Tomato spotted wilt virus particle assembly

studying the role of the structural proteins *in vivo*

Promotor:
Prof.dr. R. W. Goldbach
Hoogleraar in de Virologie
Wageningen Universiteit

Co-promotor:
Dr. ir. R. J. M. Kormelink
Universitair Docent bij de leerstoelgroep Virologie
Wageningen Universiteit

Promotiecommissie:
Prof. dr. P.J.M. Rottier (Universiteit Utrecht)
Dr. F. J.M. van Kuppeveld (Radboud Universiteit Nijmegen)
Prof. dr. A.J.W.G. Visser (Wageningen Universiteit)
Prof. dr. A.M.C. Emons (Wageningen Universiteit)

Dit onderzoek is uitgevoerd binnen de onderzoekschool Experimentele Plantwetenschappen

Tomato spotted wilt virus particle assembly

studying the role of the structural proteins *in vivo*

Marjolein Snippe

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. M. J. Kropff,
in het openbaar te verdedigen
op vrijdag 17 februari 2006
des namiddags te half twee in de Aula.

M. Snippe
Tomato spotted wilt virus particle assembly: studying the role of the structural proteins *in vivo*.
Thesis Wageningen University, The Netherlands
With references – with summaries in English and Dutch
ISBN 90-8504-326-3

Contents

Chapter 1

General introduction

This chapter has been published in a modified version as

Marjolein Snippe, Rob Goldbach and Richard Kormelink (2005). Tomato spotted wilt virus particle assembly and the prospects of fluorescence microscopy to study protein-protein interactions involved. Advances in virus research **65**:63-120

For the animal-infecting members of the *Bunyaviridae*, the site of virus assembly was identified as the Golgi complex long time ago. However, for the plant-infecting bunyavirus tomato spotted wilt virus (TSWV) this has long been an enigma, even though most cytopathological structures related to the assembly of TSWV were already observed in infected plants decades ago. It was only a couple of years ago that the site of particle assembly was identified as the Golgi complex. Whereas for the animal-infecting counterparts, particle assembly occurs by budding of ribonucleoproteins into the vacuolised lumen of the Golgi complex, for TSWV particles arise as the result of the wrapping of Golgi cisternae around ribonucleoproteins, leading to the formation of so-called doubly-enveloped virus (DEV) particles. Fusion of these DEVs to each other and likely to ER-derived membranes lead to formation of large vesicles in which mature TSWV particles accumulate and are retained. Unlike what occurs in plant cells, TSWV is secreted from its insect vector (thrips) cells, in which the virus also replicates. The formation of DEVs has never been observed in thrips cells and the secretion of TSWV from these cells is thought more to resemble the infection and release route of animal-infecting bunyaviruses from mammalian cells. This duality in assembly routes of TSWV, retention (plant cells) versus secretion (thrips cells), likely reflects the adaptation of TSWV to infect plant cells while retaining the capacity to infect its insect vector. It is generally assumed that the glycoproteins, that form the spikes on the lipid envelope of the virus particle, are the major determinants in the process of virus assembly. Hence, various efforts have been made to further characterise these proteins and study their maturation and interactions to other (non)structural proteins that lead to particle assembly.
During the last decade virological research is slowly shifting from the molecular biology to more cell biology focussed studies. The latter is more and more making use of bio-imaging which is made possible due to an increasing number of various fluorescence microscopy techniques being developed that not only allow visualisation of proteins in a living cell, but also investigation of protein-protein interactions, protein folding studies etc.
In the first part of this chapter, the current status of TSWV particle assembly and the involvement of structural proteins in this process will be described and compared to what is known for the animal-infecting counterparts. The second part will describe more recently developed fluorescence microscopy techniques that enable the analysis of *in vivo* protein-protein interactions and how some of these techniques can be employed in virus research.

Bunyaviridae **and the genus** ***Tospovirus***

Tomato spotted wilt virus (TSWV) is the type species of the genus *Tospovirus* within the family *Bunyaviridae*. Tospoviruses are restricted to plants as their host, whereas all other members of this family infect animals. The latter ones are classified in 4 genera, i.e. *Orthobunyavirus*, *Nairovirus*, *Hantavirus* and *Phlebovirus*. Most members of the *Bunyaviridae* are arthropod-borne viruses, hantaviruses being the exception as they lack an arthropod vector (Table 1; for recent reviews see Elliott, R.M. (1996) in "*The Bunyaviridae*"). At this point it should be noted that in this thesis, the term "bunyavirus" will be used to refer to

any member of the *Bunyaviridae*, and not only to members of the genus *Orthobunyavirus*.

Family	Genus	Type species	Arthropod Vector
Bunyaviridae	Orthobunyavirus	Bunyamwera	Mosquitoes
	Phlebovirus	Rift Valley fever	Mosquitoes, Ticks, Phlebotomines
	Nairovirus	Crimean Congo haemorrhagic fever	Mosquitoes
	Hantavirus	Hantaan	None
	Tospovirus	Tomato spotted wilt	Thrips

Table 1: The *Bunyaviridae* family

At this moment 14 different tospovirus species, and one tentative (Hassani-Mehraban *et al.*, 2005), have been recognised, of which TSWV has the broadest host range and has become most widely distributed. The cumulative host range of tospoviruses encompasses over 1000 different plant species, mostly *Compositae* and *Solanaceae* (Peters, 1998). High yield losses have been reported in many different, economically important agricultural and ornamental crops (Goldbach and Peters, 1994). Among these are dicots as well as monocots. Tospoviruses are exclusively transmitted by a limited number of phytophagous thrips (order *Thripidae*), in a propagative manner (Wijkamp *et al.*, 1993). So far 10 different species have been demonstrated to act as vector, with *Frankliniella occidentalis* (also known as California thrips, Western flower thrips, Alfalfa thrips) as the most important one.

Particle morphology and expression strategy of the viral genome

Tospoviruses are relatively easy to identify in infected tissues due to their unique particle morphology. Like all other members of the *Bunyaviridae*, virions are spherical lipid-bound particles, 80-120 nm in diameter, covered with spike projections consisting of two glycoproteins Gn and Gc (Figure 1, left panel). The core consists of pseudo-circular ribonucleocapsids (RNPs; Figure 1, right panel), each consisting of a viral RNA segment tightly packaged by the nucleocapsid protein (N) and minor amounts of a large protein (L), the putative viral RNA-dependent RNA polymerase (Mohamed, 1981; Mohamed *et al.*, 1973; Tas *et al.*, 1977; Van den Hurk *et al.*, 1977; Van Poelwijk *et al.*, 1993; Verkleij and Peters, 1983). All members of the *Bunyaviridae*, including the tospoviruses, replicate in the cytoplasm of the cell.

The viral genome consists of three single-stranded, linear RNA segments, which according to their sizes have been denoted small (S) RNA, medium (M) RNA, and large (L) RNA, in analogy to the animal-infecting bunyaviral genomes (De Haan *et al.*, 1989; Van den Hurk *et al.*, 1977; Verkleij *et al.*, 1982).

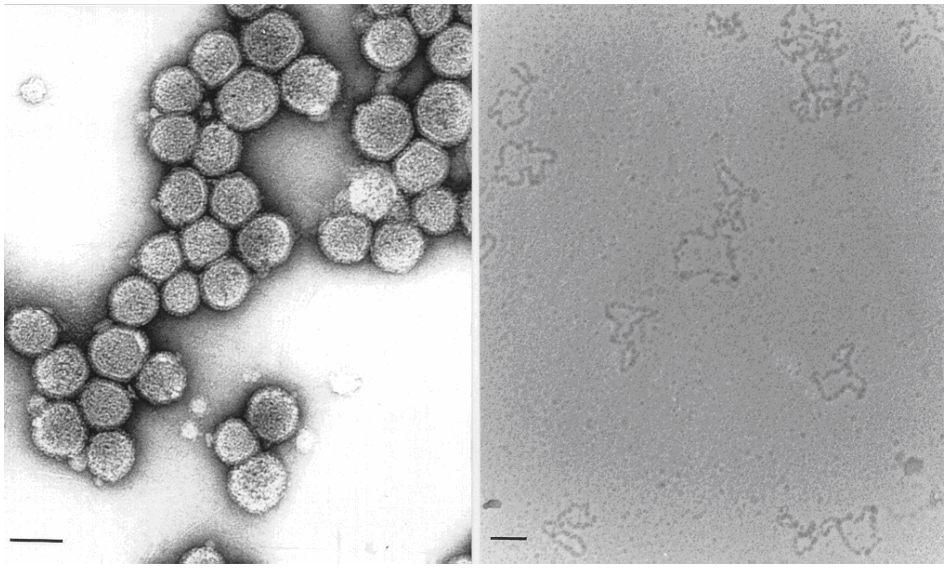


Figure 1. Electron micrograph of purified TSWV particles (left) and TSWV RNPs (right). Size bar indicates 100 nm.

Sequence data have resulted in a genomic organisation and expression strategy as depicted for TSWV in figure 2 that is so far representative for all tospoviruses.

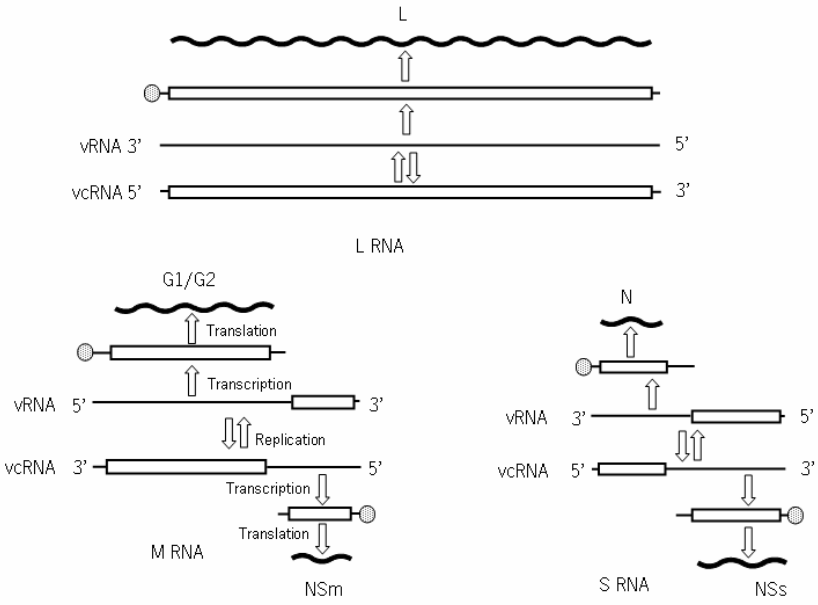


Figure 2. Organisation and expression strategy of the TSWV genome. The highly conserved terminal sequences are indicated by a black box. The small dotted circles at the 5' end of ((sub)genomic-length) viral transcripts represent non-viral leader sequences and result from transcription initiation by cap-snatching.

Purified virus preparations contain all three genomic segments and viral (v) RNA is often observed in excess over viral complementary (vc) RNA strands, which may possibly reflect the amounts synthesised during replication (Bouloy *et al.*, 1973/1974; Elliott, 1990; Eshita *et al.*, 1985; Gentsch *et al.*, 1977; Ihara *et al.*, 1985a; Kormelink *et al.*, 1992b; Objieski *et al.*, 1976; Objieski and Murphy, 1977; Pettersson and Kääriäinen, 1973). Encapsidation of viral mRNAs into virus particles appears not to take place.

The L RNA segment of TSWV is of complete negative polarity. It contains one large open reading frame (ORF) in the viral complementary (vc) strand, coding for a protein of 331.5 kDa (De Haan *et al.*, 1990), the putative RNA dependent RNA polymerase. The TSWV M RNA is of ambisense polarity, containing two non-overlapping ORFs, one in the viral (v) strand coding for a non-structural protein (NSm) of 33.6 kDa, the putative cell-to-cell movement protein, and one in the vc-strand, encoding the precursor to the glycoproteins of 127.4 kDa (Kormelink *et al.*, 1992a, 1994; Storms *et al.*, 1995). The TSWV S RNA is also of ambisense polarity, encoding a non-structural protein (NSs) of 52.1 kDa in the v-strand that acts as a suppressor of silencing, and the nucleoprotein (N) of 28.9 kDa in the vc-strand (Bucher *et al.*, 2003; De Haan *et al.*, 1990; Takeda *et al.*, 2002).

Expression of the viral genes occurs by the synthesis of (sub)genomic sized mRNAs which discriminate from full-length (anti)genomic RNA strands by the additional presence of a non-viral leader sequence at the 5'-ends, generally 10-20 nucleotides in length (Figure 2; Duijsings *et al.*, 1999, 2001; Kormelink *et al.*, 1992c; Van Knippenberg *et al.*, 2002; Van Poelwijk *et al.*, 1996). This sequence is the result of cap-snatching, a process by which the viral polymerase, encompassing an endonuclease activity, cleaves off capped-RNA leader sequences from cellular mRNA molecules to use these as primers for transcription initiation on the viral genome (Braam *et al.*, 1983; Plotch *et al.*, 1981; Ulmanen *et al.*, 1981), a feature that is common for all members of the *Bunyaviridae*, as well as the *Arenaviridae*, *Orthomyxoviridae*, and the floating genus *Tenuivirus* (Bishop *et al.*, 1983; Bouloy *et al.*, 1990; Braam *et al.*, 1983; Caton and Robertson, 1980; Collett, 1986; Dhar *et al.*, 1980; Garcin and Kolakofsky, 1990; Huiet *et al.*, 1993; Patterson and Kolakofsky, 1984; Plotch *et al.*, 1981; Raju *et al.*, 1990; Ramirez *et al.*, 1995; Shimizu *et al.*, 1996; Simons and Pettersson, 1991; Ulmanen *et al.*, 1981). In contrast to transcription initiation, not much is known on transcription termination. Bunyaviral transcripts are not polyadenylated, nor do they share a conserved sequence motif that may act as a transcription termination signal. For both the ambisense TSWV M and S RNA segments the intergenic region, highly rich in A- and U-stretches and thereby able to form a hairpin structure, this hairpin has been suggested to play a role in this process (De Haan *et al.*, 1991; Kormelink *et al.*, 1992a; Maiss *et al*, 1991). In this respect, it is interesting to mention that recently, TSWV transcripts contain a predicted stem-loop structure at their 3' end that may be involved in stimulation of viral mRNA through 5'-3' terminal interaction, as has been observed for the 3'-end structural feature of several plant-infecting RNA viruses lacking a poly(A)-tail (Fabian and White, 2004; Gallie, 1998; Gallie and Kobayashi, 1994; Leonard *et al.*, 2004; Matsuda and

Dreher, 2004; Meulewaeter *et al*., 2004; Neeleman *et al*., 2001). Hairpin structures are also likely present in some, but not all other bunyavirus genera.
The promoters for transcription and replication of the viral genome are embedded within the conserved terminal end sequences, as has been demonstrated with reverse genetics systems by the analyses of replicational and transcriptional activity of minigenomes harbouring mutagenised termini for the Bunyamwera orthobunyavirus, Rift Valley fever and Uukuniemi phleboviruses (Dunn *et al*., 1995; Flick and Pettersson, 2001; Flick *et al*., 2002, 2004; Lopez *et al*., 1995; Prehaud *et al*., 1997).

Protein functions
L protein (RNA-dependent RNA polymerase). Based on sequence similarities with putative and proven RNA polymerases from other negative-stranded, segmented RNA viruses, e.g. influenza viral PB1 protein and the Bunyamwera L RNA encoded RNA polymerase, the TSWV 331.5 kDa protein represents the putative viral RNA-dependent RNA polymerase. The sequence similarity is confined to five types of consensus sequences which are characteristic for RNA polymerases that display RNA template specificity, the so-called polymerase motifs (Poch *et al*., 1989; Tordo *et al*., 1992). The TSWV L protein is not being processed and can be detected in purified virus and RNP preparations (Van Poelwijk *et al*., 1993).
In vitro replicase and transcriptase activity of the RdRp have been demonstrated for purified, detergent-disrupted virus preparations of TSWV, La Crosse, Lumbo, germiston, Uukuniemi and Hantaan virus and in some cases depends on the additional presence of the translation machinery to support ongoing transcription (Bellocq *et al*., 1986; Bellocq and Kolakofsky, 1987; Bouloy and Hannoun, 1976; Chapman *et al*., 2003; Gerbaud *et al*., 1987; Patterson *et al*., 1984; Raju and Kolakofsky, 1986a and b; Ranki and Pettersson, 1975; Schmaljohn and Dalrymple, 1983; Van Knippenberg *et al*., 2002 and 2004; Vialat and Bouloy, 1992).
The L protein has been suggested to encompass several enzymatic activities such as polymerase and endonuclease. Depending on the circumstances (e.g. presence or absence of viral/host factors) the polymerase may exhibit transcriptase or replicase activity (Van Knippenberg *et al*., 2002). A free pool of soluble N protein is assumed to play a role in this, based on observations made for influenza virus (Beaton and Krug, 1984, 1986). As the L protein does not possess methyl transferase activity required for capping of viral transcripts, but bunyaviruses instead use cap-snatching to provide their transcripts with an m^7G-ppp cap structure, the L protein likely contains a cap-binding domain to retrieve capped cellular mRNA molecules as cap-donors. It is not unlikely that the L protein contains additional domains that play a role in other processes than transcription/replication, e.g. host range determinants or matrix function similar as to the M protein of many other negative strand RNA viruses, in light of the large size differences observed between L proteins of different genera.

Glycoproteins. The amino-acid sequence of the TSWV glycoprotein precursor contains 8 potential N-linked glycosylation sites and several hydrophobic domains (Figure 3) of which some act as a signal sequence and others as transmembrane

domains. The precursor is processed to render the glycoproteins G1 and G2. G1 (78 kDa) is located carboxy-terminal and G2 (58 kDa), amino terminal within the precursor. To avoid any confusion in the comparison with functional homologs of the bunyaviral glycoproteins, they are more generally referred to as Gn (amino-terminal) and Gc (carboxyterminal). A low, but significant sequence homology has been observed with the glycoprotein precursor of *Orthobunyavirus* members Bunyamwera, snowshoe hare, La Crosse and germiston virus (Figure 3; Kormelink *et al.*, 1992a). This homology is mainly restricted to a stretch of amino-acids within Gc. Based on this observation and the additional homology between the respective L proteins, tospoviruses are most closely related to orthobunyaviruses.

