrane. This type of docking is based on the fact that the transmembrane sequence of Gc follows domain III in the primary sequence. Additionally, the region preceding the loop that interconnects domains I and III is in hantaviruses, by prediction, a transmembrane helix [97]. Instead of being a true transmembrane helix this region probably only prefers hydrophobic environment thus forcing the domain to be a peripheral membrane domain. Curiously, we observed in SPOT peptide array that the domain III of Gc is also capable of binding to N protein (an unpublished observation). The slightly different tilt between the domains I and III in TBEV

E and SFV E1 results in the fact that TBEV E protein fits to the density map better. The docking of two TBEV E molecules into the spike density map shows that there might be connections between the molecules in the region of domains I and II (Figure 13). If the actual arrangement would be such, the disruption of the viral membrane would likely stabilize the connection between the two Gc molecules. Alternatively the Gc of hantaviruses could be slightly different in structure, that is, there would be a tilt between the domains I and II. This tilt would enable two Gc molecules to actually fill the interspike region completely and allow the formation of a tight homodimer. In this type of situation the fusion loop of Gc would be covered by another Gc instead of Gn molecule. This would be supported by our results on the biochemical properties of Gc.

How is the spike complex assembled at the ER and/or Golgi? In the case of UUKV the folding of Gn occurs faster than the folding of Gc as judged by either incorporation into the newly formed virions or by biochemical characteristics [185,280]. The folding rates of UUKV Gn and Gc were roughly 10 and 60 minutes respectively, and the formation of complexes containing both proteins were observed roughly after 30 minutes [280]. The newly formed UUKV Gn was suggested to oligomerize with a previously synthesized and already folded Gc and these complexes were interpreted by the authors to represent heterodimers [280]. The spike-forming glycoproteins of influenza virus (HA) and VSV (G) fold in minutes and trimerize in approximately 10 minutes [51,62-64,104,183], which is roughly the same time as the time required for the folding of UUKV Gn [280]. As both UUKV glycoproteins have been reported to exist as homodimers when extracted from virions [307], the results of Persson *et al*. could also be explained by the requirement of homodimerization (or oligomerization) of glycoproteins prior to making the Gn and Gc contacts. Hypotheses based on the requirements for hantavirus glycoprotein folding and localization are depicted in Figure 14. The fact that the maturation of Gn as compared to Gc is more rapid in the case of UUKV [185,280] suggests that the formation of a tetrameric Gn complex would precede the formation of the hetero-oligomeric complex. However, it is known that the transport from ER to Golgi requires the co-expression of Gn and Gc [60,312,332,348], and thus the formation of Gn-Gc complex could somehow alter the conformation of Gn (or both proteins). It is tempting to speculate that the incorporation of Gc (either a monomer or a dimer) to the tetrameric Gn complex would make the signals in Gn-CT available at the cytoplasmic face of the ER membrane (as shown in Figure 14B), thus promoting the transfer to the Golgi complex.