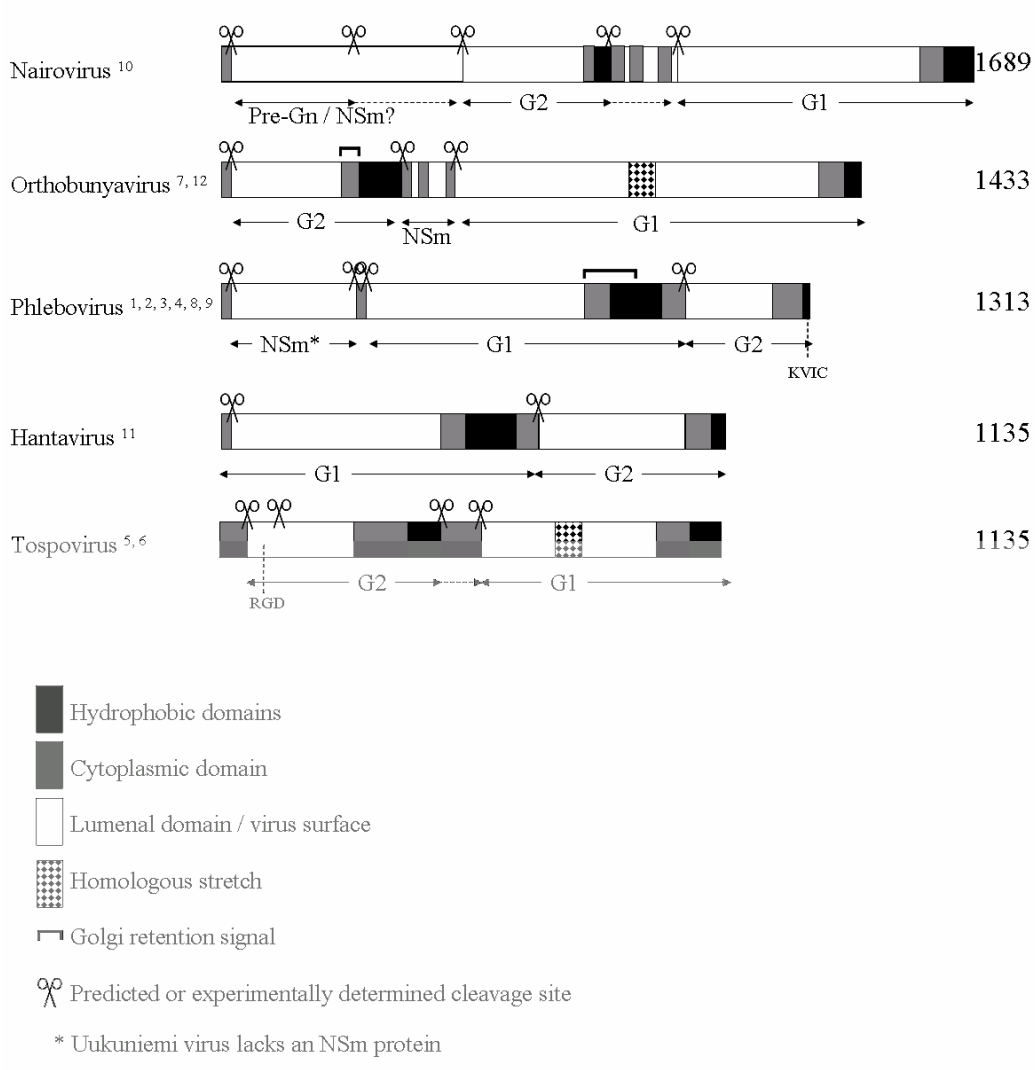


Figure 3. Topology of bunyaviral glycoprotein precursor proteins. Numbers refer to amino acid residues. The sequences of the prototypes of the genera have been used in all cases, except for the Phlebovirus genus where the sequence of Punta Toro virus has been used.
1: Andersson *et al*, 1998; 2: Collet *et al.*, 1985; 3: Gerrard and Nichol, 2002; 4: Ihara *et al.*, 1985b; 5: Kikkert *et al.*, 2001; 6: Kormelink *et al*, 1992a; 7: Lees *et al.*, 1986; 8: Matsuoka *et al.*, 1996; 9: Rönnholm and Pettersson, 1987; 10: Sanchez *et al.*, 2002; 11: Schmaljohn *et al.*, 1987; 12: Shi *et al.*, 2004

Both Gn and Gc are type I membrane spanning proteins and have been shown to be glycosylated when expressed in insect cells or mammalian cells (Kikkert *et al.*, 2001; Kormelink, 1994).
Although the presence of several N-glycosylation sites were predicted for both Gn and Gc, no N-linked glycosylation was detected in Gn from virus particles purified from infected plants. With respect to O-linked glycosylation, opposing data have been presented (Naidu *et al.*, 2004; Whitfield *et al.*, 2004). To date, no evidence for O-linked oligosaccharides of the animal-infecting members of the *Bunyaviridae* has been obtained, but various reports have appeared on N-linked glycosylation (Antic *et al.*, 1992; Lappin *et al.*, 1994; Shi and Elliott, 2004).
Both glycoproteins mature by co-translational cleavage from the precursor, a process in which ER residing proteases are assumed to be involved. For TSWV, cleavage of the signal sequence has been predicted to occur at amino acid residue 35 from the N terminus (Von Heijne, 1986; Figure 3). The exact N-terminus of Gc has so far remained unknown. For TSWV, as well as the animal-infecting counterparts, cleavage sites have been predicted and some confirmed, but not much is known on the signal peptidase involved. For Hantaan virus the glycoprotein precursor is cleaved at a conserved pentapeptide WAASA, which was found absolutely conserved for all hantaviruses (Löber *et al.*, 2001). For Crimean-Congo hemorrhagic fever (CCHF) nairovirus both Gn and Gc are preceded by tetrapeptides RRLL and RKPL, respectively, which led to the suggestion that endoplasmatic reticulum-residing SKI-1 or related proteases are involved in their processing (Sanchez *et al.*, 2002). Additional studies revealed that only Gn processing requires subtilase SKI-1, and Gc does not (Vincent *et al.*, 2003). Previously, the surface glycoproteins of Lassa virus, a member of the *Arenaviridae* family, were also shown to be cleaved at an RRLL motif into an N- and C-terminal glycoprotein by the cellular subtilase SKI-1/SP1 (Lenz *et al.*, 2000 and 2001).
Analysis of the amino acid sequence of the TSWV glycoprotein precursor, furthermore, revealed the presence of an Arg-Gly-Asp (RGD)-motif, a putative cell attachment site, within Gn immediately downstream the predicted signal cleavage site (Figure 3; Kormelink *et al.*, 1992a; Law *et al.*, 1992). It is recognised that the tospoviral envelope glycoproteins are major determinants for the insect transmission and specificity (Wijkamp, 1995), as they may interact with cell-surface receptors on the insect midgut epithelium, the first site of virus infection (Nagata *et al.*, 2002; Tsuda *et al.* 1996; Ullman *et al.*, 1992, Wijkamp *et al.*, 1993), and thereby mediate virus uptake by the thrips (Bandla *et al.*, 1998). Hence it was speculated that the RGD motif in Gn of TSWV would be involved in the attachment of tospovirus particles to the thrips midgut prior to acquisition (Kormelink *et al.*, 1992a). However, no RGD motif is present in Gn of other tospoviruses. Furthermore, virus-overlay assays have demonstrated that in case the RGD motif in TSWV Gn would be responsible for binding to a potential 94 kDa thrips cell receptor, this would probably not occur in the midgut, as the 94 kDa thrips protein was absent from these tissues (Kikkert *et al.*, 1998). On the other hand, studies performed by Bandla *et al.* (1998) showed that both Gn and Gc were involved in virus attachment to the thrips midgut, and they identified a potential receptor

protein of about 50 kDa. More recently, another study again confirmed Gn as the candidate viral ligand for binding to a midgut epithelium receptor (Whitfield *et al.*, 2004).
Also for the animal-infecting bunyaviruses only limited information is available on the role of both glycoproteins during the initial infection of a cell. For California encephalitis (CE) virus, neutralisation of Gc with monoclonal antibodies, or trypsinisation resulted in loss of infectivity both in mosquitoes and in culture (Hacker *et al.*, 1995). Furthermore, Gc was shown to undergo conformational changes (Pekosz and González-Scarano, 1996) necessary for low pH-mediated entry into cell cultures (Hacker and Hardy, 1997). For La Crosse virus, a Gc variant was selected that was restricted in the ability to infect mosquitoes when ingested, but not when injected intrathoracically (Sundin *et al.*, 1987), whereas after reversion the capacity to infect after ingestion was regained, suggesting a Gc requirement for interactions at the midgut level. Other studies by Ludwig *et al.* (1989 and 1991) demonstrated the requirement of La Crosse Gc for infection of cell cultures. Altogether, indicating an important role for Gc, possibly as the viral attachment protein, in the initial uptake of bunyaviruses in the midgut. Surprisingly, the Gc glycoprotein of all tospoviruses and orthobunyaviruses share a highly conserved peptide sequence in the core of this protein (Figure 3) which supports the idea of functional homologs and, furthermore, of an involvement of TSWV Gc in the actual attachment during receptor-mediated uptake in vector midgut cells (Pekosz *et al.*, 1995), a function that other studies would attribute to TSWV Gn.

NSm. Only during the very early stages of a TSWV infection in plant tissues, even prior to the appearance of virus particles, NSm protein is expressed and shown to form tubular structures that extend from plasmodesmata into newly infected cells. These tubules are also formed upon transient expression of NSm in protoplasts in the absence of any other viral protein (Kormelink *et al.*, 1994; Storms *et al.*, 1995). In addition to its capacity to form tubules, NSm is found associated to non-enveloped nucleocapsid aggregates (Kormelink *et al.*, 1994). For these reasons it has been suggested that the NSm protein is implicated in the cell-to-cell movement of non-enveloped infectious ribonucleocapsid structures of TSWV. This hypothesis is supported by results from microinjection studies of fluorescent dyes into parenchyma cells of transgenic plants expressing the NSm protein, which reveal a modification of the size exclusion limit of plasmodesmata (Storms *et al.*, 1998). The NSm protein has, furthermore, been shown to interact with N and to reversibly and in a non-specific manner bind to ssRNA and the panhandle structure (Soellick *et al.*, 2000). The interaction with N was suggested to explain the observed NSm association to RNPs.
For members of the *Orthobunyavirus*, *Phlebovirus* and *Nairovirus* genera the existence of an NSm protein is suggested based on the topology, cleavage products and maturation of the glycoprotein precursor. However, since tospovirus genomes code for the NSm protein in a separate ORF, while the animal-infecting bunyaviruses encode it as part of the glycoprotein precursor, and the tospoviral NSm protein is required for viral movement in plant tissues, it is unlikely that the

NSm protein of the animal-infecting viruses is functionally or structurally equivalent to the tospoviral NSm. For Bunyamwera orthobunyavirus the NSm protein of has been suggested to play a role in virion formation, based on its Golgi localisation (Lappin *et al.*, 1994; Nakitare and Elliott, 1993). For the La Crosse orthobunyavirus NSm protein, though, such localisation profile has not been observed (Bupp *et al.*, 1996).

NSs. The NSs protein is normally found distributed either dispersed throughout the cytoplasm or accumulating in fibrillar inclusion bodies in the cytoplasm of infected cells. A correlation has been observed between the amounts of NSs expression and virulence of tospovirus isolates (Kormelink *et al.*, 1991). However, the function of NSs has long been unknown also due to the absence of any sequence homology to other viral or host proteins. Recently, the NSs protein has been demonstrated to act as suppressor of RNA silencing (Bucher *et al.*, 2003; Takeda *et al.*, 2002).
Whereas the nairo- and hantavirus members do not seem to encode an NSs protein, orthobunyaviruses and phleboviruses code for an NSs protein overlapping or in ambisense arrangement with the N ORF, respectively. One or more different functions, such as downregulation of viral RNA synthesis, viral evasion of the host's anti-viral interferon response as well as counteracting the effect of siRNA, are performed by the NSs proteins of different genera and differ between genera (Bouloy *et al.*, 2001; Bridgen *et al.*, 2001; Haller *et al.*, 2000; Kohl *et al.*, 2003; Lopez *et al.*, 1995; Soldan *et al.*, 2005; Weber *et al.*, 2001, 2002).
For several bunyaviruses the NSs protein has furthermore been found associated with polysome fractions or the 40S ribosomal subunit (DiBonito *et al.*, 1999; Simons *et al.*, 1992; Watkins and Jones, 1993), which would suggest an involvement in translation. For TSWV, this observation has not been made, but the protein has been hypothesised to play a role in translation, possibly by functioning in a manner homologous to poly-A binding protein (Van Knippenberg, 2005).

Nucleocapsid protein (N). Among tospoviral proteins the nucleocapsid (N) protein is the least conserved (15-25% sequence identity) and is being used as a discriminative factor for taxonomic classification. For the animal-infecting bunyaviruses, serological relations are an important criterion for classification. The N protein is likely a multifunctional protein involved in several processes. The protein encapsidates viral RNA, not viral mRNA, and it is required for transcription and replication of the viral genome. In the latter processes the N protein is hypothesised to be involved in the switch from transcription to replication and in addition to act as anti-terminator of transcription, similar as observed for the N protein of influenza virus (Beaton and Krug, 1984, 1986) and vesicular stomatitis virus (Patton *et al.*, 1984). In relation to these features, RNA-binding properties as well as homo-oligomerisation capacity have been described for the N protein. Precise details on both features remain as yet unclear. For TSWV, both N and C-terminal halves of the protein, as well as the extreme carboxy-terminal region, exhibited non-specific RNA affinity in a cooperative manner (Figure 4; Richmond *et al.*, 1998), but this would not explain the apparent viral (anti)genomic RNA-

specific encapsidation. However, no protein binding studies were performed on RNA constructs that included the panhandle structure. For Hantaan virus N an RNA binding domain that specifically interacted with viral RNA was mapped to a central conserved region of 42 amino acids within the N protein (Figure 4; Xu *et al.*, 2002). Of this region the first half contains the major determinants for RNA binding, whereas the second half exhibits specificity for viral sense RNA in the presence of the first half. The latter was suggested to explain the specific packaging of viral genomic and not antigenomic RNA into virions. The specific interaction of Hantaan virus vRNA by the N protein, furthermore, seemed to involve the 5' non-coding region of viral RNA (Severson *et al.*, 1999, 2001). More recently, trimeric forms of Hantaan virus N were shown to exhibit specific affinity for the panhandle structure, formed by the complementary sequences of the 5' and 3' end of the viral genomic RNA (Mir and Panganiban, 2004) whereas monomeric and dimeric forms of N only showed RNA affinity in a non-specific manner.

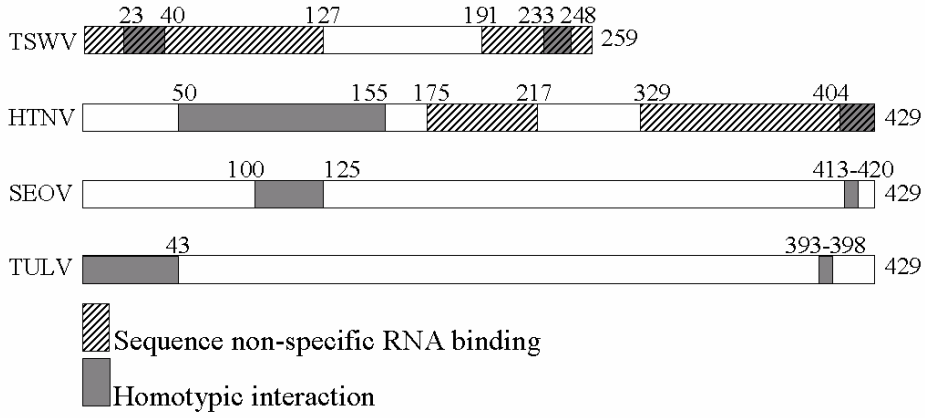


Figure 4. Topology of the N protein of representatives from the different genera of the *Bunyaviridae*.

The fact that bunyaviral transcripts are never observed encapsidated by N protein, and transcripts lack the ability to form a panhandle structure, a specific affinity of the (trimeric) N protein for the panhandle seems very plausible in order to discriminate between genomic RNA and mRNA, or even sequence non-specific single stranded RNA. On the other hand, Atomic Force microscopy on TSWV RNPs, purified from CsCl gradients, revealed that the panhandle structure of RNPs is free from any protein, including N (Kellman *et al.*, 2001). Thus, the panhandle could very well represent the encapsidation signal for (anti)genomic RNA segments but at the ultimate end to some extend remain free of N. In this way the terminal sequences of (anti)genomic RNA molecules would still be accessible for e.g. the replicase/transcriptase complex or other (viral) proteins.

Regarding the (*in vitro*) oligomerisation capacity of N, different suggestions have been for the organisation of such interaction. Whereas initially a "head-to-tail" interaction was proposed for TSWV N (Uhrig *et al.*,1999), more recent data support the idea of a "tail-to-tail" and "head-to-head" organisation (Kainz *et al.* 2004). This has also been suggested for hantavirus N (Alfadhli *et al.*, 2001;

Kaukinen *et al.*, 2001; 2003; Yoshimatsu *et al.*, 2003). From these studies it was clear that neither N-terminal nor C-terminal tags to the N protein seem to interfere in these interactions, suggesting that these sequences are not required in a sterical tight conformation. Most studies on (tospovirus and hantavirus) *in vitro* N multimerisation resulted in the identification of an N- and a C-terminal region essential for interaction (Figure 4). Marked exceptions are Hantaan virus and Seoul virus, for which a C-terminal and a more internal region have been found (Kaukinen *et al.*, 2003).

Morphogenesis

In the host. Enveloped viruses usually obtain their lipid membranes by budding through one of the cellular membranes. When budding occurs at the plasma membrane, virion particles are directly released in the extracellular space. Viruses of the *Bunyaviridae* obtain their lipid envelop from intracellular membranes, i.e. the Golgi complex, which requires a final secretion from the cell after transport of the viruses within vesicles to the plasma membrane. For several animal-infecting viruses, the process of virus assembly has been studied more extensively (Booth *et al.*, 1991; Elliott, 1990; Gahmberg *et al.*, 1986; Jäntti *et al.*, 1997; Kuismanen *et al.*, 1982, 1984; Lyons and Heyduk, 1973; Murphy *et al.*, 1973; Rwambo *et al.*, 1996; Salanueva *et al.*, 2003; Smith and Pifat, 1982).

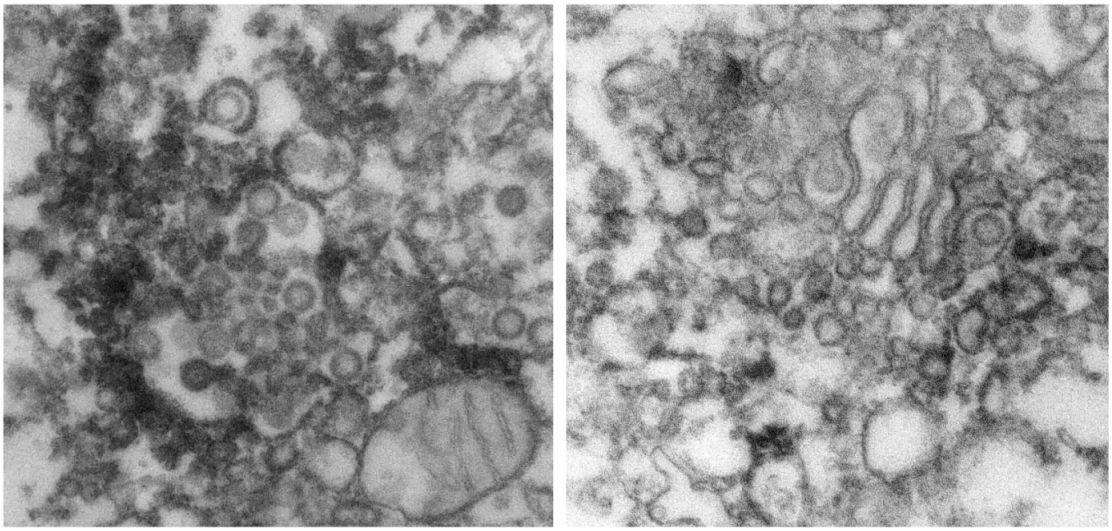


Figure 5. Presence of Uukuniemi virus particles and budding of RNPs as observed in nucleocapsid-positive 15°C intermediates. Clusters of cytoplasmic vesicles, vacuoles and tubules are observed, of which the vacuoles contain virus particles and budding virus (left and right panel; with kind permission from Esa Kuismanen). For similar pictures and more detailed explanation see Jäntti *et al.* (1997).

Although many of the details of the budding process are still poorly understood, it is generally assumed that the viral glycoproteins accumulate and concentrate at specific foci in smooth-surfaced membranes of the Golgi complex together with the viral ribonucleoproteins (RNPs). Simultaneously, Golgi cisternae vacuolise extensively allowing RNPs to bud into the lumen of the Golgi and leading to the formation of singly enveloped virus particles in the Golgi complex (Figure 5). The budding process is thought to be triggered by a specific interaction between the cytoplasmic tail of one of the glycoproteins (Andersson *et al.*, 1997a) and a

structural component of the RNP, for which the nucleoprotein is a candidate protein. The site of virus assembly is dictated by the viral glycoproteins that during their maturation accumulate and retain in the Golgi compartment due to the presence of a Golgi retention signal. Final release of virus particles from the cell was observed to occur predominantly by fusion of virus containing vesicles with the plasma membrane (Murphy *et al.*, 1973; Salanueva *et al.*, 2003; Smith and Pifat, 1982). In only a few cases have animal-infecting bunyaviruses been observed to bud at the plasma membrane (Anderson and Smith, 1987; Murphy *et al.*, 1973; Ravkov *et al.*, 1997).

For the plant-infecting tospoviruses, *in casu* TSWV, the process of particle morphogenesis has long been an enigma. Early electron microscopical studies (Milne, 1970; Ie, 1971) using infected leaf tissues, already revealed most typical structures associated with tospovirus infections that were observed, such as viroplasm (VP), nucleocapsid aggregates (NCA), paired parallel membranes (PPM) doubly enveloped particles (DEV), and singly enveloped particles (SEV) clustered within ER membranes.

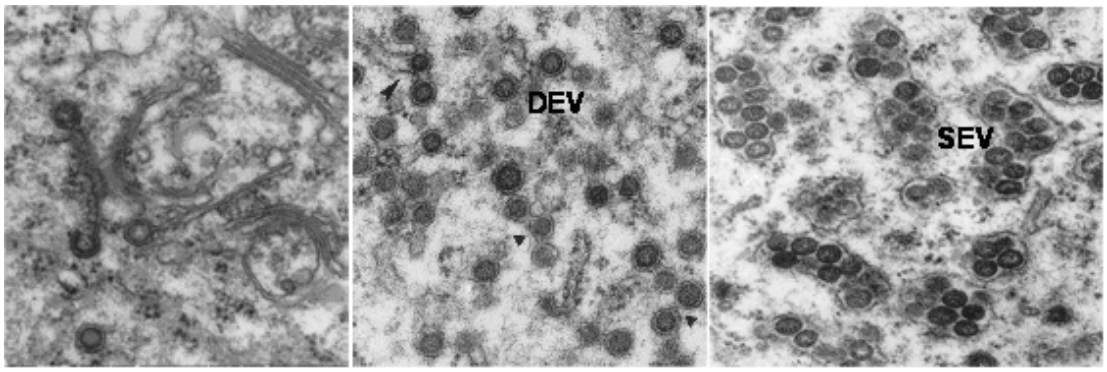


Figure 6. Assembly of TSWV enveloped particles by enwrapment of RNPs with Golgi cisternae (left panel). Fusion of DEVs to each other (middle panel) and ER-derived membranes leading to accumulation of mature virus particles (SEVs) in large vesicles (right panel).

Doubly-enveloped virus particles (DEVs) were found in the cytoplasm but only during early stages of infection (Francki *et al.*, 1985; Kitajima *et al.*, 1992; Lawson *et al.*, 1996; Milne, 1970). This founded the hypothesis that these structures formed an intermediate stage of particle morphogenesis, as a result of budding of nucleocapsids from parallel membranes. Based on their morphology, the paired parallel membranes have been suggested to originate from the Golgi system (Lawson *et al.*, 1996). Subsequent fusion of several DEVs was proposed to finally result in the accumulation of (singly-enveloped) virus particles (SEVs) in clusters within the ER (Kitajima *et al.*, 1992; Milne, 1970). This maturation pathway has been substantiated to be the mode of TSWV particle morphogenesis, by a detailed study using a plant protoplast infection system (Figure 6; Kikkert *et al.*, 1997 and 1999). By using ER and Golgi-specific markers these studies have shown that envelopment of nucleocapsids, leading to the formation of DEVs, occurs by enwrapment of parallel membranes derived from modified Golgi membranes (Kikkert *et al.*, 1999). In a later stage of the maturation, DEVs disappear by fusion to each other and ER-derived membranes, a process possibly facilitated by the presence of an ER retrieval signal that may be exposed after processing of Gn in the Golgi apparatus (Figure 6; Kikkert, 1999). Mature virions accumulated and

retained within subcellular (ER-derived) compartments, serve as the source from which thrips acquire the virus by feeding prior to transmission. Although the maturation of TSWV particles as described, is somewhat different from that of the animal-infecting bunyaviruses, the involvement of the Golgi system is a common feature of both the plant- and animal-infecting bunyaviruses.

In the arthropod vector. Tospoviruses are propagatively transmitted by their insect vector thrips, i.e. after acquisition and subsequent dissemination in the thrips body the virus not only circulates but also multiplies in the vector before being transmitted (Wijkamp *et al.*, 1993). Thrips only transmit the virus if this is acquired during the first or second larval instar but they do not transmit the virus transovarially. Thrips do not seem to suffer from the infection and have been suggested to respond with an immune response upon infection, based on the observed upregulation of several genes coding for anti-microbial peptides, lectins and receptors that activate the innate immune response (Medeiros *et al.*, 2004). So far, still only limited information is available on the life cycle of tospoviruses in the thrips vector and in particular on stages involving virus assembly. Despite this lack of detailed information, an important difference in the morphogenesis pathway of TSWV in thrips cells versus plant cells has emerged. Whereas TSWV particles are clearly retained intracellularly, probably ER-derived vesicles in plant cells, they are only observed in and secreted from thrips salivary gland cells, not in the least to allow transmission of the virus by thrips feeding on plants.
Immunolocalisation studies of TSWV in viruliferous thrips *F. occidentalis* have shown the presence of the N and NSs proteins in the salivary glands and in muscle cells associated with the midgut epithelium, the NSs protein being indicative for virus replication (Ullman *et al.*, 1992 and 1993; Wijkamp *et al.*, 1993). Whereas the N protein is found in confined areas designated as viroplasm, the NSs protein is dispersed throughout the cytoplasm. Amorphous inclusions have additionally been observed in infected thrips cells and can be labelled with antiserum directed to the Gc glycoprotein (Ullman *et al.*, 1995). Mature virus particles are observed only in low amounts in saliva vesicles, but in numerous amounts in the salivary gland ducts, suggesting that the salivary glands are the major site of replication. The presence of virus particles in saliva secretory vesicles supports the idea of virus particles being released during secretion of saliva into the ducts. Although this is an indication that the Golgi system may be involved in the assembly of virus particles in thrips, no actual virus assembly in the Golgi complex has been observed yet. Instead, budding of virus from the basal plasma membrane of midgut epithelial cells has been suggested to occur (Nagata *et al.*, 2002) but these observations have not been repeated and remain rather vague. In thrips that have acquired TSWV while feeding on infected plants during the adult stage, viral proteins have only been observed in the midgut epithelium, and not beyond these cells, indicating that dissemination of the virus to the hemocoel is blocked, and a midgut escape barrier likely exists in adult thrips (Ullman *et al.*, 1992). Immunofluorescence studies a.o. on *Thrips setosus* larvae following acquisition of TSWV, have shown that during the initial stages the virus first accumulates in the anterior part of the midgut, and gradually spreads to the entire midgut, before it

finally spreads to the other organs during later developmental stages of the thrips (Filho *et al*., 2002; Nagata *et al*., 2002; Tsuda *et al*., 1996).
For tospoviruses, and all other bunyaviruses, the glycoproteins are the major determinants for infection of the vector and transmission as discussed before (section "glycoproteins"). So far, no receptors have been identified and characterised to which the glycoproteins bind that lead to receptor-mediated endocytosis and subsequent infection.

RNP formation
In the formation of virus particles, RNPs serve as the first intermediate. This formation is enabled due to the specific affinity of the viral N protein for viral (anti) genomic RNA in concert with the capacity to oligomerise. Viral transcripts, though, are not encapsidated by the N protein (Kormelink *et al*., 1992b), which can be explained in light of recent observations made for hantavirus N protein, in which a trimeric form of the N protein showed high affinity for the panhandle structure of the viral genomic RNA (Mir and Panganiban, 2004), a structure that is absent from (bunyavirus) mRNA molecules.
Once viral RNA is encapsidated by N protein functional RNPs, i.e. RNPs that contain all components to initiate a virus multiplication cycle, are formed. Based on *in vivo* reconstitution analyses of several bunyaviruses (Blakqori *et al*., 2003; Dunn *et al*., 1995; Flick and Pettersson, 2001; Flick *et al*., 2003a and b; Jin and Elliott, 1991, 1993; Lopez *et al*., 1995), a transcriptional/replicational active, and thereby functional RNP minimally consists of viral RNA encapsidated by N protein and supplemented with a few copies (catalytic amounts) of the L protein. Whether this RNP represents the entity that becomes membrane bound, or whether a (non)structural viral protein and /or host protein is additionally required to trigger RNP envelopment is not known. On this point it is interesting to mention that cytoplasmic RNPs of Rift Valley fever virus (RVFV) and TSWV were shown to contain (higher) amounts of NSs protein than RNPs purified from enveloped virus particles (Michele Bouloy pers. comm.; Van Knippenberg, 2005). Furthermore, TSWV cytoplasmic and particle RNPs exhibited different replicational and transcriptional activities (Van Knippenberg *et al*., 2002). This, altogether, would point towards the existence of RNPs with different compositions. Whether the NSs protein, next to acting as a suppressor of silencing, is also involved in the selection and subsequent preparation of cytoplasmic RNPs for envelopment, on which moment NSs is released, remains speculative. Also the RdRp protein has been implicated as a possible matrix protein and triggering the envelopment of RNP by Golgi membranes. Very recently, a transcription factor has been identified from the vector thrips *F. occidentalis*, named FoTF, which stimulates TSWV replication and binds to the TSWV L protein (Medeiros *et al*., 2005) but also to Gc. This protein was observed to co-localise with L and Gc in ER and Golgi vesicles in thrips cells suggesting a possible role in particle assembly, e.g. by recruiting the L protein to virus particles (Medeiros, pers. comm.).
Next to the cytoplasmic and virus-derived RNPs that are normally distinguished, a (sub)population of cytoplasmic RNPs has been observed during the very early stages of the infection, prior to the appearance of virus particles, that can be

discriminated by the additional association of NSm. As discussed earlier, the NSm protein facilitates the cell-to-cell movement of non-enveloped infectious RNP structures of TSWV through plasmodesmata (Kormelink *et al*., 1994; Storms *et al*., 1995 and 1998) and allows viral spread. These RNPs do not need to become membrane bound as it otherwise would have the contrary effect. After all, enveloped tospovirus particles are 80-120 nm in diameter and thereby too large to pass plasmodesmata, which have an effective size exclusion limit of only 5 nm. In conclusion, it is evident that various compositions of RNP are observed, and so far the exact composition of RNPs to become membrane bound remains unclear.

Prior to the actual envelopment, RNPs need to locate and concentrate around dilated Golgi membranes where the glycoproteins are retained and accumulate. In cells infected with Uukuniemi virus, RNPs are observed in a punctuate pattern throughout the cytoplasm, but also increasingly in the Golgi region (Kuismanen *et al*, 1984). For Crimean Congo hemorrhagic fever (CCHF) virus RNPs were observed to perinuclear regions in infected cells (Andersson *et al.*, 2004a). The additional interaction and co-localisation of CCHF RNPs with interferon-induced MxA in the perinuclear region (Andersson *et al.*, 2004a) was proposed as the mechanism to make RNPs unavailable for the process of replication and envelopment. Similar observations were earlier made on La Crosse, Dugbe and several hantaviruses in which MxA was suggested to inhibit virus replication by sequestering RNPs to the perinuclear region (Bridgen *et al*., 2004; Flohr *et al*., 1999; Frese *et al*., 1996; Kanerva *et al*, 1996; Kochs *et al*., 2002). Whether targeting and final localisation of RNPs at the Golgi complex is the result of an early interaction with the glycoproteins, is unknown. So far, transient expression studies of various bunyavirus N proteins have primarily shown a perinuclear localisation. hantavirus N protein expressed as a membrane-associated protein in the perinuclear region (Ravkov and Compans, 2001) and La Crosse virus N formed perinuclear complexes, suggested to be caused by MxA (Reichelt *et al*., 2004). For CCHF and Black Creek Canal virus (BCCV), transiently expressed N protein co-localised with actin microfilaments in infected cells. The use of inhibitors of actin filament polymerisation confirmed the requirement of actin filaments for targeting of CCHF N protein to perinuclear regions of mammalian cells (Andersson *et al.*, 2004b). Since in none of these expression studies a co-localisation with the Golgi complex was observed, with the exception of BCCV N protein (Ravkov and Compans, 2001; Ravkov *et al*., 1998), the presence or interaction with additional viral elements (RNA, L protein, (non)structural proteins?) seems required for proper Golgi targeting. The perinuclear accumulation of N/RNPs has in several cases been suggested to represent aggresomes (Andersson *et al*., 2004b; Kochs *et al*., 2002), structures that are related to the microtubule organising centre (MTOC) and suggested as aggregation sites for misfolded proteins (Johnston *et al*., 1998). On the other hand, the aggresomes pathway has also been suggested to be exploited by viruses in order to concentrate structural proteins at virus assembly sites (Heath *et al*., 2001).

Although several papers have reported on the interaction of bunyaviral N proteins with a variety of cellular proteins (Lee *et al*., 2003; Li *et al*., 2002), e.g. SUMO-1,

Daxx (a Fas-mediated apoptosis enhancer), the biological significance of these still remains to be elucidated.

Maturation of the glycoproteins

Since the glycoproteins dictate the site of virus assembly, many studies have been performed on expression of glycoproteins to identify and characterise domains within these proteins required for specific steps in the virus assembly. For several bunyaviruses, the glycoproteins were expressed in mammalian cell systems from a precursor or from constructs representing the individual glycoproteins (Figure 4). For TSWV it has been shown that the N-terminal protein Gn (G2) of the glycoprotein precursor could be transported to and retained in the Golgi system on its own, though with decreased efficiency, which suggested the presence of a Golgi retention signal. The C-terminal protein Gc (G1) expressed on its own was unable to leave the ER, but in the presence of Gn was able to exit the ER and translocate to the Golgi (Kikkert *et al.*, 2001). These data strongly suggested heterodimerisation of Gn and Gc for proper targeting and maturation. In the presence of tunicamycin, an inhibitor of glycosylation, the glycoproteins were retained in the ER which suggested that the absence of N-linked glycans probably resulted in aberrant folding of the proteins leading to a hampered transport from the ER to the Golgi complex. An association with ER-residing chaperone proteins (such as calnexin and calreticulin) at this stage is likely, as already observed for Uukuniemi virus glycoproteins during an infection in BHK cells (Veijola and Pettersson, 1999) as well as for most other (viral and non-viral) glycoproteins (Braakman and Van Anken, 2000). More or less similar observations were made during heterologous expression studies of Gn and Gc from Uukuniemi and Punta Toro phleboviruses, and Bunyamwera orthobunyavirus (Chen and Compans, 1991; Chen *et al.*, 1991; Lappin *et al.*, 1994; Matsuoka *et al.*, 1988; Rönnholm, 1992). For hantaviruses, the situation seems to be slightly different as both Gn and Gc remain in the ER upon individual expression, whereas Golgi localisation requires co-expression of both glycoproteins (Ruusala *et al.*, 1992; Shi and Elliott, 2002; Spiropoulou *et al.*, 2003).

For Uukuniemi phlebovirus, the Golgi retention signal was mapped to the cytoplasmic tail of Gn, i.e. amino acid residues 10 to 40 downstream the transmembrane domain (TMD). Neither the ectodomain nor the extreme carboxy terminus and TMD flanking these sequences were required for Golgi retention (Figure 3; Andersson *et al.*, 1997b and 1998). For two other phleboviruses, i.e. Punta Toro virus and Rift Valley fever virus, the Golgi retention signal was mapped to the junction region of the TMD and the cytoplasmic tail (Figure 3; Gerrard and Nichol, 2002; Matsuoka *et al.*, 1994). On the other hand, for Bunyamwera orthobunyavirus the Golgi retention signal completely resided in the TMD of Gn (Shi *et al.*, 2004), so viruses within the *Bunyaviridae* seem to have developed different strategies for Golgi retention. Alignment of these Golgi retention signals did not reveal any sequence homology, suggesting that the retention signal rather involves a conformational structure. The role of a TMD in Golgi retention in general still remains a matter of debate, as for several Golgi-membrane residing proteins the size of the TMD has been shown to be crucial for

Golgi retention, not only in vertebrate cells but also plant cells (Brandizzi *et al*, 2002; Gerrard and Nichol, 2002; Munro, 1998), whereas in other cases the TMD does not seem to be involved at all (Misumi *et al.*, 2001).
Although there are several discrepancies between the bunyaviruses with respect to Golgi targeting and signals involved, the observation that TSWV glycoproteins contain information necessary and sufficient for their transport to and retention in the Golgi system of mammalian cells, not only supports the notion of the putative evolution of an ancestral animal-infecting bunyavirus into the plant-infecting tospoviruses, but also the idea that protein trafficking and targeting machinery is homologous among all eukaryotes. On this point it is believed that cellular transport and retention signals are not only conserved among closely related organisms, but are similar if not identical for all eukaryotes (Bar *et al.*, 1996; Kermode, 1996).
It is still a matter of debate how the glycoproteins after being transported to the Golgi, finally are incorporated in virus particles. For Uukuniemi virus, they were first reported to do so as heterodimeric complexes assembled in the ER (Persson and Pettersson, 1991). However, in later studies Gn and Gc were each observed to associate into homodimeric complexes in mature virus (Rönka *et al.*, 1995). The Gn protein of Sin Nombre virus was also found in monomeric and stable, SDS-resistant, multimeric forms, with the dimer being the only form present late in infection (Spiropolou *et al.*, 2003). Conversely, Punta Toro virus Gn was found as a heterodimer with Gc, but not as a homodimer (Matsuoka *et al.*, 1996). Recently Gn homodimers were also observed in TSWV particles (Whitfield *et al.*, 2004). The biological importance of homodimerisation still is unknown.
Although it is not known which of the two, Gn or Gc, cytoplasmic tails interacts with the RNPs prior to envelopment, it has been postulated that for Uukuniemi virus the cytoplasmic tail of Gn is involved. Since of the cytoplasmic tail of Gc is rather short, i.e. only 5 amino acids, the tail of Gn was proposed as the target for interaction (Andersson *et al.*, 1997a). In contrast, TSWV Gn and Gc proteins have more or less equal sized cytoplasmic tails.

Fluorescence microscopy

During the last decade bio-imaging has slowly gained enormous attention due to the possibilities to study different viral processes in tissue and living cells. Whereas previously, and still, *in vitro* methods such as (co-)immunoprecipitation, overlay blots, gel shift essays, subcellular fractionation and sucrose gradient centrifugation have proved very valuable to study protein dynamics and localisations (Andersson and Pettersson, 1998; Bandla *et al.*, 1998; Gutiérrez-Escolano *et al.*, 2000; Kikkert *et al.*, 1998; Lappin *et al.*, 1994; Medeiros *et al.*, 2000; Persson and Pettersson, 1991; Rönkä *et al.*, 1995; Ruusala *et al.*, 1992; Veijola and Pettersson, 1999), the spectrum of *in vivo* tools to detect and analyse proteins have long remained more limited, and as a result detailed knowledge based on *in vivo* studies. This is slowly changing with the ongoing development of more elegant and sophisticated fluorescence tools and the significance of these will, no doubt, continue to increase its influence on virus research. To understand

the way viruses infect their host cell, it is very helpful if not essential to follow the fate of different viral components during infection. A major advantage of using fluorophore fusion proteins (i.e. proteins fused to a fluorescent group such as green fluorescent protein – GFP) is that the protein of interest can be studied in living cells or even in whole living plants. Another advantage is that it offers the possibility of investigating proteins such as glycoproteins and other membrane proteins that are difficult if not impossible to study using for example yeast-two-hybrid.

For some years, fluorescent tags have been applied to follow the intra- as well as intercellular localisation of (viral) proteins to study virus infection (Bosch *et al.*, 2004; Brideau *et al.*, 1999; Silva *et al.*, 2002; Silva, 2004) or expression and localisation patterns of individual proteins (Andersson and Pettersson, 1998; Dalton and Rose, 2001; Kochs *et al.*, 2002) as well as, for instance, the effect of a viral infection on the intracellular membranes (Carette *et al.*, 2000). Recently a couple of new methods have been developed that allow for more detailed investigation of features such as *in vivo* protein-protein interaction (Immink *et al.*, 2002; Larson *et al.*, 2003; van Kuppeveld *et al.*, 2002), protein-nucleic acid interaction (Murchie *et al.*, 2003), phosphorylation (Sato *et al.*, 2002), cleavage (Bastiaens and Jovin, 1996; Xu *et al.*, 1998), apoptosis induction (Xu *et al.*, 1998), DNA and RNA folding (Katiliene *et al.*, 2003), protein folding and conformational changes (Rhoades *et al.*, 2003; Truong and Ikura, 2001). In addition, these properties can be studied *in vivo*, allowing for spatial as well as temporal observation (e.g. where in the cell, and when during a viral infection does the interaction occur).

A few methods which recently have come into use for virological research will be explained below. A promising method for analysing processes involving three proteins is three-way or two-step FRET (Galperin *et al.*, 2004; Liu and Lu, 2002; Watrob *et al.*, 2003). Because the method is still being developed, it will not be discussed here. The same goes for other methods such as homotransfer or energy migration FRET (emFRET) (Lidke *et al.*, 2003) and photochromic FRET (pcFRET) (described in Giordano *et al.*, 2002), both efficient techniques to study protein-protein interactions and protein dynamics.

Fluorescence Resonance Energy Transfer (FRET)

FRET (Fluorescence resonance energy transfer, or Förster resonance energy transfer) is the phenomenon where energy is transferred from a donor fluorophore to an acceptor fluorophore (Figure 7). This energy transfer occurs through dipole-dipole interactions and will only take place if the donor and acceptor fluorophore are in very close proximity (typically 10-100 Å, which is in the same range as protein dimensions) and is therefore a good indicator for direct protein-protein interactions (Pollok and Heim, 1999). Another prerequisite for FRET is that the absorption spectrum of the acceptor has considerable overlap with the emission spectrum of the donor. FRET couples are, for example, CFP – YFP (Cyan and Yellow fluorescent protein, respectively); Cy3-Cy5; GFP with RFP; GFP or YFP with HcRed and, somewhat less optimal due to the weak fluorescence intrinsic of BFP as well as damaging UV excitation, BFP - GFP (Bastiaens and Jovin, 1996;

Erickson *et al.*, 2003; Pollok and Heim, 1999). Different methods have been developed to measure FRET. Since measurement of sensitised emission of acceptor fluorescence is very sensitive to cross-talk or bleedthrough of the signal, either hardware or software should be employed to correct for this background signal. Hardware can be optimised using narrow filter sets both for excitation and for detection of the fluorophore; software can correct for background signals by using algorithms to compare signals from cells expressing only donor and only acceptor with signals from the sample (Gordon *et al.*, 1998; Nagy *et al.*, 1998; Xia and Liu, 2001; a comparison of different methods can be found in Berney and Danuser, 2003).

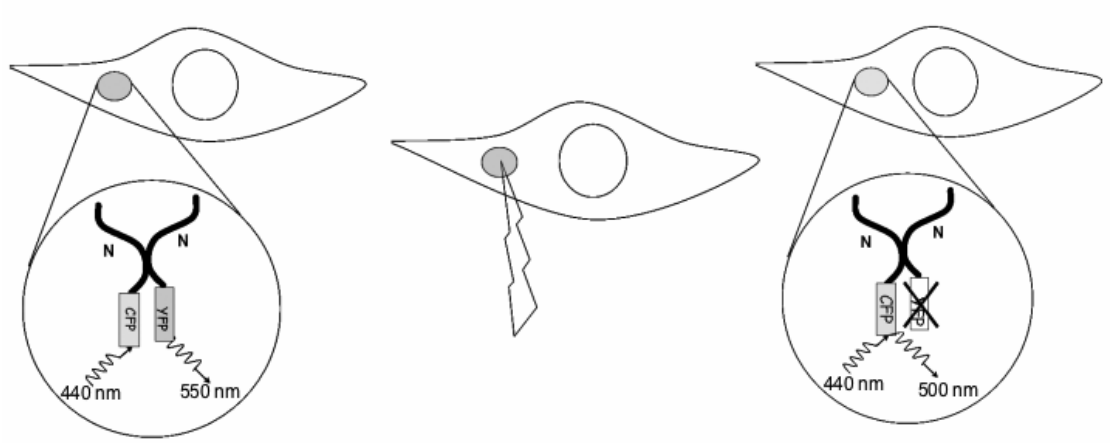


Figure 7. Principles of FRET and acceptor photobleaching. In a region where FRET occurs, CFP emission is significantly quenched due to energy transfer to YFP. After photobleaching, YFP is no longer functional, leading to restored CFP fluorescence.