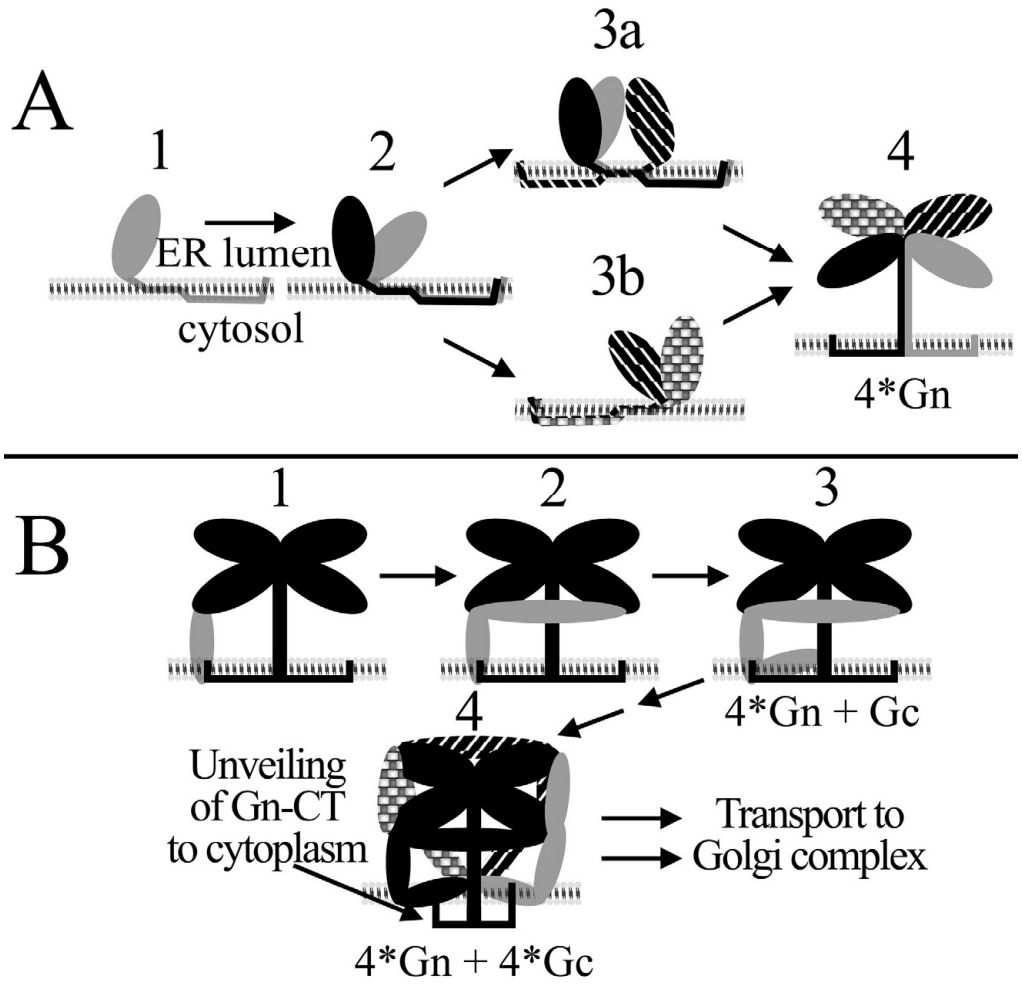

Figure 14. *Hypothesis on the assembly of the spike complex. In A (1) the first Gn molecule synthesized lies on the ER membrane via membrane-association of the putative coiled-coil region. (2) This Gn forms a homodimer with a newly synthesized Gn molecule. (3) The spike assembly proceeds either via (a) step-wise addition of Gn monomers or (b) by dimerization of two Gn homodimers until (4) the tetrameric Gn spike assembly is formed. In B (1-3) the tetrameric Gn complex acts as a chaperone for the folding of nascent Gc by interacting initially with the N-terminal residues of Gc (as shown in Figure 8 of I) and eventually with all the domains of Gc. (4) Repetition of the steps 1-3 eventually results in the formation of a spike complex with 4 units of both Gn and Gc. Formation of this complex induces the unveiling of Gn-CTs that target the complex to the cis-Golgi. In panel A only Gn molecules are shown (indicated by grey, black, dashed and grid color patterns). In panel B Gn molecules are colored black and the individual Gc molecules are highlighted by grey, black, dashed and grid color patterns.*

As depicted in Figure 14, the formation of tetrameric Gn assembly would precede the folding of Gc. When the tetrameric Gn complex is formed, the amino acids of Gc being synthesized protrude to the ER lumen and interact with the Gn tetramer. For UUKV it has been shown that the presence of more than 50 (but less than 98) residues of Gc are required for successful cleavage of the GPC [10]. In our hypothesis the initial spike complex containing four units of both Gn and Gc would have to keep Gc in a semi-stable conformation. When facing another Gc molecule in the same conformation the Gn-Gc interaction would be lost, resulting in the formation of a tight Gc homodimer between the spikes, as schematically depicted in Figure 15. Thus in the intact virion the interactions inside the homodimeric Gc, instead of the Gn-Gc interaction, would be responsible for hiding of the fusion loop thus preventing premature fusion. Alternatively to the

hypotheses presented in Figure 14, Gn and Gc could initially form a heterodimer that would stay in oligomerization competent form in the ER membrane. The increased concentration of such complexes would trigger the formation of tetrameric complexes. The observations made with UUKV indicate that Gn and Gc do not come from the same transcript and this would argue against this route of oligomerization. The newly synthesized Gc could also lie on the ER membrane and mature together with another Gc molecule, and thus directly form a homodimeric Gc complex that would interact with the tetrameric Gn complex. The assembly and oligomerization of the spike complex is an interesting subject for further studies.