A more direct method of direct FRET determination is spectral imaging spectroscopy or SPIM. In this method, a fluorescence spectrum of the region or whole cell is recorded upon excitation of the donor fluorophore. The spectrum is compared to negative controls. In case FRET occurs, the relative donor emission can be seen to decrease, while acceptor emission increases (Immink *et al.*, 2002; Pouwels, 2004; van Kuppeveld *et al.*, 2002).

Another method to measure FRET is acceptor photobleaching (Figure 7). This method exploits the phenomenon that the fluorescence of the donor fluorophore is quenched if FRET takes place. Comparing the donor fluorescence before and after irreversibly destructing the fluorescence and energy absorbing capacity of the acceptor, gives a clear indication of FRET. Destruction is carried out by exposing the fluorophore to an intense laser beam.

Yet a different measuring method is FLIM (fluorescence lifetime imaging; Figure 8), in which the fluorescence lifetime of the donor fluorophore is measured on a picosecond scale. If FRET occurs, the fluorescence lifetime of the donor is decreased compared to its normal lifetime (Gadella, 1999).

FRET has been used in the recent past for investigation of (viral) proteins, for example the homomultimerisation of viral proteins (Larson *et al.*, 2003; van Kuppeveld *et al.*, 2002), protein folding dynamics (Rhoades *et al.*, 2003; Truong and Ikura, 2001) and enzyme activity (Bark and Hahn, 2000; Violin *et al.*, 2003; Yoshizaki *et al.*, 2003).

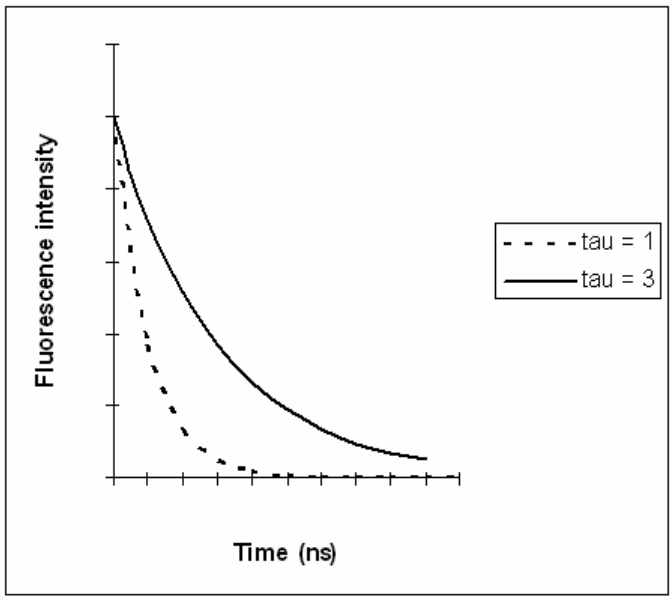


Figure 8. Principle of FLIM. The fluorescence life time is indicated in nanoseconds (ns). In the additional presence of an acceptor fluorophore, the fluorescence lifetime of the donor decreases (from tau=3 ns to tau=1 ns).

A method related to FRET is bioluminescence resonance energy transfer (BRET) (Xu *et al.*, 1999). In this technique, the donor of a FRET couple is replaced by a luciferase. Energy transfer takes place under the same circumstances as described for FRET, but no potentially damaging laser light is required for excitation. As a consequence, autofluorescence and photobleaching no longer occur. In addition, BRET has been reported to be significantly more sensitive than FRET, possibly due to much lower background signals from direct excitation of the acceptor fluorophore (Arai *et al.*, 2001).

Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Loss In Photobleaching (FLIP)

Fluorescence recovery after photobleaching (FRAP) is a valuable technique to study the structure of biological membranes and to measure movement (e.g. diffusion) of proteins in a living cell. It is especially useful to gain insight in the movement of membrane or membrane-bound proteins (Sekar and Periasamy, 2003). In FRAP, the fluorescent molecules in a specific region are bleached (using an intense laser pulse). The non-bleached molecules outside this region might move into the bleached area resulting in a (partial) recovery of the fluorescence. The recovery curve yields information about the diffusion coefficient of the fluorophore and the proportion of mobile fluorophore molecules. Moreover, diffusion can be distinguished between flow-driven processes from the shape of the recovery curve (Sciaky *et al.*, 1997; White and Stelzer, 1999). FRAP may prove helpful to determine the mobility of proteins in membranes such as the cell membrane. The method has recently been employed to elucidate the interaction between molecules of CPMV viral movement protein and the cell membrane of protoplasts (Pouwels, 2004), to study raft formation in influenza virus infection (Shvartsman *et al.*, 2003) and the mechanisms of protein retention in the ER and the dynamics of protein-folding complexes in native ER membranes using the glycoprotein of vesicular stomatitis virus (VSV-G) (Nehls *et al.*, 2000).

In fluorescence loss in photobleaching (FLIP), a region is repeatedly bleached and the disappearance of fluorescence in the non-bleached areas is monitored. If all proteins can diffuse towards the region that is subjected to bleaching, this will eventually lead to a loss of fluorescence throughout the cell.
This method can be a especially valuable to gain insight in ER-Golgi trafficking (White and Stelzer, 1999), to demonstrate the continuity of intracellular organelles (Cole *et al.*, 1996; Köhler *et al.*, 1997) and to study nuclear transport and ER retention (Imreh *et al.*, 2003; Shimi *et al.*, 2004).

Fluorescence Correlation Spectroscopy (FCS)
Fluorescence correlation spectroscopy is a method that can be used to determine the diffusion rate of fluorescently labelled proteins. This diffusion rate gives information about molecular interactions in a very sensitive way: in principle, molecular interaction of a single fluorescent ligand with a macromolecule can be determined and fully quantified. In addition, transport properties as well as chemical properties such as association-dissociation kinetics can be investigated using FCS (Kinjo and Rigler, 1995; Rauer *et al.*, 1996; Visser and Hink, 1999).
The principle of FCS is that a small open-volume element is created by a focused laser beam. Fluorescent molecules moving through this confocal volume are excited and fluorescence is measured with a fast photon detector (Figure 9).

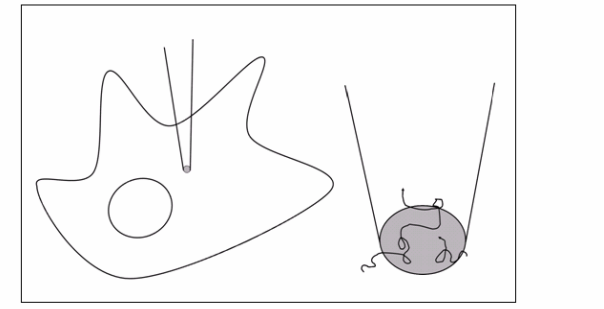


Figure 9. Principle of Fluorescence correlation spectroscopy (FCS).

Especially at low concentrations, the Brownian motion of the molecules results in relative large fluctuations of the fluorescence intensity. The fluctuations can be auto-correlated to give information about the average time the fluorescent molecule needs to pass through the volume element. This speed of movement is directly related to the diffusion coefficient of the molecule, which, in turn, is related to its size. Thus, conclusions can be drawn about the state of the protein, i.e. its presence as a free molecule or as part of a large complex. In addition to the diffusion coefficient, analysis of the autocorrelation signal also yields the molecular concentration of fluorescent molecules. Using these parameters together, the state of a binding equilibrium can be calculated. FCS has proven useful in the investigation of protein-protein and protein-lipid interaction as well as DNA dynamics (Berland *et al.*, 1996; Bonnet *et al.*, 1998; Pramanik *et al.*, 2000). The method and applications have been reviewed by Eigen and Rigler (1994); Hink *et al.* (2002); and by Hess *et al.* (2002).

Bimolecular fluorescence complementation (BiFC)

A relatively new method is to tag two ends of one protein (to study folding of that protein) or two proteins (to study their interaction) with two non-fluorescent fragments of a fluorescent protein. When brought together, the fragments complement each other to render a fluorescent protein. Since fluorescence is only generated if the two fragments are allowed to behave as one, the signal is not disturbed by background fluorescence and therefore, no complex processing of the data is required. The method is sensitive enough to enable studying of proteins in a cellular environment. The method was first described in mammalian cells by Hu *et al.* (2002) and has recently been described in plant cells as well, for dimerisation of both nuclear proteins and cytoplasmic proteins (Walter *et al.*, 2004). By using several different GFP variants, the technique was extended to multicolour BiFC. With this technique, several protein interactions can be monitored simultaneously in the same cell (Grinberg *et al.*, 2004; Hu and Kerppola, 2003).

Acknowledgements

The authors wish to thank Mark Hink for critically reading the manuscript, Ricardo Medeiros for providing (unpublished and in press) data and Esa Kuismanen for providing an EM-micrograph on Uukuniemi virus assembly. The work was supported in part by the Dutch NWO-ALW and EU- RTN grant HPRN-CT-2002-00262.

Outline of this thesis

As documented in the preceding sections of this chapter, it has been shown that TSWV particle assembly occurs by enwrapment of RNPs at the Golgi complex. Furthermore, virus particles accumulate and are retained in large (ER-derived) vesicles in plant cells, whereas they are secreted from salivary gland cells into the gland ducts in the vector thrips. More recently, as a first step to identify these critical differences between the viral replication cycle in the two cell types, TSWV Gn and Gc were expressed and analysed in mammalian cells. These studies revealed that Gn and Gc exhibit a similar trafficking behaviour as their counterparts in animal-infecting bunyaviruses, i.e. Gn translocates to the Golgi but Gc requires the presence of Gn in order to exit the ER. Hence, it was suggested that Gn and Gc heterodimerise to become translocated to the Golgi where they are retained due to the presence of a Golgi-retention signal in Gn. Interactions between the cytoplasmic tail of Gn and/or Gc with a component of the RNPs, for which the N protein is a candidate protein, is generally assumed to subsequently trigger the actual envelopment of the RNPs.

The work described in this thesis started from here and was especially aimed to identify interactions between the structural proteins involved in particle assembly. In order to get a first insight of the final stage of particle assembly, the presence of homodimers, heterodimers or oligomers of the structural proteins as occurring in mature virus particles and RNPs were analysed and described in Chapter 2. In Chapter 3, the feasibility of Förster Resonance Energy Transfer (FRET) microscopy for *in vivo* studying of the interactions between the TSWV structural proteins was investigated using the cytosolic N protein as a first target protein. These studies not only demonstrated the feasibility of FRET microscopy to study viral protein interactions in living cells but also allowed intracellular localisation of these interactions. Hence, in Chapter 4 FRET microscopy was further exploited to identify the interactions between Gn, Gc and N.

Previous work had demonstrated that Gn contains a Golgi retention signal. In Chapter 5 this domain was mapped and further analysed. While performing Gn and Gc expression analyses, Gc was found to induce fusion of mammalian cells. This phenomenon was investigated and described in Chapter 6. Finally, in Chapter 7 a model is presented for the particle assembly of TSWV in which the different steps are discussed in light of the findings presented in the preceding chapters as well as literature data.

Chapter 2

Protein composition of tomato spotted wilt virus particles

Abstract
In this chapter protein composition and interactions have been studied both in enveloped virions and ribonucleotide particles (RNPs) of tomato spotted wilt virus (TSWV). Using Western blot analysis under reducing and non-reducing conditions the occurrence of homo-oligomeric forms of the nucleocapsid protein N in both virion and RNPs was demonstrated. Virus particles were found to contain monomeric as well as homodimeric forms of the glycoproteins Gn and Gc, in addition to heterodimers of both glycoproteins. Moreover, Gc, but no Gn, was found bound to RNPs even after rigorous purification. Surprisingly, the non-structural protein NSs was found in significant amounts in purified RNPs while detected in much lower amounts in virions.

Marjolein Snippe, Dick Lohuis, Janneke Saaijer, Rob Goldbach and Richard Kormelink

Introduction

Tomato spotted wilt virus (TSWV), like all other members of the *Bunyaviridae*, has spherical, enveloped particles encompassing a tripartite RNA genome. The viral envelope contains two types of glycoproteins Gn and Gc that are processed from a single precursor protein (Kormelink *et al.*, 1992a). Within the virion, the viral genome is encapsidated by multiple copies of the nucleocapsid (N) protein and this complex, together with 10-20 copies of the viral RdRp (L protein), forms the ribonucleoprotein (RNP) complex (van Poelwijk *et al.*, 1993). RNPs are infectious units and associate with the viral movement protein to enable cell-to-cell transport throughout the plant (Kormelink *et al.*, 1994; Storms *et al.*, 1995). For plant to plant spread TSWV is vectored by a limited number of thrips species and this requires RNPs to become enveloped by a membrane containing the two viral glycoproteins Gn and Gc of approximately 58 and 78 kDa, respectively (Mohamed *et al.*, 1973; Nagata *et al.*, 2000). Despite some general insight in the particle morphology and protein content, data concerning the protein-protein interactions underlying the TSWV virion architecture are rather limited. Over the past years homotypic interactions between N protein copies as well as interactions between N and RNA have been demonstrated (Kainz *et al.*, 2004; Richmond *et al.*, 1998; Uhrig *et al.*, 1999), but little is known about interactions between the viral RNPs and the envelope glycoproteins. As Gn is required for Golgi targeting of Gc, heterotypic interactions are assumed to exist between the two glycoproteins at some point during viral infection (Kikkert *et al.*, 2001). In cells expressing the two glycoproteins from most, if not all bunyaviruses, either resulting from infection or from transient expression, heterodimers are consistently observed (Chen and Compans, 1991; Antic *et al.*, 1992). Furthermore, interactions between the glycoproteins and viral RNPs are assumed to trigger RNP envelopment in the formation of enveloped viral particles. However, it is not clear what interactions are underlying the architecture of the stable enveloped virus particle. Also it has remained undetermined whether there are any differences between RNPs as packaged in mature virions versus those found in the cytoplasm of infected cells. Previous research has pointed to a difference in replicational and transcriptional activity of virus particles versus cytoplasmic RNPs and the absence or presence of associated NSs molecules have been implicated herein (van Knippenberg *et al.*, 2002; van Knippenberg, 2005).

In order to gain more knowledge about the interactions between the proteins present in TSWV virions, as well as those in viral RNPs, purified enveloped virus as well as RNP preparations were subjected to protein analyses in the presence and absence of a reducing agent

Materials and methods

Virus purification

TSWV isolate BR-01 was used and maintained in *Nicotiana rustica* plants by mechanical inoculation and thrips transmission. Virus particles were isolated at 4°C from systemically infected *N. rustica* leaves as described by Kikkert *et al.* (1997) with the modification that the resuspension buffer consisted of 0.01M KH_2PO_4 and 0.01M Na_2SO_3, pH 7.0 (described by van Knippenberg *et al.*, 2002). In

short, infected leaves were ground in extraction buffer, filtered through cheesecloth and centrifuged at 10,000 g. The pellet was homogenised in resuspension buffer and centrifuged at 8,000 g after which the supernatant was collected and pelleted at 100,000 g, homogenised in resuspension buffer and centrifuged over a sucrose gradient. The band containing the virus particles was collected and diluted in resuspension buffer and virions were pelleted by centrifugation and resuspended in sterile double distilled water. Integrity of enveloped particles was verified by electron microscopy (not shown).

RNP purification

Ribonucleocapsids (RNPs) of TSWV isolate BR-01 were purified from systemically infected leaves of *N. rustica* essentially as described by de Ávila *et al*. (1990) with the modification that the extraction buffer consisted of 0.1M Tris-HCl instead of 0.01M, as described by van Knippenberg *et al*. (2002). Briefly, infected leaves were homogenised in extraction buffer and filtered through cheesecloth. The extract was centrifuged at 1,000 g and the supernatant was collected and centrifuged at 50,000 g. The pellet was resuspended in resuspension buffer (0.01M Tris-HCl pH 7.9, 0.01M EDTA, 0.01 M sodium sulphite and 1% Nonidet P40) and centrifuged at 8,000 g. The supernatant was centrifuged on a 30% sucrose cushion at 125,000 g. The pellet was resuspended in citrate buffer (0.01M, pH 6.0) and centrifuged at 3,000 g. This step was repeated 4-5 times with fresh citrate buffer. The supernatants were pooled and stored at -20 °C. This fraction will be referred to as “semi-purified RNPs”.

For further purification, cesium sulphate was added to the pooled supernatants (1.65 g/5.5 ml). After overnight (16h) centrifugation at 120,000 g, the band containing the RNPs was collected, dialysed against citrate buffer and stored at -20 °C.

Protein sequencing

Virus preparations were resolved on a 10% SDS-polyacrylamide gel in protein sample buffer (Laemmli, 1970) in the presence of 10% (v/v) β-mercaptoethanol and transferred to a PVDF membrane (Biorad) using CAPS buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) pH 11 and 10% (v/v) methanol). The membrane was sequentially rinsed with water, 20% methanol, water and 100% methanol and proteins were visualised with Coomassie Brilliant Blue staining. The proteins of interest were cut out of the membrane and sent for N-terminal sequencing by the Edman degradation protocol at the sequencing facility of UvA (Universiteit van Amsterdam).

Low pH treatment of virions

Purified virus was incubated in 100 mM sodium phosphate buffer at pH 5.8 and 37°C for 3 hours (as described by Whitfield *et al.*, 2005).

Protein gel electrophoresis and Western blotting

Samples were mixed with protein sample buffer (Laemmli, 1970) with or without 15 mM DTT, boiled for 5 minutes and resolved on a 10% SDS-polyacrylamide gel.

After electrophoresis, proteins were blotted onto a PVDF membrane (Millipore) using a semi-dry blotting procedure in tris-glycine buffer (48 mM Tris base, 39 mM glycine, 0.0375% SDS and 20% (v/v) methanol). Blots were rinsed with TBS-T (0.05 M Tris base, 0.2 M NaCl, 0.1% tween-20, pH 7.5) at room temperature. Subsequently, the blots were blocked overnight in 1.5% (w/v) skimmed milk powder in TBS-T at 4°C, washed a few times with TBS-T and incubated with antiserum diluted in incubation buffer (TBS-T with 0.2% skimmed milk powder) for 1-2 hours at 37°C. After washing with TBS-T, the blots were incubated in incubation buffer with secondary antibody conjugated to alkaline phosphatase for 1-2 hours at 37°C and developed using 400 µl BCIP/NBT stock solution (Roche) in 20 ml AP-buffer (0.1M Tris base, 0.1M NaCl and 0.05M $MgCl_2$).

Results

Determining the N-terminus of the glycoproteins

Virions are known to contain the structural proteins L, N, Gn and Gc, the two glycoproteins being produced by co-translational processing from a common precursor protein. The exact cleavage sites in the precursor and therefore the exact first and last amino acids of Gc and Gn, respectively, have remained unknown. Also, the positioning of the smaller cleavage product of Gn, previously often referred to as G2b but hereafter to be called Gn*, frequently found in virion preparations (Tas *et al.*, 1977; Goldbach and Peters, 1996), has remained unresolved. Hence, prior to interpreting protein blots potentially containing oligomeric forms of the various proteins, first the two glycoproteins as well as Gn* were subjected to N-terminal amino acid sequencing. Thus it was shown that Gn starts at position 36 of the precursor (KVEIIRG) whereas its smaller cleavage product Gn* starts at position 86 (IREEKS) (Figure 1). These results demonstrate

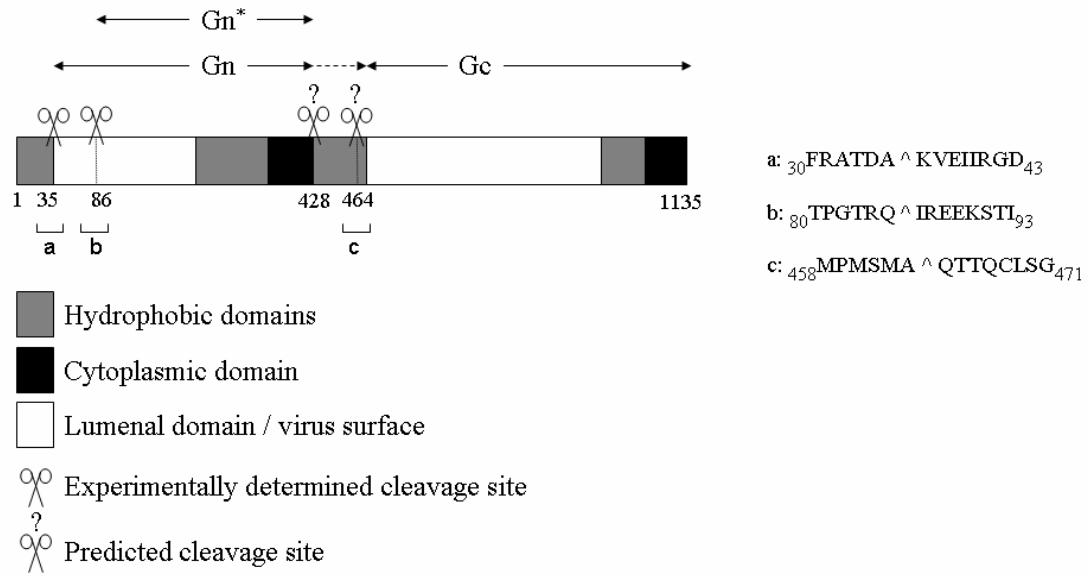


Figure 1. Topology of the TSWV glycoprotein precursor. The amino acid positions of the (predicted) cleavage sites in the precursor are indicated by numbers and sequences around the cleavage sites shown (a, b, c) where a caret (^) represents the position where cleavage takes place.

that the 35 amino acid signal peptide is indeed removed from the propeptide during processing, as has been predicted previously (Kormelink *et al.*, 1992a). The theoretical difference in molecular mass between Gn and Gn* is 5.6 kDa and this fits with their apparent molecular sizes observed on protein gel of 58 and 52 kDa, respectively. The N-terminal sequence of Gc could not be determined as this protein appeared to be blocked. As the other viral glycoprotein species on the same blot were not blocked, blocking possibly occurred *in vivo* rather than during the blotting procedure and the N-terminal amino acid is most likely a glutamine, for which blocking occurs in approximately 80% of the cases (Muysers, pers. comm.; Mozdzanowski *et al.*, 1997; Fischer and Spies, 1987). For this reason, Gc is postulated to start at amino acid position 464 within the precursor protein (Figure 1).

Protein analysis of virions and RNPs

In order to determine their protein content and the oligomerisation states of the respective structural proteins, preparations of virus particles and RNPs were analysed on Western blot having separated the proteins in either the presence or absence of a reducing agent. Two types of RNP preparations were analysed, i.e. a semi-purified and a more rigorously purified fraction, using a Cs_2SO_4 gradient to remove any substance not specifically bound to the RNPs.

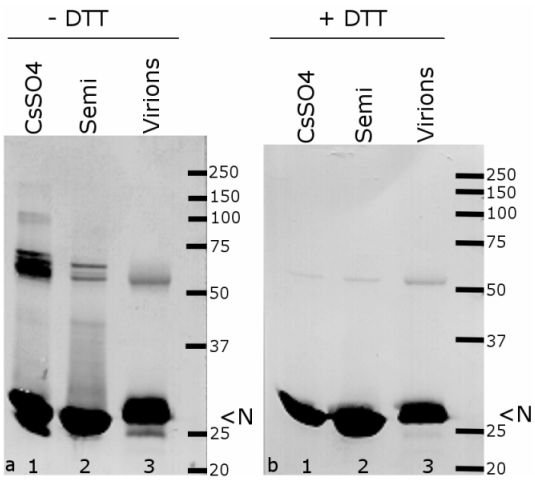


Figure 2. Protein analysis of virus and RNP preparations using antiserum against N. Blots contain RNPs purified with Cs_2SO4 (lanes 1), semi-purified RNPs (lanes 2) and virus preparations (lanes 3) in the absence (a) or presence (b) of DTT. Sizes of the molecular weight marker are indicated in kDa.

Semi-purified cytoplasmic RNPs analysed for the N protein contents revealed the presence of monomeric (30 kDa) N and dimers of N both in the presence and absence of DTT. In the absence of DTT two bands were seen around 60 kDa that resolved into a single protein species upon DTT addition and may represent different conformational forms (Figure 2a, b lanes 2). As no other protein species around this position were visible when the blots were probed for the other viral proteins, the bands likely represent N dimers. The differences in mobility, resulting in various discrete bands rather than a single one, may be due to different conformations. Higher order multimers were occasionally observed, mainly in the absence of DTT (Figure 2a, lane 2). When semi-purified cytoplasmic RNPs were analysed for their glycoprotein (Gc and Gn) contents, both Gc (78 kDa)

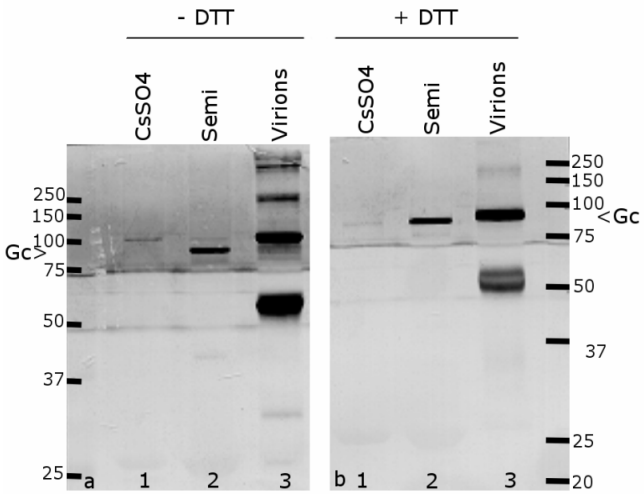


Figure 3. Protein analysis of virus and RNP preparations using antiserum against Gc. Blots contain RNPs purified with Cs_2SO4 (lanes 1), semi-purified RNPs (lanes 2) and virus preparations (lanes 3) in the absence (a) or presence (b) of DTT. Sizes of the molecular weight marker are indicated (kDa).

and Gn (58 kDa) were detected regardless of the presence of DTT. The band assigned to Gc consistently appeared as a doublet or sometimes as a triplet (Figure 3a and b, lanes 2; Figure 4 lanes 2 and 6; Figure 5 lanes 2 and 5). Finally, monomers of NSs (52 kDa) were clearly detected (Figure 6a and b, lanes 2).

Cs_2SO_4-purified cytoplasmic RNPs were found to contain monomeric as well as dimeric N in either the presence or the absence of DTT. Similarly to what was observed for semi-purified RNPs, the various bands around the expected position of N dimers in the absence of DTT rendered a single band after DTT was added (Figure 2a and b, lanes 1). Furthermore, higher multimers were occasionally seen

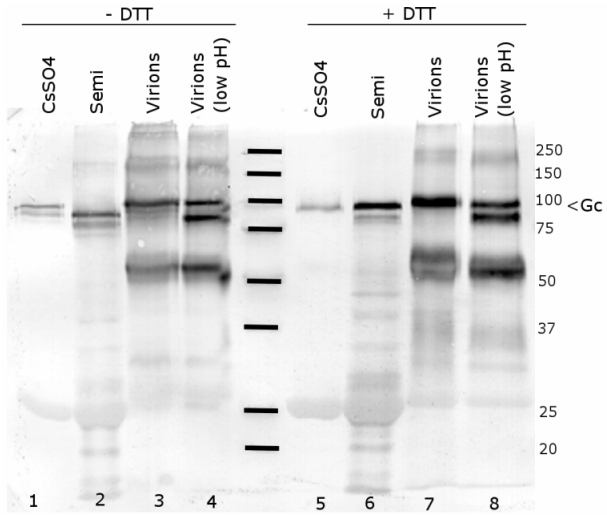


Figure 4. Protein analysis of virus and RNP preparations using antiserum against Gc. Blot contains RNPs purified with Cs_2SO4 (lanes 1 and 5), semi-purified RNPs (lanes 2 and 6), virus preparations (lanes 3 and 7) and virus preparations after exposure to a low pH (lanes 4 and 8) in the absence (1-4) or presence (5-8) of DTT. Sizes of the molecular weight marker are indicated (kDa).

in the absence of DTT (Figure 2a, lane 1). Upon analysis of the glycoprotein content of Cs_2SO_4-purified cytoplasmic RNPs, monomers of Gc were seen in both the absence and presence of DTT (Figure 3a and b lanes 1; Figure 4 lanes 1 and 5). In contrast, whereas semi-purified RNP fractions contained considerable amounts of Gn (Figure 5, lanes 2 and 5), no Gn could be detected after Cs_2SO_4 purification (Figure 5 lanes 1 and 4), indicating that specific binding of RNPs to the viral envelope likely involves Gc but not Gn. In addition, the NSs protein was also found to be present which in the absence of DTT resulted in a smear (Figure 6a

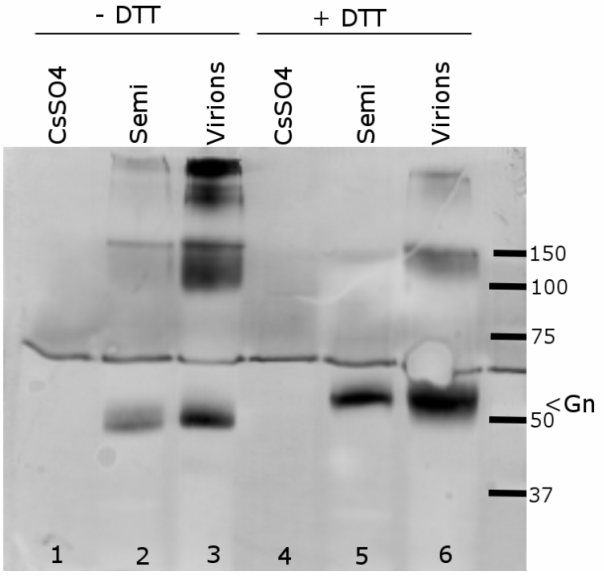


Figure 5. Protein analysis of virus and RNP preparations using antiserum against Gn. Blot contains RNPs purified with Cs_2SO4 (lanes 1 and 4), semi-purified RNPs (lanes 2 and 5) and virus preparations (lanes 3 and 6) in the absence (1-3) or presence (4-6) of DTT. Sizes of the molecular weight marker are indicated (kDa).

and b, lanes 1). This suggested that a specific association exists between RNPs and NSs that is not disrupted by rigorous (i.e. cesium sulphate) purification.

Enveloped virus particles were analysed and showed the presence of both monomeric and dimeric forms of N. In partial analogy to the results of the RNP analyses, in the absence of DTT a more diffuse N dimer band was present that appeared as a single protein species after DTT addition (Figure 2a and b, lanes 3). Higher multimeric forms were occasionally detected at lower amounts. Gc was detected in a monomeric form as well as a higher molecular weight form of around 180 kDa that may represent heteromultimeric complexes involving Gc (Figure 3a and b lanes 3; Figure 4 lanes 3 and 7). In addition, a faster migrating protein was observed around 55 kDa that likely represents a breakdown product. Gn was seen

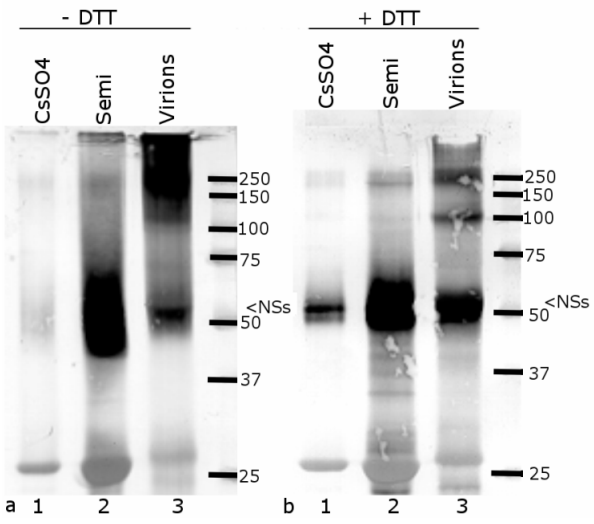


Figure 6. Protein analysis of virus and RNP preparations using antiserum against NSs. Blots contain RNPs purified with Cs_2SO4 (lanes 1), semi-purified RNPs (lanes 2) and virus preparations (lanes 3) in the absence (a) or presence (b) of DTT. Sizes of the molecular weight marker are indicated (kDa).

as a monomer and in addition showed a discrete band migrating at around 140 kDa on top of a diffuse band migrating at 120-140 kDa (Figure 5, lanes 3 and 6). Based on the estimated size and the absence of this band in blots probed band of around 140 kDa is visible in virus particles as well as semi-purified RNPs in the absence

of DTT. As a similar band was sometimes seen in blots screened for Gc (not shown), this signal may represent heterodimers of Gn and Gc. Larger complexes (>250 kDa) that reacted with antisera against N, Gc, Gn and NSs were visible in all blots, suggesting the presence of large hetero-oligomeric complexes involving Gn, Gc, N and / or NSs. These complexes were clearest in the absence of DTT. Finally, as observed for semi-purified RNPs, monomeric and oligomeric forms of NSs were also detected in (or associated with) virus samples although in lower quantities compared to RNP preparations (Figure 6a and b, lanes 3).

pH dependent cleavage of virion-associated Gc

To assess the origin of the various Gc bands around the position of Gc monomers (78 kDa) (see Figures 3 and 4), virus samples were subjected to a reduced pH to induce cleavage of Gc as described by Whitfield and co-workers (2005). After incubation, two clear protein species were visible of which the lower co-migrated with the one seen in semi-purified RNP preparations, indicating a cleaved form of Gc may be present in cytoplasmic RNPs (Figure 4, lanes 4 and 8).

Discussion

When TSWV viral proteins were separated in the absence of a reducing agent, Western blot analysis revealed, next to a very prominent amount of N monomers, the presence of stable N dimers, both in RNPs and in virions. N was also present in the form of trimers, but to a lesser extent, indicating dimers are either more resistant or more prevalent than trimers. This is surprising in view of reports on several hantaviruses, where trimers are reportedly the most stable forms of N (Alfadhli *et al.*, 2001; Kaukinen *et al.*, 2001; Mir and Panganiban, 2004). For hantavirus, N trimers are probably an important intermediate as this is the form in which the nucleocapsid proteins display enhanced as well as viral sequence specific RNA affinity (Mir and Panganiban, 2004). On the other hand, the building block of Bunyamwera virus appears to be monomeric rather than trimeric N (Elliott, pers. comm.). Together, these data suggest a genus-specific mechanism may exist for RNP assembly.

Surprisingly, the non-structural protein NSs (52 kDa) was encountered in both RNP and virion preparations. In RNPs this protein was detected in either the presence or absence of DTT. In the absence of DTT however, the bands were much more diffuse. Although it can not be excluded that some NSs was present due to a non-specific co-purification during the extraction procedure, the presence of some NSs in RNPs even after Cs_2SO_4 purification, indicates a strong and specific interaction. In preparations of enveloped virus particles, several bands were observed that may represent homo- or heterodimers of NSs with, for instance, N. The relatively large amount of NSs present in semi-purified RNP preparations as compared to enveloped virus purifications fits an earlier suggestion that NSs may be a factor modulating replication and transcription (van Knippenberg, 2005).

When samples were tested for the presence of Gn, Gc and homo- or hetero-oligomeric forms of these proteins, in virus as well as semi-purified cytoplasmic RNPs Gn-Gc heterodimers were detected, as well as homodimers of Gn. This is in

agreement with recent reports on the occurrence of Gn dimers as well as heterodimers in purified TSWV virions (Whitfield *et al*., 2004). Since in this latter case the blot was not probed with anti-Gc antibody the presence or absence of Gc homodimers could not be determined. For other members of the *Bunyaviridae*, very little has been reported on this subject. For Uukuniemi virus, even conflicting results have been published that may be due to the use of different experimental techniques allowing for preferential detection of homo- or hetero-oligomers. Even the *in vitro* formation of complexes, during or after virus purification, cannot be excluded. Whereas initially sucrose gradient centrifugation experiments led to the conclusion that in virions the two glycoproteins are present exclusively as heterodimers (Persson and Pettersson, 1991), subsequent analyses demonstrated the absence of stable heterodimers or –oligomers in virus particles (Rönkä *et al.*, 1995). In contrast, these experiments showed that in Uukuniemi virions Gc is present as pH-insensitive homodimers while Gn is present in monomeric as well as homodimeric forms above pH 6.4, dissociating completely into monomers below pH 6. These data, too, were obtained using gradient centrifugation, in combination with chemical cross-linking and immunoprecipitation. In a similar way, the occurrence of homodimers and higher –multimers and indications for the presence of non-covalently linked hetero-oligomers was also demonstrated in purified La Crosse virions (Pekosz and González-Scarano, 1996).

To gain more insight into the processing of the two glycoproteins, they were subjected to N-terminal amino acid sequencing. The impossibility to determine the N-terminal sequence of Gc is likely the result of blocking of the N-terminal amino acid. This may have happened either *in vivo* or *in vitro* but since the protein has received the same treatment as (unblocked) Gn, it is likely that blocking in this case has occurred during the *in vivo* stage. In vivo blockage occurs predominantly if the first (N-terminal) amino acid is a glutamine, an amino acid known to cyclise in around 80% of the cases when present at the N-terminus (Muysers, pers. comm.; Mozdzanowski *et al.*, 1997; Fischer and Spies, 1987). This would indicate that Gc starts with the glutamine at amino acid position 464 from the precursor, which would also fit perfectly with the presence of a proteolytic cleavage site directly upstream of residue 464, as predicted by SignalP (Bendtsen *et al*., 2004) cleaving Gc off the hydrophobic sequence postulated to act as a signal sequence for Gc (Kormelink *et al.*, 1992a; Kikkert *et al.*, 2001).

The observation that Gn starts after a hydrophobic sequence of 35 amino acids fits with the earlier prediction of a signal sequence to be released after cleavage downstream residue 35 (Kormelink *et al*., 1992a; Whitfield *et al*., 2004). The start of Gn* with an isoleucine at position 86 was a bit more surprising as Gn* was earlier speculated to result from cleavage of the hydrophobic sequence between the cytoplasmic tail of Gn and the lumenal domain of Gc (Kikkert, 1999; Figure 1, residues 428-464). It still remains to be determined if this C-terminal hydrophobic domain is present in the mature forms of Gn and Gn*. Moreover, it is not clear if the N-terminal cleavage resulting in Gn* renders a biologically functional protein as this protein lacks the RGD motif, a putative attachment site (residues 41 - 43 of the precursor).

Since for Gc, mainly in RNP fractions, various bands were visible around the position of the monomer, the possible presence of a cleaved form of Gc, recently reported to occur in virus preparations after incubation at low pH conditions (Whitfield *et al.*, 2005), was assessed.
These analyses indeed revealed that after exposure to low pH, an additional faster migrating Gc protein appeared in virus preparations. This protein is similar in size as shown by Whitfield and colleagues and comigrates with one of the faster migrating protein species consistently observed in untreated semi-purified cytoplasmic RNP preparations, indicating that these molecules result from proteolytic cleavage of Gc. This suggests that the cleaved form of Gc as present in semi-purified RNPs is not incorporated into virions but the significance of this difference remains as yet unclear. Although the peptide that is cleaved off from Gc was not identified, nor the cleavage site predicted by computer analyses, it is likely that cleavage occurs at the N-terminus of Gc since this seems the most accessible part of Gc, exposed on the outside of the virion (see Figure 1). The C-terminus of Gc consists of a small cytoplasmic tail buried in the virion. In addition, pH-dependent conformational change and / or cleavage is often seen as a first step in the process of pH-dependent fusion and hence most likely involves the outside rather than the inside of the viral membrane. This suggests cleavage after the first 100 to 150 amino acids, counted from amino acid 465 (glutamine) in the precursor, removing a peptide with a predicted mass of 11.3 to 16.7 kDa.
In summary, in this chapter we have demonstrated the existence of homodimeric and / or oligomeric interactions for N, Gc and Gn as well as heterodimeric interactions for Gn-Gc and N-Gc in the mature virion. To obtain a clearer view of the significance of these interactions, analyses of the maturation of the structural proteins after *in vivo* synthesis is required.
Furthermore, the significance of the additional presence of NSs in RNPs is far from understood. The only function assigned to TSWV NSs so far is suppression of gene silencing or RNAi and this function is performed upon transient expression as well as during viral infection (Bucher *et al.*, 2003; Takeda *et al.*, 2002). It is, however, conceivable that suppression of gene silencing is enhanced by association of NSs to RNPs or, alternatively, that viral RNA is better protected by direct association with NSs. This last possibility is not very likely as NSs interferes with early steps in RNAi rather than directly with RNA breakdown. Another possibility would be that the association of NSs to RNPs concerns a second function of NSs, for instance involving translational events, as suggested previously (van Knippenberg, 2005). Further experiments, for instance replacing NSs by a different known silencing suppressor in a reconstituted viral infection, may render a better understanding of the role(s) of NSs during TSWV infection.

Acknowledgements
We are grateful to Dr. R.M. Elliott for sharing his unpublished results and to Dr. A.O. Muijsers for N-terminal protein sequencing. This work was financially supported by the Netherlands organisation for Scientific Research, section Earth and Life Sciences (NWO/ALW).