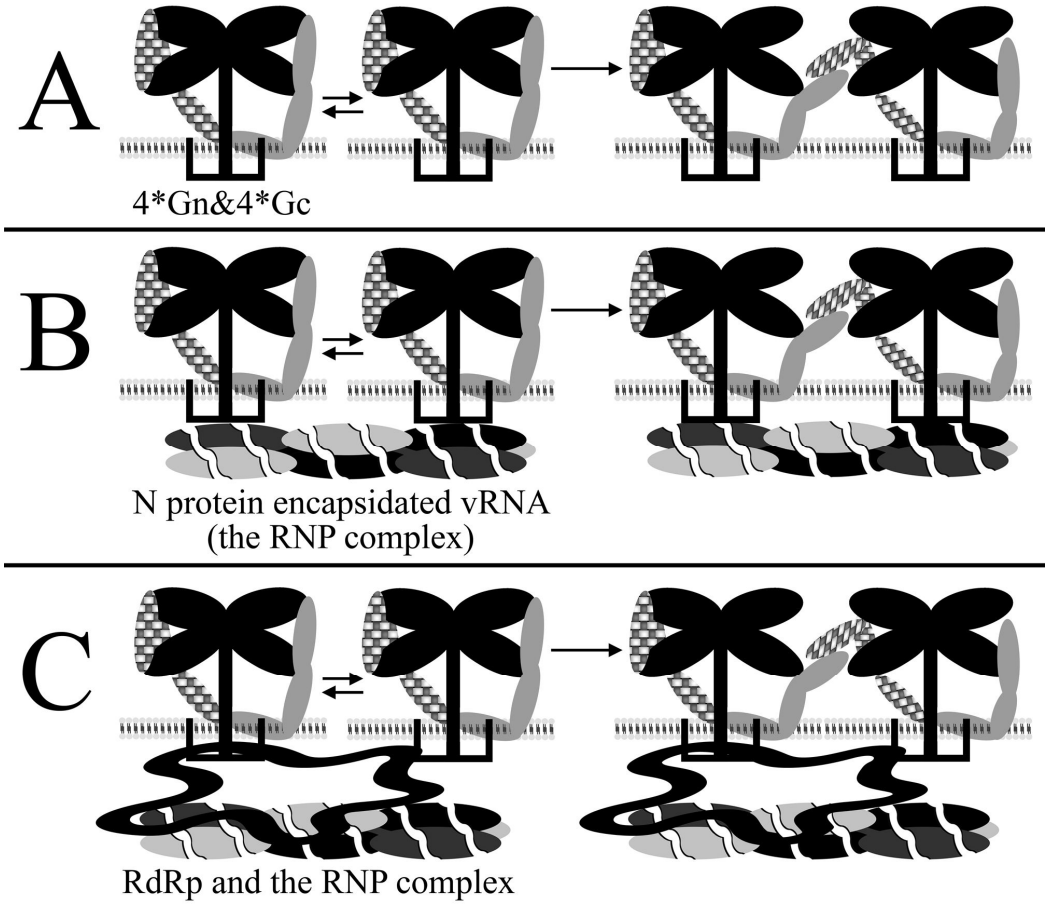

Figure 15. *Oligomerization of the spike complexes at ER-Golgi intermediate compartment (ERGIC) or cis-Golgi. The oligomerization of the spike complexes could be triggered (A) in a concentration-dependent manner, (B) as mediated by contacts to RNP or (C) by interactions to both RdRp and the RNP.*

The fusogenic potential of Gc

Hantavirus-infected cells have been demonstrated to form syncytia (or polykaryons) when subjected to acidic pH [18,232,261,400,401]. Also the successful entry of hantaviruses and the subsequent infection of host cells requires low pH [147,294,295]. The low pH activation of fusion protein is typically associated with class II viral fusion proteins [166,341], but also class I and III viral fusion proteins may require low pH for activation [61,388]. The fusogenicity has in hantaviruses been mapped to the GPC [261,401]. Furthermore, bioinformatics and mutational studies have shown that this feature resides in the Gc [47,97,366]. Class II viral fusion proteins exist as either hetero-

or homodimers in their prefusion conformation, and the fusion loop is covered by interactions between the glycoproteins [388].

We observed that both Gn-Gc and Gc-Gc interactions were pH-sensitive. Subjection of the glycoproteins to pH of 6.2 resulted in partial dissociation, and at pH 6.0 the dissociation of both contacts was evident. This indicates that the fusion event is most likely initiated at the late endosome (pH 5.5 to 6.0) [313]. Based on our data we could not conclude, whether it is the homo- or the hetero-oligomeric contacts of Gc that are responsible for preventing premature fusion. We did not observe homotrimeric complexes of Gc after the low-pH treatment, most likely because the experiments were carried out in conditions devoid of viral membrane. The prefusion state of class II viral fusion protein is described to be analogous to a loaded spring, which after triggering the fusion results in the formation of a stable trimeric complex via conformational changes [388]. Thus we studied, whether a low pH treatment of virions would result in complete inactivation of viruses. We observed a reduction in the virus titer after the low pH treatment, but did not see complete inactivation and assumed the decrease to be due to the aggregation of viruses at low pH, described for instance for UUKV [268]. Also the presence of a target membrane could contribute to the finalization of changes in conformation, thus explaining the resistance of virions towards acidic pH.