Chapter 3

Intracellular localisation and homotypic interactions of TSWV nucleocapsid protein in living cells

Abstract

The tomato spotted wilt virus structural nucleoprotein (N) was fused to either yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP) and transiently expressed in Baby hamster kidney (BHK21) cells. Like wild type N protein, both fluorophore fusion proteins showed a cytoplasmic localisation pattern with perinuclear accumulation, and were observed to form aggregates indicating that the fusions did not affect the intracellular behaviour of N. The perinuclear accumulation was still observed when N protein was expressed in the presence of brefeldin A, but was abolished after the addition of either cytochalasin D or colchicine, suggesting the involvement of both actin filaments and microtubules for perinuclear targeting. The homotypic interaction of N was subsequently studied in vivo using FRET (fluorescence resonance energy transfer) and FLIM (fluorescence lifetime imaging microscopy) methods. In sensitised emission experiments, energy transfer was observed to take place from CFP to YFP when CFP-N was co-expressed with YFP-N, strongly indicating homotypic interaction of the N proteins. This was confirmed by acceptor photobleaching studies as well as by FLIM experiments. All three methods showed interactions taking place, not only in the aggregates in the perinuclear region, but also throughout the cytoplasm.

This chapter has been published in a modified form as:
Marjolein Snippe, Jan Willem Borst, Rob Goldbach and Richard Kormelink (2005). The use of fluorescence microscopy to visualise homotypic interactions of tomato spotted wilt virus nucleocapsid protein in living cells. J. Virol. Meth. **125**: 15-22.

Introduction

Tomato spotted wilt virus (TSWV) is the type species of the Tospovirus genus within the family Bunyaviridae (Elliott, 1990 and 1996). The tospoviruses are the only plant-infecting members of this virus family. Like all members of the Bunyaviridae, TSWV consists of enveloped, spherical particles of 80-120 nm in diameter containing a tri-partite RNA genome. The L segment is of negative polarity and codes for the viral RNA-dependent RNA polymerase (de Haan *et al.*, 1991), whereas both the M and S segments are of ambisense polarity. The M RNA codes for the glycoprotein precursor GP in the viral complementary strand and the movement protein NSm in the viral strand (Kormelink *et al.*, 1992a). The S RNA encodes in viral complementary sense the nucleoprotein (N) and in viral sense the non-structural protein NSs, which is involved in suppression of gene silencing (Bucher *et al.*, 2003; de Haan *et al.*, 1990; Takeda *et al.*, 2002). The genomic RNA molecules are tightly associated with the N protein and small amounts of the viral polymerase to form infectious ribonucleoproteins (RNPs) (van Poelwijk *et al.*, 1993).

Infectious RNPs are assumed to concentrate at the Golgi complex where the two glycoproteins Gn and Gc are retained. Mature virus particles are then formed through enwrapment of RNPs by a Golgi stack to acquire a lipid membrane containing Gn and Gc (Kikkert *et al.*, 1999). It is hypothesised that interaction of N with the cytoplasmic tail of Gn triggers the process of wrapping, based on the observation that purified RNP preparations often show a co-purification of Gn (unpublished results). Prior to the assembly of mature virus particles, RNPs are formed by the association of N protein with viral RNA. TSWV N protein has been demonstrated to non-specifically bind ssRNA in vitro in a co-operative manner (Richmond *et al.*, 1998). However, no common RNA binding motif has been identified so far. Moreover, N-N interactions have been shown to occur using the yeast-two-hybrid system (Uhrig *et al.*, 1999). Studies on related hantaviruses, both in vivo and in vitro, have yielded similar results (Alfadhli *et al.*, 2001; Kaukinen *et al.*, 2001).

Although it is very likely that many of the steps within the process of RNP and virus assembly require interaction between the viral structural proteins, studies on protein-protein interactions involving the viral glycoproteins have so far been hampered due to the absence of proper systems to investigate these interactions.

Förster or Fluorescence Resonance Energy Transfer – FRET is a technique that enables protein studies in living cells and is in principle applicable to any protein, including glycoproteins. FRET-based techniques have the advantage, above common approaches like co-immunoprecipitation and yeast-two-hybrid, that they not only enable a study on homo- and heterodimeric interactions of proteins in living cells, but also provide insight in the intracellular localisation of these complexes. The principle of FRET lies in the non-radiative transfer of energy between a donor fluorescent molecule (CFP) and an acceptor molecule (YFP), when the molecules are in very close proximity (<10 nm) (Gadella *et al.*, 1999; Sekar and Periasamy, 2003). This principle can be used for in vivo studying of e.g. protein folding (Rhoades *et al.*, 2003), protein cleavage (Bastiaens and Jovin, 1996; Xu *et al.*, 1998), phosphorylation (Sato *et al.*, 2002, Violin *et al.*, 2003) and

protein-protein interactions (Immink *et al.*, 2002; Larson *et al.*, 2003). If, as is the case in this work, the two fluorophores are fused to two separate molecules, FRET will in practice take place only when the proteins tagged with the fluorophores interact. If FRET takes place, a decrease in donor fluorescence as well as an increase in acceptor fluorescence can be observed. This phenomenon is called sensitised emission. In order for energy transfer to occur, it is essential that the emission spectrum of the donor fluorophore has a spectral overlap with the absorption spectrum of the acceptor, as illustrated for the CFP-YFP FRET couple in Figure 1a. In order to measure FRET, and thus observe protein-protein interaction, several methods can be used: (1) sensitised emission: the observation of YFP emission upon excitation of CFP; (2) acceptor photobleaching, after which the increase in donor fluorescence will give an indication of the FRET efficiency; and (3) Fluorescence Lifetime Imaging Microscopy (FLIM), in which the fluorescence lifetime of the donor fluorophore (CFP) is measured (Gadella, 1999; Hink *et al.*, 2002; Xia and Liu, 2001).

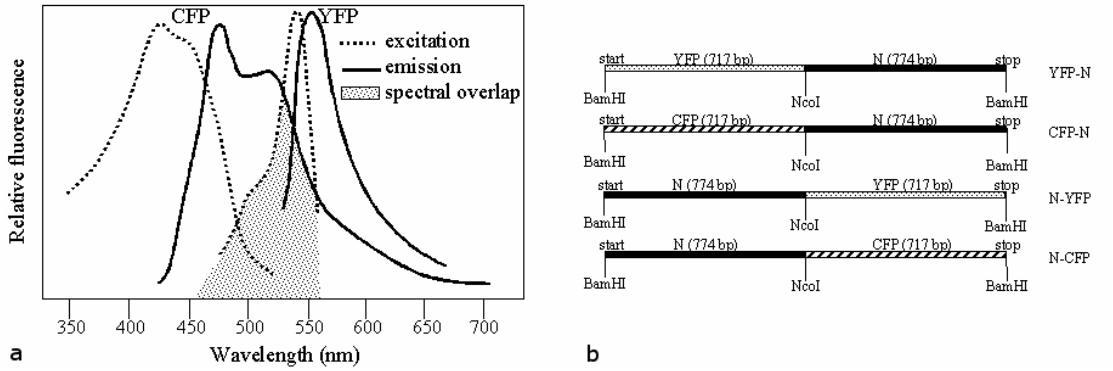


Figure 1 a. Absorption and emission spectra of CFP and YFP. FRET can occur because of the overlap of the donor (CFP) emission wavelength with the acceptor (YFP) excitation wavelength.
b. Schematic representation of the fusion constructs used during analyses. CFP / YFP fluorophores were fused in frame at the position of the start codon (CFP-N / YFP-N) or the stop codon (N-CFP / N-YFP) of the TSWV N protein, after translation resulting in an N protein that is, respectively, N- or C-terminally fused to the fluorophore.

As a first step to dissect TSWV protein-protein interactions between the structural N, Gn and Gc proteins involved in the envelopment of RNPs, N-fluorophore fusion proteins were transiently expressed in mammalian cells and analysed for their in situ localisation behaviour and, with the use of fluorescence microscopy techniques, for in vivo protein-protein interactions. The results described here suggest that homotypic N interactions occur dispersed throughout the cytoplasm and that eventually accumulation of large N aggregates takes place, predominantly in a perinuclear region. Targeting of the N protein to this region seems to involve actin filaments as well as microtubules.

Materials and Methods

Cell culture

Baby hamster kidney (BHK21) cells were maintained at 37°C with 5% CO2 in Glasgow MEM culture medium (Invitrogen) supplemented with 10% foetal calf

serum (FCS), 26 g/l tryptose phosphate broth (Sigma), penicillin (100 U/ml) and streptomycin (100 µg/ml).

Constructs
The pCS2 plasmid (Rupp *et al.*, 1994) was used for cloning and expression in BHK21 cells. The BamHI site in the multiple cloning site was used to insert the DNA coding for the fusion proteins described below.
The TSWV N gene was amplified from TSWV isolate BR-01 by PCR using primers carrying a BamHI restriction site and a start codon in the forward primer (p19), and an NcoI site and no stop codon in the reverse primer (Rev-Nco-N), or an NcoI site in the forward primer (Fwd-Nco-N) and a BamHI site and a stop codon in the reverse primer (p15). Restriction sites used are underlined; start and stop codons are depicted in bold letters; capitals are identical to the target sequence.

p19: cccggatccATGTCTAAGGTTAAGCTCACTAAGG
Rev-Nco-N: gggccatggcAGCAAGTTCTGCGAGTTTTGCC

Fwd-Nco-N: ccatggATGTCTAAGGTTAAGC
p15: cccggatccTCAAGCAAGTTCTGCGAGTTTTG

Likewise, genes of CFP and a pH-insensitive form of YFP were amplified from plasmids pMON-ECFP and pMON-EYFP, kindly provided by G. van der Krogt, using primers Fwd-Bam-Y with Rev-Nco-Y, and Fwd-Nco-Y with Rev-Bam-Y.

Fwd-Bam-Y: cccggatccgATGGTGAGCAAGGGCG
Rev-Nco-Y: ccatggCTTGTACAGCTCGTCC

Fwd-Nco-Y: gggccATGGTGAGCAAGGGCGAGGAGC
Rev-Bam-Y: cccggatccatatgTTACTTGTACAGCTCGTCCATGCC

By a three-point ligation, inserts were ligated into the vector. The resulting expression vector contained a gene coding for TSWV N protein, which is fused either C-terminally or N-terminally to one of the fluorophores (denoted here as N-CFP or N-YFP and CFP-N or YFP-N, respectively). Experiments with wild type N protein were performed using the fragment resulting from a forward primer with a BamHI site in combination with a reverse primer also containing a BamHI site. A schematic representation of the constructs is given in Figure 1b.

Transfection
Cells were grown on coverslips to 60-70% density in 35-mm dishes.10µg of each plasmid to be transfected was mixed in the presence of 20µl of lipofectamine (Invitrogen), in a total of 200 µl serum free medium. After incubation at room temperature for 45 minutes, cells were washed once with serum free medium. The DNA-lipofectamine mixture was added on top of the cells and the cells were left at room temperature for 10 minutes. Next, 800µl of serum free medium was added and incubation was prolonged for 5-6 hours at 37°C with 5% CO_2. Finally, the

medium was removed and replaced by 1.5 ml of medium containing 10% FCS. Cells were incubated at 37°C with 5% CO2 until further analysis.

Treatment of cells with biochemicals
Brefeldin A (BFA) was purchased from Sigma and a stock solution of 5 mg/ml in ethanol was made and stored at -20ºC. Following transfection, cells were incubated in 1 µg/ml BFA for 19 hours prior to analysis. Colchicine was purchased from Duchefa and a stock solution of 40 mg/ml (100 mM) in ethanol was made and stored at room temperature. Following transfection, cells were incubated in 100 µM colchicine for 19 hours prior to analysis. Cytochalasin D was purchased from Sigma and stored at –20 ºC in a stock solution of 5 mg/ml (10 mM) in ethanol. Following transfection, cells were incubated in cytochalasin for 19 hours and subsequently analysed.

Indirect fluorescence analysis
Coverslips with attached cells were fixed in 96% ethanol, washed with PBS for 15 minutes and blocked with 5% BSA in PBS for 45 minutes. Polyclonal antiserum against N was diluted in 1% BSA in PBS and incubated for 1 hour at room temperature. The cells were washed with PBS and incubated with secondary antibodies conjugated to FITC (fluorescein isothiocyanate) for 45 minutes at room temperature. Cells were washed overnight with PBS and examined in a Zeiss LSM510 confocal laser scanning microscope. As a (trans-) Golgi marker, we used a galactosyl transferase protein C-terminally fused to EYFP (Llopis *et al.*, 1998). As a marker for the ER-Golgi intermediate compartment (ERGIC), a monoclonal mouse antibody against human ERGIC-53 protein was used (Schweizer *et al.*, 1998) and visualised using a secondary antibody conjugated to Rhodamin Red-X, purchased from Molecular Probes.

FRET analysis
Preparations of cells on coverslips were mounted on microscope slides and observed using a Zeiss LSM510 confocal laser scanning microscope based on an Axiovert inverted microscope with a 40× oil immersion objective with a numerical aperture of 1.3. The Argon laser was used to excite both fluorophores. CFP excitation was provided by the 458-nm laser line using 8 - 20% of laser power and detected by using an HFT 458/514 main dichroic splitter, an NFT 515 dichroic splitter acting as a short-pass filter, and a band-pass 470- to 500-nm filter. The pinhole was set at 164 µm for N-CFP and at 66 µm (1 airy unit) for CFP-N. YFP was excited at 514 nm with 1% of laser power and detected with the same HFT 458/514 main dichroic splitter, the same NFT 515 dichroic splitter acting as a long-pass filter, and a band-pass 535- to 590-nm filter. The pinhole for YFP detection was set at 76 µm (1 airy unit). Operating in a multi-tracking mode allowed cross talk-free CFP and YFP images to be collected without changing filters. YFP bleaching was performed by exposing a pre-defined region to 10 pulses of the 514-nm laser line set at 100%.
Fluorescence Lifetime Imaging Microscopy (FLIM). FLIM was performed using a Biorad Radiance 2100 MP system in combination with a Nikon TE 300 inverted

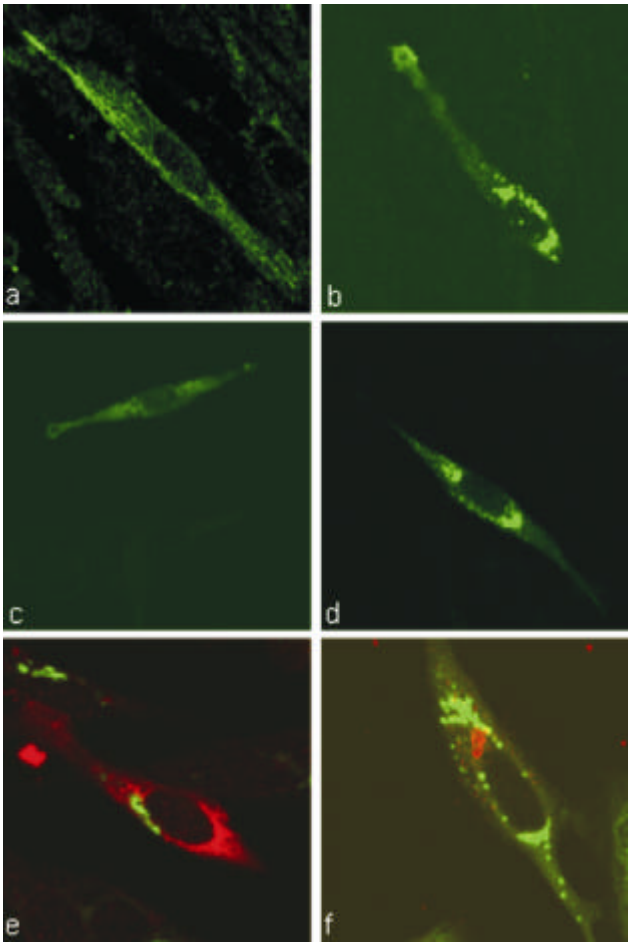


Figure 2. Time course analysis of wild type TSWV N protein (wt N) and N-YFP expressed in BHK21 cells. Images were taken at 4 (a, c) and 24 (b, d, e, f) hours post transfection. Both N (a, b) and N-YFP (c, d) are observed dispersed throughout the cytoplasm early in transfection. Upon longer incubation, both proteins are gradually observed to form clusters that are ultimately most abundantly localised near the nucleus. Panels e and f show N (e) and N-YFP (f) with a marker for the trans-Golgi system (e; marker in green, N in red) or for the ERGIC (f; ERGIC in red, N-YFP in green).

microscope. Two photon excitation pulses were generated by a Ti:Sapphire laser (Coherent Mira) that was pumped by a 5 W Coherent Verdi laser. Pulse trains of 76 MHz (150 fs pulse duration, 860 nm centre wavelength and pulse energy) were produced. The excitation light was directly coupled into the microscope and focused into the sample using a CFI Plan Apochromat 60 x water immersion objective lens (N.A. 1.2) Fluorescent light was detected using the non-descanned single photon counting detection, which is the most sensitive solution for two-photon imaging. For the FLIM experiment the Hamamatsu R3809U MCP PMT was used, which has a typical time resolution around 50 ps. CFP emission was selected using a 480DF30 nm band-pass filter. Images with a frame size of 64*64 pixels were acquired using the Becker and Hickl SPC 830 module (Borst *et al.*, 2003). The average count rate was 2.104 photons/s for an acquisition time of 90 seconds. From the intensity images obtained, complete fluorescence lifetime decays were calculated per pixel and fitted using a double exponential decay model. The lifetime of one component was fixed to the value found for CFP-N (2.5 ns).

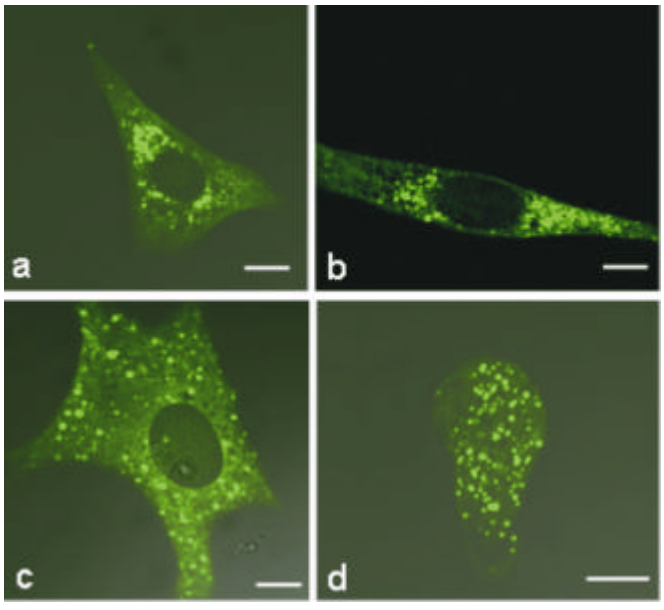


Figure 3. Fluorescence images of BHK21 cells transfected with N-YFP and treated with BFA (panel b), colchicine (c) or cytochalasin D (d). Panel a is an untreated control transfection. The disturbing effect of both colchicine and cytochalasin D on the perinuclear translocation of N-YFP is clearly visible, indicating the involvement of actin filaments as well as microtubules in the peri-nuclear targeting. Bars indicate 20 (a, b, c) or 10 (d) µm.

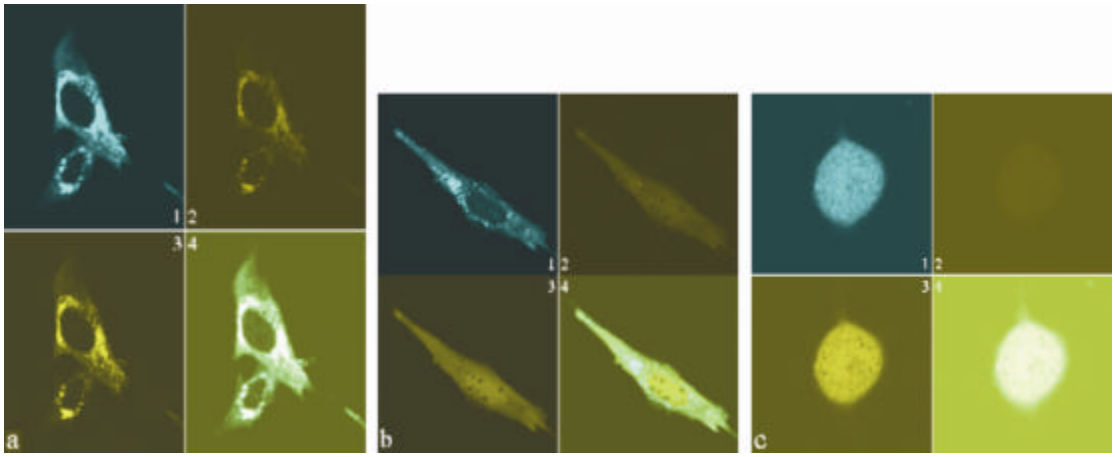


Figure 4. Fluorescence images of BHK21 cells co-transfected with a: CFP-N and YFP-N; b: CFP-N and wt-YFP; c: wt CFP and wt YFP. Panels represent signals of 1: CFP; 2: YFP fluorescence due to FRET; 3: YFP fluorescence after YFP excitation; 4: merge of 1, 2 and 3. The signal in panel a2 (YFP signal due to sensitised emission) is absent in both controls, indicating that interaction does indeed take place between the N proteins.