The role of free thiols in the viral fusion proteins has been recognized [316]. Additional factors such as cellular receptor could also contribute to the activation of viral fusion protein, as suggested for alphaviruses [316]. Thus it is tempting to speculate that free thiols would also play a role in hantavirus fusion. Indeed, thiol reactive NEM is able to block the infectivity of hantaviruses [356]. Even though the exact mechanism of this inactivation is not known, the inactivation likely occurs at stages after the internalization of the virus, since NEM-treated viruses are still able to bind cells [356]. The ectodomain of Gc contains a conserved CxxC motif that is found in protein disulphide isomerases [82,316]. This motif or active site could during the fusion reaction contribute to the release of the fusogenic activity of Gc. Thus the target of inactivation using NEM could be this motif as suggested by Strandin *et al*. [356]. Further experimental work is required to characterize the components or factors required for successful fusion.

Interaction between spike and RNP

The lack of a matrix protein among the family *Bunyaviridae* members has created the dogma that the Gn-CT would act as a surrogate matrix protein of these viruses. In addition to interacting with the RNP, the matrix proteins of other enveloped viruses have been shown to actively participate in the regulation of replication [253,357,386]. This is in the case of other RNA viruses mediated via direct interactions with RNA [49,52,75,76,386,395]. The Gn-CT is in natural situation located on the cytoplasmic face of the ER membrane. Quite recently the partial structure of Gn-CT was solved using NMR [79]. The structure represents ~50% of the whole Gn-CT and this region forms a tandem CCHC ZF domain [79]. The ZF domains are typically found in proteins that mediate interactions to DNA and RNA [52,230]. The ZF domain of Gn-CT did not bind nucleic acids [79], but was rather suggested to mediate the binding to N protein [381]. Our results performed using co-IP and synthetic peptides indicated that the flanking regions, but not the ZF domain itself mediates the binding to N protein. We suggested,

based on peptide data and the fact that Gn and Gc co-immunoprecipitated from a fresh virus lysate (Figure 1 in III), that actually both Gn and Gc might be required for the interaction. Based on our data, we created a hypothesis (presented in Figure 14) that the formation of the Gn-Gc hetero-oligomer induces a conformational change in Gn. This change in conformation makes the Gn-CT available for interactions from the cytoplasmic face, as shown in Figure 16.

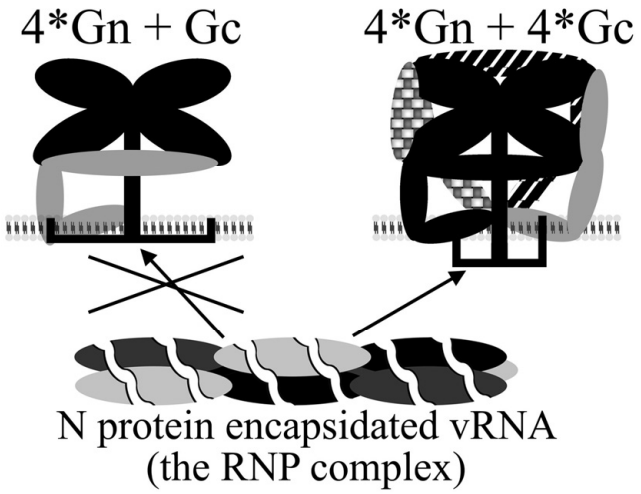

Figure 16. *A hypothesis on the requirements for the RNP-spike interaction. The Gn-CTs become exposed only in the fully assembled spike complex that contains four units of both Gn and Gc. This complex is able to interact with the RNP via Gn- and Gc-CTs.*

While describing the Gn-CT–RNP interaction, we observed that Gn-CT was able to directly bind nucleic acids. After performing a carefully planned set of new experiments, we confirmed that Gn-CT is able to interact with both RNA and DNA [355]. The interaction between Gn-CT and nucleic acids is enigmatic; however, very recently it was shown that the Gn-CT of RVFV [283] and CCHFV [80] are able to bind nucleic acid. Our initial hypothesis (depicted in Figure 7 of III) suggested that N protein together with the packaged vRNA forms an interaction platform, which mediates the binding to Gn-CT. The ZF found in the Gn-CT of hantaviruses is a classical type CCHC ZF that is commonly involved in either interactions with DNA/RNA or with proteins [230]. The interaction with DNA/RNA requires the presence of three such domains, but the Gn-CT contains only two [79]. On the other hand, once formed, the tetrameric Gn complex would contain eight classical ZF domains. This type of organization would also meet the criteria for DNA/RNA binding. The classical ZF domain can also mediate protein-protein interactions and for instance protein oligomerization [230]. However, Estrada *et al*. did not report presence of ZF oligomers [79] and thus ZF domain is unlikely to drive the oligomerization of Gn.

Fine mapping of the nucleic acid binding site in Gn-CT [355] showed the regions involved in the binding to largely overlap with the Gn-CT–RNP interaction sites described in III. Because of the oligomerization requirement of ZF domain and the fact that we used peptides to study the interaction, we cannot rule out the possibility of ZF contributing to the interaction with nucleic acids (especially in the case of tetrameric Gn complex). The nucleic acid-binding region was using peptides found to locate to the very carboxyl terminus of Gn-CT [355], and to partially overlap the third N protein binding site of Gn-

CT. What would be the role for the nucleic acid binding ability of the Gn-CT? Some viral matrix proteins are reported to regulate replication via binding to nucleic acids [52,75,76,357,395]. Using both synthetic Gn-CT of PUUV and the C-terminal peptide of Gn-CT Tomas Strandin was able show that the binding to nucleic acids induces the formation of a double helix (Strandin T, an unpublished observation). This indicates that the Gn-CT could potentially drive the annealing of RNA. In order to meet each other the 5' and 3' ends of the vRNA the Gn-CT should most likely be in complex with the RdRp. If true, this could mean that Gn-CT is directly involved in the regulation of transcription and replication. It is tempting to speculate that Gn-CT would act in concert with the RdRp to trigger the assembly of progeny virions. This, together with the hypothesis that Gn-CT becomes available only after forming a complex with Gc, would ensure that all the structural components are present in the progeny virion.

Assembly of virions

The assembly of family *Bunyaviridae* viruses has most thoroughly been studied for phleboviruses UUKV and RVFV [92,134,268,331]. The results obtained using phleboviruses as a model for the whole family *Bunyaviridae* may, however, be slightly misleading since the glycoproteins of only phleboviruses are capable of forming VLPs without other viral proteins [113,223,254,270,271]. This is most likely due to the fact that the Gn and Gc of phleboviruses can form icosahedra via contacts between these proteins as described by cryo-EM [92,134,268,331]. No such symmetry has, however, been applied to the viruses of the other genera of the family. If the glycoproteins are incapable of forming a polyhedron through their contacts, as in the case of hantaviruses, it is highly likely that additional components are required to form VLPs. Indeed, in the case of hantaviruses it has been reported that the expression of both S and M genome segments is a necessity for VLP formation [23,84,200,287].

The assembly of family *Bunyaviridae* virions is suggested to take place at the Golgi complex [35,115,167,192,228,229,308,334]. The site of assembly has not been resolved for hantaviruses, and based on glycoprotein localization both Golgi and plasma membrane have been suggested [310,332,333,348]. Additionally, the formation of so-called viral factories has been demonstrated for some members of the family [260,315]. The studies on the assembly of hantaviruses have been hampered by the lack of efficient reverse genetics system such as described for UUKV [85,87] and RVFV [283]. There are reports for such systems also for hantaviruses [84,287]; however, their usage is complicated and has thus not been generally established. Both the RdRp and N protein of hantaviruses have been described as membrane associated [187,298], and this suggests the budding and replication to require a membraneous environment such as found in the ERGIC or the Golgi compex.

The results on the interactions between Gn and Gc together with the spike structure indicated that the glycoproteins are able to form highly ordered lattices on a membrane. The angles between spikes on the virion surface suggested that the interactions between the glycoproteins would create curvature to the lattice formed. The formation of spherules would, however, likely require additional components. Successful assembly of infectious virions requires the presence of all structural components: N, Gn, Gc, RdRp and the vRNA. How does the virus ensure this? One possible scenario is that the Gn-CT

exposed to the cytoplasm, as a result of Gn-Gc interaction (as shown in Figures 14-16), is in contact with the RdRp and perhaps regulates transcription. This type of arrangement would bring all the structural components to close proximity to one another.

The virus has three genome segments. This raises a question: how does the virus ensure that all three segments are included into the progeny virions? The most straightforward way to ensure this would be that all three segments could bind one RdRp molecule. The structures of viral polymerases have been studied extensively, and by similarity to known domain structures the RdRp of hantaviruses is unlikely to have three binding sites for nucleic acids. An alternative explanation would be that the RdRp would also form a homo-oligomeric complex. In fact for RVFV it has been shown [398] that the RdRp is functional only when oligomerized. Thus the encapsidation of the three genome segments could be via communication through RpRp complexes. This might be achieved by signaling via alteration of conformation as the result of complex formation between Gn-CT, vRNA and the RdRp. The RdRp would in this hypothesis preferably exist as a homotrimer. After formation of the first complex with all the components, the replication in this complex would halt, which would induce termination of transcription in the two other RdRp molecules. This complex would then act as a nucleation center for the assembly process. The assembly would be driven by interactions between the spike complexes, perhaps recruited via spike-RNP interaction. The Gc in the spike complex of 4 Gn and 4 Gc molecules would be in a “metastable” state, which would be released when in contact with a similar molecule of the neighboring spike. After the initial contact between two spikes, the contacting Gc proteins of the spikes would form a homodimer, thus interconnecting the two spikes. This process would continue, driven by spike-RNP interactions, and would eventually lead to the budding of the newly synthesized virion. The hypotheses described above are illustrated in Figure 17.