Results

Time course analysis of N-YFP and wild type N protein in BHK cells

The expression and localisation of transiently produced N and N-YFP were analysed in order to verify that the in vivo behaviour of the N protein is not altered by fusion with a fluorophore. N-YFP was constructed by fusing YFP to the C-terminus of N, just before the stop codon of N, as depicted in Figure 1b. The constructs were cloned into the PCS2+ plasmid, downstream of a CMV promoter, and transfected into BHK21 cells. Transfected cells were analysed 4, 8, 12 and 24 hours after transfection. Both wild type N and N-YFP appeared throughout the cytoplasm of BHK cells from 4 hours after transfection and both were gradually observed to form clusters (Figure 2 a-d). These clusters were ultimately most abundant close to the nucleus, but did not co-localise with specific markers for the trans-Golgi system or the ER-Golgi intermediate compartment (ERGIC) (Figure 2 e-f).

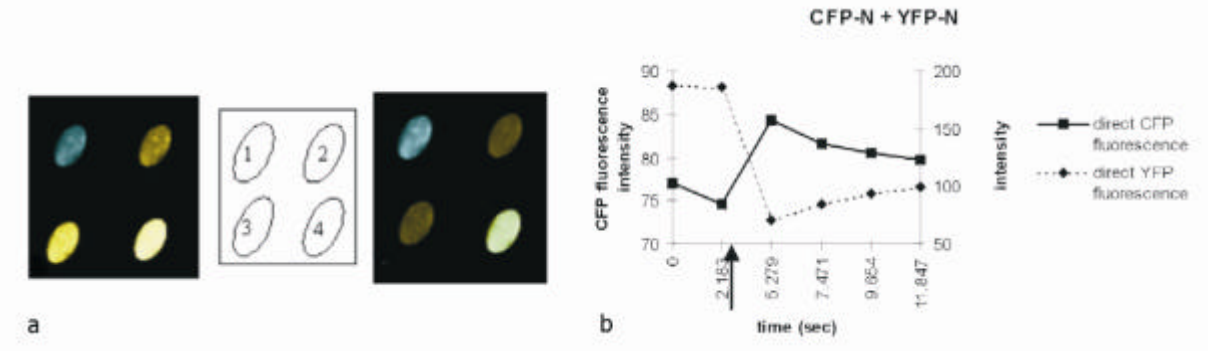


Figure 5 a. Region of a co-transfected cell before (left) and after (right) YFP bleaching. 1: direct CFP emission; 2: YFP signal due to sensitised emission; 3: direct YFP emission; 4: merge of 1, 2 and 3. As the two YFP signals practically disappear, the CFP signal increases visibly, indicating that before bleaching energy transfer, and thus interaction, was taking place between both proteins.
b. CFP and YFP fluorescence intensities (in arbitrary units) of a region in a cell co-transfected with CFP-N and YFP-N, measured before and after YFP bleaching. The arrow indicates the bleaching pulse. A decrease in YFP fluorescence, caused by bleaching, coincides with a significant CFP fluorescence increase, an indication for the occurrence of FRET before photobleaching.

Intracellular translocation of N

Upon treatment of the cells with BFA, hardly any difference in the localisation pattern of N-YFP was observed (Figure 3, panel b). In contrast, incubation in the presence of either colchicine or cytochalasin D, inhibitors of microtubule assembly and actin filament polymerisation, respectively, rendered an obvious change in the localisation pattern. Whereas small clusters continued to be formed, no perinuclear accumulation was found to occur (Figure 3, panel c and d), suggesting both microtubules and actin filaments are essential to obtain perinuclear accumulation of N.

FRET analyses of homotypic interaction by indirect YFP excitation

Since fluorophore fusion to the N protein did not appear to affect the behaviour of this viral protein in vivo, the fusion proteins were used for further research using FRET microscopy. Fusion constructs of N and CFP or YFP were made as described in Materials and Methods, and as schematically depicted in Figure 1b. As a first step to investigate in vivo N-N interactions, cotransfections of different combinations of N-fluorophore fusions were carried out. Figure 4 shows the situation after co-transfection of CFP-N and YFP-N. A clear FRET signal from YFP-N can be seen caused by energy transfer from the donor (CFP-N) to the acceptor (YFP-N) (Figure 4a, panel 2). FRET was also clearly observed after cotransfection of CFP-N and N-YFP (data not shown). N-CFP always rendered low fluorescence intensities and was therefore not used in sensitised emission experiments. Almost no (background) signal was detected when control combinations were used (Figure 4b and c).

FRET analyses of homotypic interaction by acceptor-photobleaching

In order to support the observations made with FRET microscopy that indicated N-N interactions, a second experiment was performed using acceptor photobleaching. During this experiment, emission of CFP-fusion proteins was measured in the presence of an intact versus a photodestructed (bleached) form of the acceptor (YFP fusion). Destruction of YFP was performed by exposure to a laser pulse of 514 nm using 100% of laser power (Materials and Methods), which resulted in an irreversibly destructed form of YFP. This form of YFP was therefore not able to function neither as a fluorophore nor as a fluorescence energy acceptor. Under these circumstances, a fused CFP would not be able to transfer its fluorescence resonance energy to YFP. It was anticipated that in case the previously observed interaction between CFP-N and YFP-N was genuine, inactivation of YFP-N by photobleaching would result in an increase of CFP-N emission. After bleaching, leading to almost complete disappearance of the YFP-N signal (Figure 5a, right panel, region 2 and 3), the CFP-N signal had indeed visibly increased (Figure 5a, right panel, region 1, versus left panel, region 1). No increase in CFP emission was observed whenever combinations with negative controls were used.

The intensities of the CFP-N and YFP-N signals were measured and set out in a graph (Figure 5b). From this graph, it was again obvious that upon YFP bleaching (indicated by the arrow), a drop in YFP emission occurred that co-incided with a

clear increase in CFP fluorescence. In time, a recovery of the YFP signal was observed, likely due to the mobility of YFP-N, since the cells were not fixed in these experiments. The same measurements were carried out for all combinations of the different fusion proteins and the results of these experiments are summarised in Figure 6. With the exception of the couple N-CFP + N-YFP, at least 3 independent measurements were performed. Due to the very low fluorescence of N-CFP, further analysis of N-CFP + N-YFP was not performed; however, interaction between these two fusion proteins was observed as well (Figure 6). As a control, cells transfected with only N-CFP did not show any increase in CFP fluorescence after bleaching. Routinely, in this case a slight decrease of CFP fluorescence was observed, which is likely to be caused by unintentional bleaching of CFP.

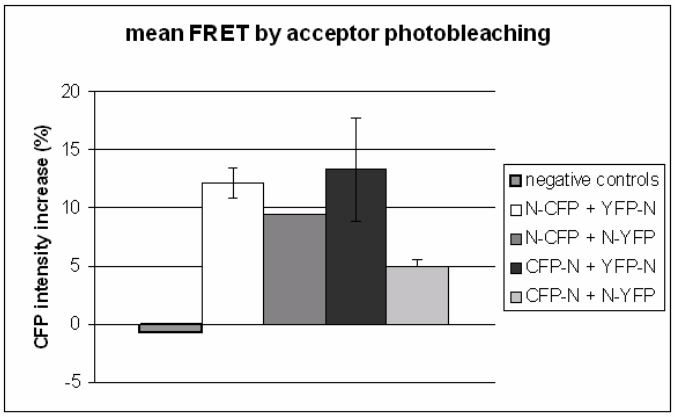


Figure 6. Relative FRET efficiency of combinations of the fusion proteins as measured by CFP increase after YFP bleaching. Relative FRET efficiencies were calculated as follows: Efficiency (%) =100*(intensity after bleaching - intensity before bleaching) / intensity before bleaching. Intensities are in arbitrary units; bars represent standard deviation. Negative controls include N-CFP (single transfection), wt CFP + wt YFP, and CFP-N + wt YFP. All combinations show a significant increase in CFP fluorescence upon YFP photobleaching.

FRET analyses of homotypic interaction using FLIM

To further substantiate the data obtained by sensitised emission and acceptor photobleaching experiments, fluorescence lifetime measurements were performed on all different combinations of N- and C-terminal fluorophore fusions to N. These experiments confirmed the interaction found using the other methods by revealing a clear decrease in N-CFP and CFP-N fluorescence lifetime when N-YFP or YFP-N was present (Figure 7). In control transfections, the mean fluorescence lifetime of the CFP fusion proteins was 2538 ps. The mean CFP fluorescence lifetime for the cell depicted in Figure 7 was 2100 ps, which amounts to a 17% decrease. Decreases found in the different co-transfections ranged from 2.4% to over 20%. This is a significant decrease, indicating very clearly interactions between the two proteins. Like in the acceptor-photobleaching experiments, FLIM measurements showed very low fluorescence intensity of N-CFP compared to CFP-N. Due to these low intensities, no accurate data were obtained for N-CFP from lifetime experiments. However interaction could also be observed with N-CFP (not shown). The average decrease in fluorescence lifetime for CFP-N in combination with YFP-N or N-YFP is summarised in Figure 8.

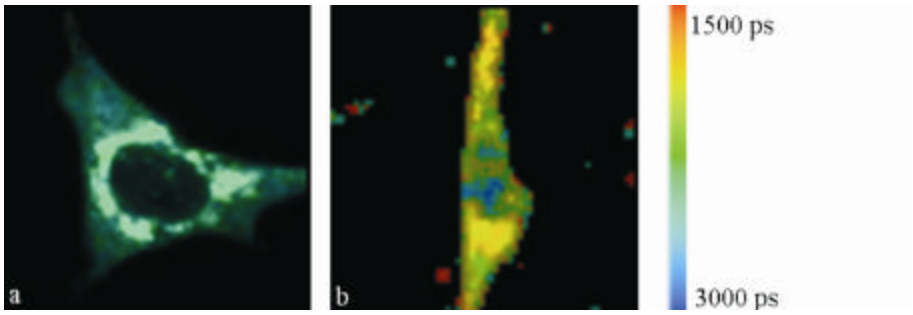


Figure 7. Fluorescence lifetime of CFP in a cell transfected with a: CFP-N, and b: CFP-N and YFP-N. A shorter fluorescence lifetime of CFP(-N) is clearly observed in the presence of YFP-N (panel b), as indicated by the pseudocolour change into yellow. The legend for the pseudocolours representing CFP fluorescence lifetime is provided in the colour scale. The decrease in lifetime is a consequence of fluorescence energy transfer and thus of interaction between the fusion proteins.

Discussion

FRET (fluorescence resonance energy transfer) and FLIM (fluorescence lifetime imaging microscopy) were employed to study homotypic TSWV N protein interaction in vivo. Based on earlier work on the assembly of TSWV, a model has been proposed in which enveloped particles arise as a result of enwrapment of RNPs at Golgi stacks that contain mature viral glycoprotein (Kikkert *et al.*, 1999).

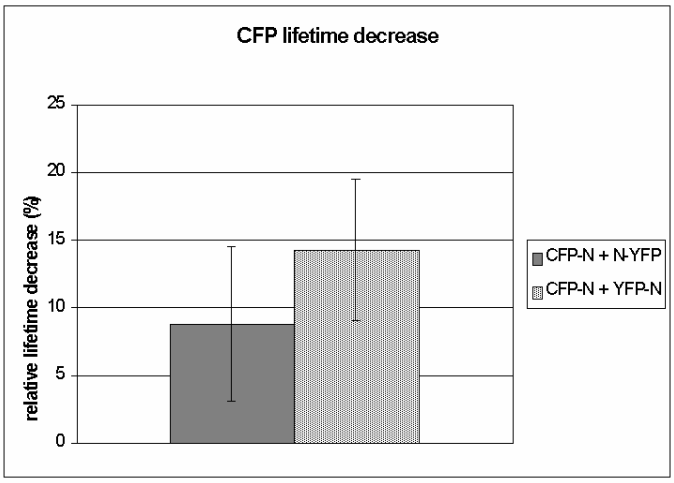


Figure 8
CFP(-N) lifetime decrease in co-transfections with N-YFP and YFP-N, as measured by FLIM. Lifetime decreases were calculated as follows:
Lifetime decrease (%) = 100*(lifetime of non-interacting CFP - lifetime of CFP in sample) / lifetime of non-interacting CFP. Bars represent standard deviation. In both cases a significant decrease in CFP fluorescence lifetime was observed.

Prior to this process, RNPs are formed and thus it is assumed that the viral N protein, which represents the major structural protein of RNPs, has affinity for (viral) ssRNA and harbours a capacity for homotypic interactions. These interactions have indeed been demonstrated in studies using the yeast-two-hybrid system (Uhrig *et al.*, 1999). Moreover, the N protein has been shown to contain non-specific ssRNA binding affinity in vitro (Richmond *et al.*, 1998). To further unravel the interactions between the TSWV structural proteins, the in vivo approach of FRET microscopy was tested. In view of the assembly process and the proven in vitro homotypic interactions for N, it was anticipated that N-N interactions would occur in vivo as well. Therefore, this protein was selected as the first and easiest (cytosolic) target to demonstrate in vivo protein-protein interactions for a TSWV structural protein using FRET microscopy. To this end,

N- and C-terminal fusions of CFP (donor) and YFP (acceptor) to the N protein were made and transiently co-expressed in BHK21 cells to be analysed by FRET microscopy. The use of the mammalian cell system was justified due to the close evolutionary relationship of TSWV with the mammalian viruses of the Bunyaviridae family. Using not only direct FRET microscopy, but also acceptor photobleaching and FLIM analyses, the occurrence of in vivo homotypic interactions of the N protein was clearly demonstrated. All combinations of N-fluorophore couples, with the exception of N-CFP, rendered clear results that indicated the occurrence of homotypic interactions of N proteins.

Furthermore, FLIM experiments and acceptor photobleaching experiments (data not shown) revealed that homotypic interactions are present not only in the perinuclear aggregates that can be observed from 8 hours post transfection onward, but also throughout the cytoplasm. This localisation pattern of N from a disperse cytosolic up to perinuclear appearance was also observed when wild type N protein was transiently expressed. This indicated that the fusions had not affected the localisation behaviour of N, next to the fact that interactions were not abolished or hindered by the presence of the fusions. Previous studies on TSWV by Uhrig *et al.* (1999) indicated a "head-to-tail" organisation of the homotypic N interaction, involving an N- and a C-terminal domain. Multimerisation into N aggregates would thus require the presence of both domains. Based on the data presented in this manuscript, it is interesting to note that in case the "head-to-tail" hypothesis would be true, the presence of a fluorophore fusion on either side of the protein does not hinder this dimerisation. However, additional domains within the N protein required for dimerisation have recently been found by Kainz *et al.* (2004), and in the additional light of data from hantavirus N multimerisation studies where a "tail-to-tail" and "head-to-head" interaction is proposed (Alfadhli *et al.*, 2001; Kaukinen *et al.*, 2001, 2003; Yoshimatsu *et al.*, 2003), the "head-to-tail" model still remains to be further investigated. FRET / FLIM techniques may become a helpful tool to study these in vivo interactions in further detail.

In view of the virus assembly taking place at the Golgi stacks, it is assumed that the viral RNPs also accumulate close to the site where Gn and Gc concentrate in the Golgi prior to envelopment. This has been confirmed by studies of a natural infection cycle, where TSWV RNPs or N protein were observed in the cytoplasm and near the Golgi complex (Kikkert *et al.*, 1999; Kitajima *et al.*, 1992). However, upon transient expression of TSWV N protein in BHK cells, the protein did not colocalise with marker proteins for either the Golgi or the ERGIC. Similar observations have been made for Crimean-Congo hemorrhagic virus (Andersson *et al.*, 2004b), but it contrasts the findings for transiently expressed hantavirus N protein (Ravkov and Compans, 2001). In both latter cases, the perinuclear localisation of viral N protein was disturbed in the presence of the actin depolymerising drug cytochalasin D (Andersson *et al.*, 2004b; Ravkov *et al.*, 1998). This was also found to be the case for transiently expressed N protein of TSWV described here. Moreover, expression in the presence of the anti-microtubular drug colchicine also rendered a cytoplasmic distribution of N-aggregates. Thus, not only actin filaments are involved in the targeting of N to a perinuclear location, but microtubules are needed as well. Although it is unclear whether the

perinuclear translocation of N represents an intermediate stage in the process of RNP envelopment, it is very well possible that co-expression of N with any of the viral glycoproteins, or any other viral (non)structural proteins, targets the RNPs to the site of envelopment, presumably the Golgi (Kikkert *et al.*, 1999, 2001). Alternatively, the presence or absence of viral RNA may influence the behaviour of the RNPs or aggregates. Future co-expression and localisation studies will help to answer these questions.

Acknowledgements

We thank Dr. R. Y. Tsien for kindly providing the trans-Golgi marker GT-EYFP and Dr. H. P. Hauri for the antibody against human ERGIC-53. We are grateful to G. N.M. van der Krogt who provided us with the plasmids encoding EYFP and ECFP, and to H. D. Lim for constructing the pCS2+N expression vector. This work was financially supported by the Dutch ALW-NWO; the equipment was purchased owing to an NWO-Groot investment grant.

Chapter 4

Tomato spotted wilt virus Gc interacts with the nucleocapsid protein *in vivo*

Abstract
Tomato spotted wilt virus (TSWV) virions consist of a nucleocapsid core surrounded by a membrane containing glycoproteins Gn and Gc. To unravel the protein interactions involved in the membrane acquisition of RNPs, TSWV nucleocapsid protein (N), Gn and Gc were expressed and analysed in BHK21 cells. Upon co-expression of Gn, Gc and N, a partial colocalisation of N with both glycoproteins was observed in the Golgi region. In contrast, upon co-expression of Gc and N in the absence of Gn, both proteins colocalised to a distinct non-Golgi perinuclear region. Using FLIM and FRET, interaction was demonstrated between N and Gc, but not between N and Gn. The interaction was only observed in the region where N and Gc accumulated. The interaction of N and Gc in a non-Golgi perinuclear region is discussed in view of TSWV particle assembly taking place at the Golgi complex.

Marjolein Snippe, Jan Willem Borst, Rob Goldbach and Richard Kormelink
To be submitted

Introduction

Members of the Bunyaviridae have membrane enveloped, spherical virus particles about 100 nm in diameter. The lipid membrane contains spike proteins that consist of the viral glycoproteins Gn and Gc (n and c refer to the amino and carboxy terminal position within the glycoprotein precursor, respectively) which are generally assumed to dictate the mode and site of virus assembly. Tomato spotted wilt virus (TSWV) is the type species of the genus Tospovirus, whose members are the only plant-infecting members of the family Bunyaviridae. For TSWV, the 58 kDa Gn is also referred to as G2 and Gc (78 kDa) as G1 (Figure 1).

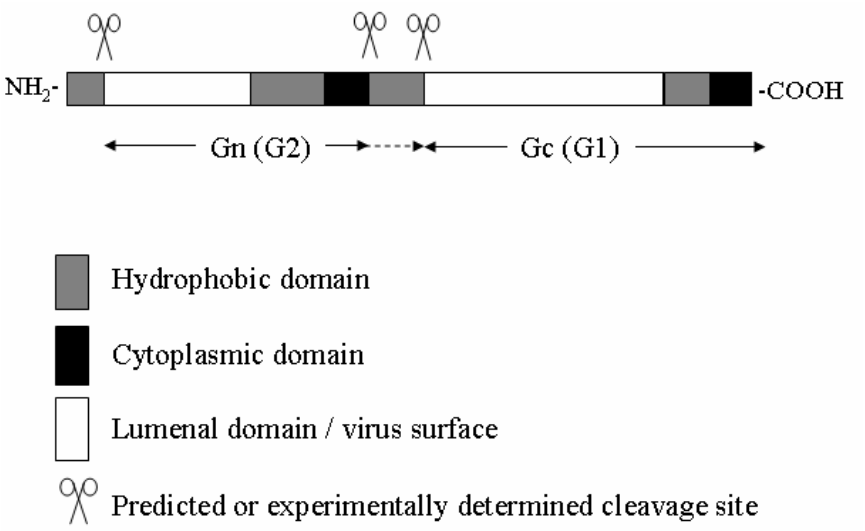


Figure 1. Topology of the TSWV glycoprotein precursor.

The assembly of virus particles first requires the formation of ribonucleoproteins (RNPs). These RNPs are complexes of viral genomic RNA, N protein and a few copies of the L protein (Elliott, 1990). For the animal-infecting bunyaviruses, the actual particle assembly involves budding of RNPs into the vacuolised lumen of Golgi stacks and, to a lower extent, the ER-Golgi intermediate compartment (ERGIC). An exception are members of the *Hantavirus* genus, which bud at the plasma membrane (Elliott, 1990; Ravkov *et al.*, 1997). Membrane envelopment of RNPs is likely triggered by interaction of a component of the RNPs with (one of) the viral glycoproteins.

In contrast to the animal-infecting bunyaviruses, TSWV particle assembly in plant cells involves enwrapment of RNPs by an entire Golgi stack, leading to the formation of doubly enveloped virus (DEV) particles. Fusion of DEVs with each other as well as with ER derived membranes results in singly enveloped virus (SEV) particles that accumulate in large vesicles (Kikkert *et al.*, 1999). Also for TSWV, the process of enwrapment is assumed to be triggered by an interaction of the RNPs with (for instance the cytoplasmic tails of) one or both of the glycoproteins.