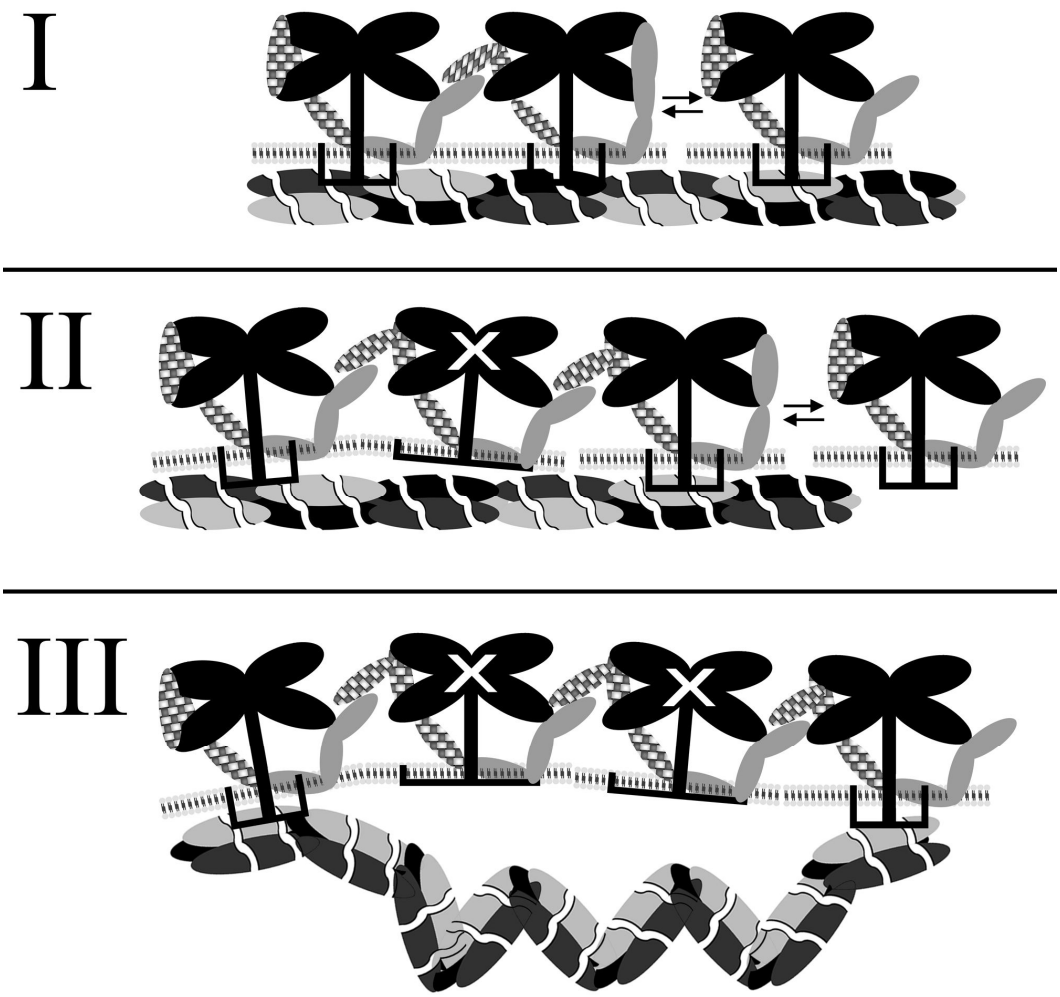

Figure 17. *Model for the assembly of hantaviruses at cis-Golgi. (I) A complex of two spikes (only two Gc molecules are shown per spike) is bound to the RNP and a third spike is brought to the complex via interactions with the RNP. The contact between the two-spike complex and the incoming spike triggers the dissociation of Gn-Gc interaction and the formation of a trimeric assembly of spikes. (II) As the oligomerization proceeds the spikes that are connected from all 4 faces (depicted by X in the spike) dissociate from the RNP due to hiding of the Gn-CT as a result of the lost of Gn-Gc contacts. (III) The process continues, creating increased curvature to the membrane and inducing the compression of the RNP. Eventually the process results in the formation roundish virions. Presumably the spikes not in contact with the RNP are more flexible in their contacts to the neighboring spikes, thus explaining the dilemma of square-shaped subunits in a spherical virion.*

The above model is highly speculative, since we have thus far only described the interactions between the glycoproteins, and between glycoproteins and the RNP. The interaction between Gn-CT and the RdRp has not been demonstrated for hantaviruses; however, the Gn-CT of phleboviruses was shown to bind the RdRp [283]. Currently no role has been assigned to the highly conserved ZF domain in the Gn-CT of hantaviruses. Due to the conservation among hantaviruses, and the fact that this domain does not bind RNA or N protein, it seems likely that it could interact with the RdRp. This putative interaction with the RdRp would be likely to regulate the functions of the RdRp, whether it would be to promote or to demote transcription remains to be seen.

Interaction of TULV with 90K/Mac-2BP

No clear consensus exists on the physiological role of 90K/Mac-2BP. It has been shown to interact with several components of the ECM such as galectins, collagens, laminins, fibronectin, β1 integrin, nidogen and CD14 [110,141,319,365,369,371,397]. On the other hand it has been suggested to have a function in the modulation of innate immunity response via induction of cytokines (ILs, IFNs and TNF-α) and major histocompatibility complex-1 (MHC-1) expression along with activation of T and NK cells [139,255,290,369]. 90K/Mac-2BP has been characterized to contain three to four domains [123,251,364]. The N-terminal domain is a scavenger receptor cysteine-rich (SCRC) domain, and the structure of this domain has been solved by X-ray crystallography [123,129,180]. Domains 2 and 3 are by prediction a combination of BTB/POZ (broad complex, tramtrac, and bric-a-brac/poxvirus and zinc finger) and BACK (BTB and C-terminal Kelch) domain ([123,251] and http://smart.embl-heidelberg.de). The C-terminal domain 4 does not have a significant similarity to any known domain family. The interactions of 90K/Mac-2BP with the ECM components have been mapped to the last three domains [251], and the N-terminal SRCR domain is currently devoid of a function. Other proteins containing the SRCR domain have been assigned to the molecular recognition of non-self components [318]; however, the SRCR of 90K/Mac-2BP has not been previously demonstrated to bind foreign antigens.

90K/Mac-2BP exists as homodimers that further oligomerize to form a huge ring-like complex under physiological conditions via domains 2 and 3 [123,251,319]. These oligomers of 90K/Mac-2BP are prone to dissociation at low pH [319]. We hypothesize that also sucrose would induce similar disintegration of the oligomers, thus forcing 90K/Mac-2BP to exist as homodimers in high sucrose concentrations. This would explain the more pronounced co-purification of 90K/Mac-2BP with TULV in OptiPrep™ as compared to sucrose.

We speculate that the SRCR domain of 90K/Mac-2BP is responsible for the binding to TULV and would thus be involved in the recognition of non-self. The level of 90K/Mac-2BP has been shown to correlate with the viral load in the case of HIV infection [57,111]. Interaction of 90K/Mac-2BP with HIV particle would be supported by the observation that galectin-1, a known ligand of 90K/Mac-2BP, promotes the binding of HIV to host cells [236,265]. If 90K/Mac-2BP would be bound to HIV particles, the divalent ligand, galectin-1, could provide a bridge between the virus and the cell surface. Our observation that anti human-90K MAb was able to inhibit TULV infection in cell culture could be related to the interactions between 90K/Mac-2BP and the ECM components. 90K/Mac-2BP could act as a viral attachment factor, promoting the binding of hantaviruses to the cell surface. As galectin-1 has been demonstrated to promote the attachment of HIV to cells [236,265], it would be interesting to test this protein also in the case of hantaviruses. This could help in determining whether the interaction between 90K/Mac-2BP and TULV is beneficial or disadvantageous for hantaviruses.

90K/Mac-2BP in acute PUUV infection

Previous studies have suggested that 90K/Mac-2BP might have a role as an immune stimulator [65,109,255,290,369,399]. IFNs have been shown to induce 90K/Mac-2BP up-

regulation [65,139], which in turn has been demonstrated to induce the production of various cytokines such as ILs and TNF-α [290,369], activation of T and NK cells [290] and MHC-1 expression [109,255]. It has also been shown that children who receive high levels of 90K/Mac-2BP in breast milk suffer less from acute respiratory infections [90]. Contrary to this finding a high baseline level of 90K/Mac-2BP is associated with faster progression of HIV infection to AIDS (acquired immunodeficiency syndrome) and with shorter survival [209].

What is the role of 90K/Mac-2BP up-regulation in acute HFRS? Acute HFRS and/or HCPS, and the up-regulation of 90K/Mac-2BP share several common features, such as induction of IL-1, IL-6 and TNF-α [27,181,204,222,267,290,296,362]. Could the induction of 90K/Mac-2BP production be the initial step in hantavirus pathogenesis? It could be speculated, based on the observed direct interaction between TULV and 90K/Mac-2BP, that 90K/Mac-2BP would be involved in the recognition of non-self. Tagging of virions with 90K/Mac-2BP could be used to promote the clearance of virus, and to activate the complement system. This would be supported by the observed correlation between the 90K/Mac-2BP level and complement activation. However, based only on the correlation one cannot draw a conclusion on which of the two events precedes the other. The fact that 90K/Mac-2BP induces T and NK cell proliferation [290] combined with the observation that increased amounts of NK cells are observed over a prolonged period following hantavirus infection [26], could suggest that 90K/Mac-2BP levels would remain high long after the acute infection. We attempted to approach this question by using the serum samples of cynomolgous macaques experimentally infected with PUUV [174,176,177,336]. Unfortunately, the kit used in measuring the 90K/Mac-2BP levels from patient samples did not cross-react with the 90K/Mac-2BP of the monkey samples. Studying these samples in the near future will provide more insight into the role of 90K/Mac-2BP in not only hantavirus, but in acute virus infection in general. How does the induction of 90K/Mac-2BP correlate to virus levels? How does the basal level of 90K/Mac-2BP correlate with the prognosis of HFRS? These are the obvious questions to be addressed in future studies.

CONCLUDING REMARKS AND FUTURE PROSPECTS

The changing weather conditions allow and even force rodents to spread into new areas. The globalization has made it possible for people to travel to more exotic locations. These factors potentially increase the amount of contacts between rodents and man, and have thus raised hantaviruses as a potential re-emerging threat to public health. The most effective means against viral infections in general is vaccination; however currently only in China are people more broadly vaccinated against hantaviruses. While the existing vaccine is generated through inactivation of “live” virus, there is a demand for a safer alternative such as a recombinant vaccine generated of components insufficient for replication. It is commonly assumed that the structural proteins of a virion, that is the antigens, are most naturally represented in a form of VLPs. VLPs by definition are incapable of replicating. Even though the epitopes and the antigens required for protective immunity against hantaviruses are rather well known, the molecular mechanisms behind the generation of hantavirus-like particles remain largely unsolved. The interactions between the structural components of virion, described in this study, are of profound importance in understanding the mechanisms of virion assembly.

There are still a number of open questions related to the assembly of hantaviruses that should be answered in future studies. How is the spike generated? The mechanism of spike generation/folding is an interesting question since it is known that both Gn and Gc are required for correct folding and subcellular targeting. Does the tetramer of Gn form by oligomerization of heterodimeric units or rather via homo-oligomerization of Gn monomers? Is the putative fusion loop of Gc covered in virions by Gn-Gc or Gc-Gc interaction? Does the virion require other factors than pH (such as a receptor or reducing conditions) for successful fusion? How do the seemingly rectangular units form a roundish virion? What drives the budding, is it the curvature generated by glycoprotein lattice, spike-RNP interaction or a combination of the previous? What is the cellular localization where the spike-RNP interaction takes place?

Even though the most effective measure against viral infections is vaccination, there is also the possibility of intervention during infection. Such therapeutic measures are typically via prevention of one or more of the key steps during the life cycle of the virus. Potential targets for intervention during the replication of enveloped RNA viruses include at least the following: binding to the receptor, release to cytoplasm (or transport to nucleus), replication and transcription, translation, assembly and release. Prevention at the stage of receptor binding can be very effective; however, the problem is that by preventing the binding of virus one might also prevent the natural ligands of the target receptor from binding and thereby potentially inducing other problems.

Inhibition of the release from endosome is also a potential target for antiviral therapy and this approach is used for instance in HIV therapy. This type of inhibition requires thorough understanding of the fusion event, and typically also structural information of the fusion protein. The peptides interacting with Gn and Gc (described in I) could potentially provide means to intervene with this step of the hantavirus entry.

Drugs targeting the transcription of the virus can potentially be very selective as is the case for HIV. In the case of hantaviruses the RdRp would be the most potential target for

this type of an intervention. Currently ribavirin, a prodrug that is metabolized to a nucleotide analog, is used in severe hantavirus cases, and in case of hantaviruses it functions by causing an error catastrophe during replication.

Preventing the formation of progeny virions by inhibiting the interaction between the Gn-CT (or the spike complex) and the RNP could also be a potential target for intervention. The Gn-CT peptides interacting with the RNP, described in III, could be used to prevent natural Gn-CT–RNP interaction. In similar fashion the peptides interacting with Gn and Gc could be used to prevent the formation of the spike complex. The problem with these approaches is that the peptides are not likely to spontaneously enter the cytoplasm, and thus conjugation to a carrier molecule would be required.

Several factors have contributed to the increased amount of contacts between people and previously less-known or even unknown pathogens. Many of these pathogens come from animals and most these zoonotic infections are caused by enveloped RNA viruses. Therefore there is a need for research aiming at understanding the basic mechanisms behind the pathogenesis and the replication of such viruses. There most likely is no universal feature that would be shared by all viruses, but it is possible that through analogy one can find broadly applicable ways to intervene viral replication.

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