In mammalian cells transiently expressing the two TSWV glycoproteins from the precursor gene, both Gn and Gc accumulate in the Golgi apparatus (Kikkert *et al.*,

2001). When expressed in the absence of other viral proteins, Gn is able to reach the Golgi, although some Gn can still be seen in the ER. On the other hand, Gc on its own shows only ER localisation, suggesting Gc is not able to exit the ER, possibly due to improper folding. Upon co-expression of Gn and Gc from individual constructs, both proteins are observed in the Golgi complex again, suggesting that Gn is capable of rescuing Gc from the ER to the Golgi complex, most likely by the formation of heterodimers (Kikkert *et al.*, 2001).
Recently, transient expression of N in mammalian cells revealed no Golgi or ERGIC localisation. Instead, the TSWV N protein is observed throughout the cytoplasm with a large fraction accumulating in a non-Golgi perinuclear region, the nature of which is as yet undetermined (chapter 3). An active actin and microtubuli system is required for this perinuclear localisation, altogether indicating that N may localise at the microtubule organising centre (MTOC) which in mammalian cells is known to be located close to the nucleus. Similar observations have been made for several of the animal-infecting bunyaviruses. In light of the observation that transiently expressed Gn and Gc accumulate and retain in the Golgi where the actual particle envelopment takes place, the question arises how RNPs or N are able to translocate from the non-Golgi perinuclear region to the Golgi complex. In order to analyse whether interactions between N and the glycoproteins play a role in this, N was expressed in the absence and presence of Gn and / or Gc in mammalian cells and its localisation behaviour analysed. Although minor differences in behaviour of the viral proteins in mammalian cells, compared to plant or thrips cells, cannot be excluded, no significant differences regarding interactions between the viral proteins are expected. The results showed a partial colocalisation of N and Gc, but not of N and Gn, in a non-Golgi perinuclear region, suggesting an interaction between N and Gc. This was supported by the existence of protein interactions as demonstrated by FRET and FLIM.

Materials and methods

Constructs

The pCS2 plasmid (Rupp *et al.*, 1994) was used for cloning and expression of the N fusion proteins as described previously (chapter 3). In analogy, Gn and Gc fluorophore fusion constructs were made. In brief, PCR products of TSWV Gc and Gn genes, containing a BamHI restriction site at the 5' end and an NcoI site at the 3' end, before the stop codon, were used in a three point ligation together with either the CFP or YFP gene containing a stop codon and a BamHI site at the 3' end and an NcoI site at the 5' end. The resulting genes encoding the fusion proteins Gc-YFP, Gc-CFP, Gn-YFP or Gn-CFP, were inserted into the pSFV1 vector (Gibco-BRL, Life Technology inc.), containing an NruI linearization site. The BamHI site of the multiple cloning site was used for insertion of the genes. The Gn/Gc precursor gene as well as the wild type Gn and Gc proteins were expressed from pSFV-GP as described by Kikkert *et al.* (2001).

Cell culture

Baby hamster kidney (BHK21) cells were maintained at 37°C with 10% CO2 in Glasgow MEM culture medium (Invitrogen) supplemented with 10% foetal calf serum (FCS), 2,6 g/l tryptose phosphate broth (Sigma), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cell densities were kept at a maximum of 70 – 80% confluency.

Semliki Forest Virus expression system For transient expression of the TSWV Gn and Gc glycoprotein constructs, the Semliki Forest virus (SFV) system was used. This system was first described by Liljeström and Garoff (1991). Recombinant pSFV constructs each containing the gene for one of the proteins of interest were linearised using NruI, followed by treatment with proteinase K to inactivate RNAses. After phenol extraction, in vitro transcription and capping was performed using the SP6 mMessage mMachine (Ambion, inc.). The quality of the transcripts was verified on a 1% agarose gel. Cells were cultured in 80 cm2 tissue culture flasks and harvested by treatment with Trypsin-EDTA (Gibco, Invitrogen), pelleted by centrifugation for 5 minutes at 130 × g and washed once with electroporation buffer (2 mM HEPES, 15 mM potassium phosphate buffer pH 7.2, 250 mM mannitol, 1 mM MgCl2). Cells were resuspended in 800 µl electroporation buffer and 150 µl was transferred to a 0.4 cm electroporation cuvette. Cells were supplied with 7.5 to 15 µl of RNA transcript, depending on the quality of the RNA. Electroporation was performed in a BioRad GenePulser II electroporator by applying 2 pulses of 650 V and a duration of 5 ms with an interval of 5 seconds. After electroporation, cells were resuspended in growth medium and seeded on microscope cover glasses in six-well plates. Cells were incubated at 37° and 10% CO2 for 20-24 hours. Cells were examined in a Zeiss LSM510 confocal laser scanning microscope. To detect proteins that were not fused to a fluorophore, an indirect fluorescence analysis was performed.

Indirect fluorescence analysis

Coverslips with attached cells were fixed in 96% ethanol for 20 minutes, washed with PBS and blocked with 5% BSA in PBS for 1 hour. Polyclonal antiserum against N, Gn or Gc was diluted in 1% BSA in PBS and incubated on the cells for 1 hour at room temperature. The cells were washed with PBS and incubated with secondary antibodies conjugated to Alexa Fluor 488 (Molecular probes, Invitrogen) or Rhodamin Red X (Nordic) for 1 hour at room temperature. Cells were washed overnight with PBS and examined in a Zeiss LSM510 confocal laser scanning microscope. As a (trans-) Golgi marker, a galactosyl transferase protein C-terminally fused to EYFP was used (Llopis *et al.*, 1998).

Fluorescence Lifetime Imaging Microscopy (FLIM)

FLIM was performed using a Biorad Radiance 2100 MP system in combination with a Nikon TE 300 inverted microscope. Two photon excitation pulses were generated by a Ti:Sapphire laser (Coherent Mira) that was pumped by a 5 W Coherent Verdi laser. Pulse trains of 76 MHz (150 fs pulse duration, 860 nm centre wavelength) were produced. The excitation light was directly coupled into the microscope and focused into the sample using a CFI Plan Apochromat 60 x

water immersion objective lens (N.A. 1.2) Fluorescent light was detected using the non-descanned single photon counting detection, which is the most sensitive solution for two-photon imaging. For the FLIM experiment the Hamamatsu R3809U MCP PMT was used, which has a typical time resolution around 50 ps. CFP emission was selected using a 480DF30 nm band-pass filter. Images with a frame size of 64*64 pixels were acquired using the Becker and Hickl SPC 830 module (Borst *et al.*, 2003). The average acquisition time was 200 seconds. From the intensity images obtained, complete fluorescence lifetime decays were calculated per pixel and fitted using a double exponential decay model. The lifetime of one component was fixed to the value found for Gc-CFP and Gn-CFP (2.6 ns).

FRET analysis by acceptor photobleaching

Cells were either observed directly or after fixation with ethanol (96%, 20 minutes at room temperature) or paraformaldehyde (4%, 20-30 minutes at room temperature). Preparations of cells on coverslips were mounted on microscope slides and observed using a Zeiss LSM510 confocal laser scanning microscope based on an Axiovert inverted microscope with a 40× oil immersion objective with a numerical aperture of 1.3. The Argon laser was used to excite both fluorophores. CFP excitation was provided by the 458-nm laser line using 3 - 25% of laser power and detected by using an HFT 458/514 main dichroic splitter, an NFT 515 dichroic splitter acting as a short-pass filter, and a band-pass 470- to 500-nm filter. The pinhole was set at 60 or 86 µm for most experiments, although diameters up to 329 µm were used incidentally in case of Gc-CFP and Gn-CFP (1 airy unit = 66 µm). YFP was excited at 514 nm with 1 - 10% of laser power and detected with the same HFT 458/514 main dichroic splitter, the same NFT 515 dichroic splitter acting as a long-pass filter, and a band-pass 535- to 590-nm filter. The pinhole for YFP detection was set at 71, 76 or 96 µm (1 airy unit = 76 µm). Operating in a multi-tracking mode allowed cross talk-free CFP and YFP images to be collected without changing filters. YFP bleaching was performed by exposing a pre-defined region to 20 pulses of the 514-nm laser line set at 95%.

Results

Effect of fluorophore fusion on the localisation of the viral proteins

Fusion of N with CFP or YFP at either the C- or the N-terminal side of the protein has recently been shown not to influence its intracellular localisation pattern. Both wild type and fluorophore fusions of N form small aggregates in the cytoplasm and large aggregates in a non-Golgi, perinuclear region (see chapter 3). In order to verify that the localisation behaviour of the glycoproteins is not altered due to a fusion with a fluorophore, the glycoproteins were fused at their carboxy termini to CFP or YFP and expressed in BHK21 cells. Like what is routinely observed for Gn, Gn-CFP localised in accumulating amounts in a perinuclear region. Upon cotransfection with the trans-Golgi marker galactosyl transferase fused to YFP (GT-YFP) (kindly provided by Dr. Tsien), this perinuclear localisation was confirmed to be the Golgi apparatus (Figure 2a-f). Likewise, fusion of CFP or YFP to Gc did not alter its ER localisation, as shown in Figure 2g-h.

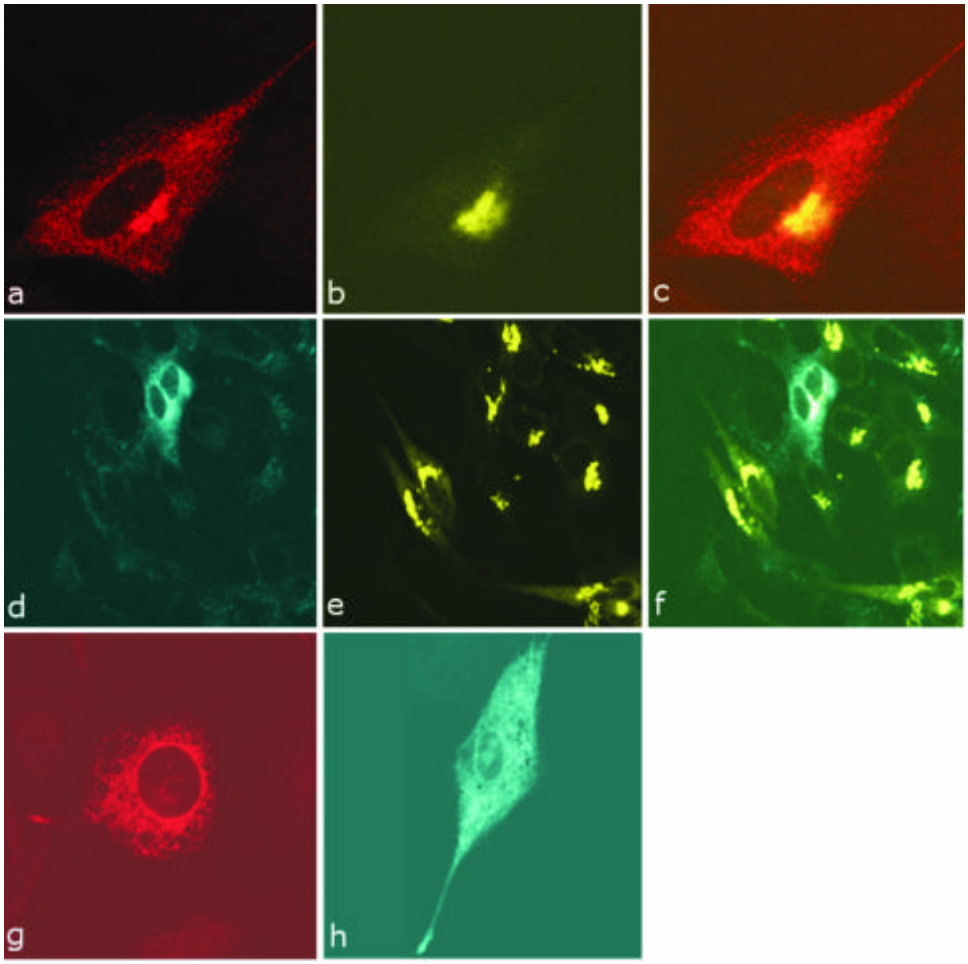


Figure 2. Expression of TSWV Gn (a-c), Gn-CFP (d-f), TSWV Gc (g) and Gc-CFP (h) in BHK21 cells. Cells were co-transfected with constructs coding for Golgi-marker GT-YFP and either wild type Gn (a-c) or Gn-CFP (d-f), or singly transfected with constructs coding for Gc (g) or Gc-CFP (h). Wild type Gn (a) and Gc (g) were immunostained and visualised using Rhodamine Red X; CFP and YFP were examined directly. Images were taken using a confocal microscope. Detection of: Gn (a); GT-YFP (b); merge of a and b (c); Gn-CFP (d); GT-YFP (e); merge of d and e (f); Gc (g); Gc-CFP (h). Wild type Gn and the Gn-CFP fusion protein show Golgi localisation, overlapping with that of GT-YFP whereas both Gc and Gc-CFP are distributed throughout the cell in a reticular pattern.

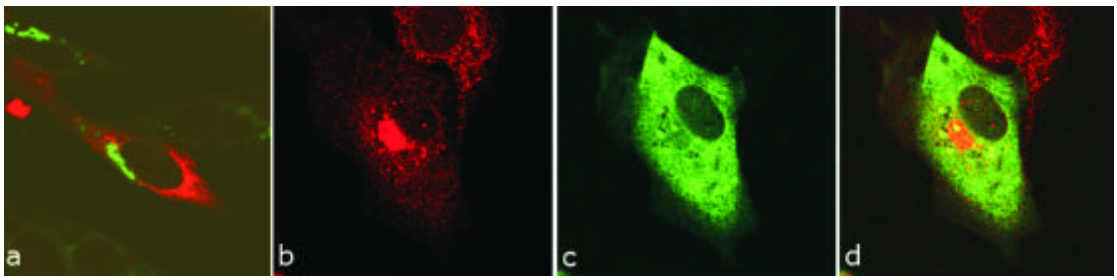


Figure 3. Expression of TSWV N in the absence (a) or presence (b-d) of the viral glycoproteins. BHK21 cells were co-transfected with constructs coding for wild type N and Golgi marker GT-YFP (a) or N-YFP and the glycoprotein precursor (b-d). Wild type N and Gn (expressed from the precursor) were immunostained using Rhodamine Red X; YFP was visualised directly. Detection of N (red) and GT-YFP (green) (a); Gn (red) (b); N-YFP (green) (c); merge of b and c (d).Wild type N accumulates at a perinuclear site clearly distinct from the Golgi complex (a), but in the presence of both glycoproteins accumulates in the Golgi region, partially overlapping with Gn.

Co-expression of N and glycoproteins

To analyse whether co-expression of N with Gn and / or Gc affected its intracellular localisation, BHK cells were co-transfected with constructs coding for the different proteins and the expressed proteins were analysed for their intracellular localisation. Whereas transient expression of N in BHK21 cells

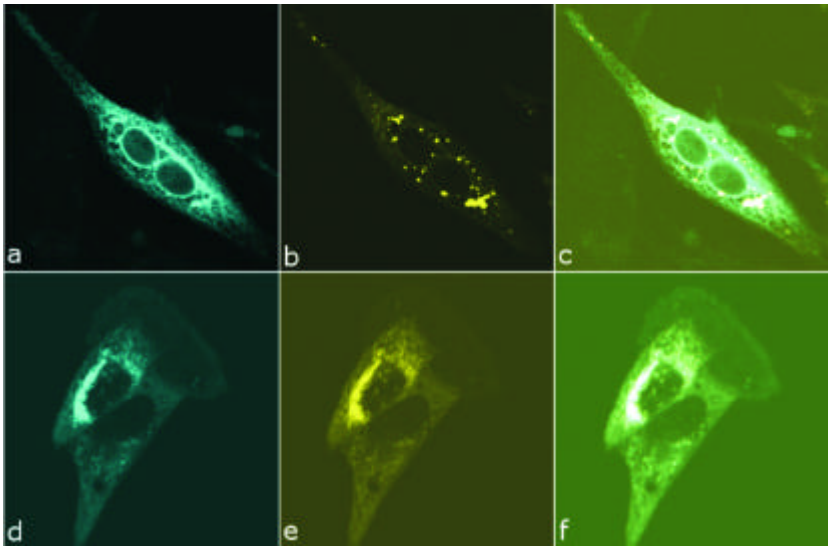


Figure 4. Co-expression of Gn-CFP and N-YFP (a-c) and Gc-YFP and CFP-N (d-f) in BHK21 cells. Cells were co-transfected with constructs coding for Gn-CFP and N-YFP (a-c), or for Gc-YFP and CFP-N (d-f) and the localisation of the proteins was readily visualised by the fluorophores. Panels show Gn-CFP (a); N-YFP (b); merge of a and b (c); CFP-N (d); Gc-YFP (e); merge of d and e (f). No clear colocalisation occurs in cells co-expressing Gn and N, whereas in cells expressing N and Gc, colocalisation is clearly visible in a perinuclear region.

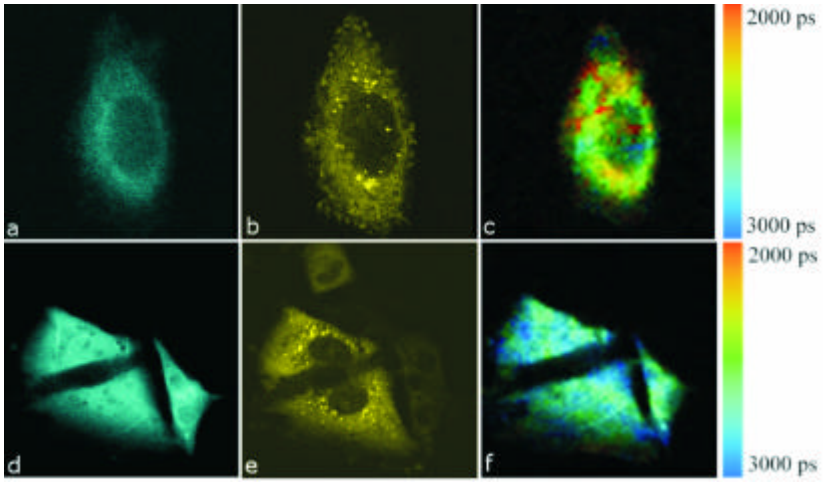


Figure 5. Fluorescence and fluorescence lifetime images of cells co-expressing GC-CFP and N-YFP (a-c) or GN-CFP and N-YFP (d-f). Cells were co-transfected with constructs coding for the two fusion proteins. Fluorescence was directly detected (a, b, d, e), whereas CFP lifetimes were measured and displayed using pseudocolours as indicated in the legend on the right (c, f). Panels show GC-CFP (a); N-YFP (b); fluorescence lifetime of GC-CFP (c); GN-CFP (d); N-YFP (e); fluorescence lifetime of GN-CFP (f). In regions where N accumulates, the lifetime of GC-CFP clearly decreases (c) whereas that of GN-CFP remains unchanged (f).

revealed accumulation in a non-Golgi perinuclear region as reported earlier (Figure 3a; chapter 3), co-expression with the glycoprotein precursor, rendered a partial colocalisation with the glycoproteins in the Golgi region (Figure 3b-d). This pattern was consistently observed in several independent experiments. Upon co-expression of N with Gn only, no clear co-localisation could be observed (Figure 4a-c) and both proteins remained localised to their distinct perinuclear areas as observed in the single expression experiments, with Gn accumulating in the Golgi (as verified by GT-YFP colocalisation; not shown). In contrast, upon co-expression of N and Gc, the localisation pattern of Gc changed from a reticular pattern to a perinuclear accumulation, in which N and Gc partially colocalised (Figure 4d-f). This was a strikingly different localisation as compared to the Golgi colocalisation of N and Gc (and Gn) when Gc was expressed from the glycoprotein precursor. Moreover, clusters of N as observed frequently throughout the cytoplasm during single expression were also less abundant in the presence of Gc, altogether suggesting an interaction between N and Gc.

FLIM analyses of interaction between N and the glycoproteins

As the localisation pattern of Gc seemed to change upon co-expression with N, and both proteins seemed to partially colocalise, our next step was to test if both proteins interacted. To this end, BHK21 cells were co-transfected with the constructs coding for an N-fluorophore fusion protein and either the Gn- or Gc-fluorophore fusion protein. Irrespective of the combination used, in repeated experiments a clear decrease in fluorescence lifetime of CFP was observed when N was co-expressed with Gc (Figure 5a-c). This decrease of CFP fluorescence lifetime (from 2600 ps to 2270 ps), visualised by red to yellow pseudocolours, was only observed at locations were N clearly accumulated and co-localised with Gc (Figure 5a-c). A change in CFP lifetime was never observed in cells co-expressing N and Gn (Figure 5d-f), nor in cells only expressing either of the proteins alone (results not shown).

FRET analysis by acceptor photobleaching

To further substantiate these observations, BHK cells were co-transfected with Gc-CFP and N-YFP or with Gn-CFP and N-YFP and analysed using acceptor photobleaching. Similarly as observed in FLIM experiments, a direct interaction, as evident from CFP fluorescence increase after photobleaching of YFP, could be observed between N and Gc in regions where N accumulated and co-localised with Gc. No CFP increase could be observed after photobleaching when N was co-expressed with Gn (Figure 6). The continuous decrease in CFP fluorescence intensity observed both before and after bleaching, was also observed in regions of the same cell which were not subjected to a bleaching pulse.

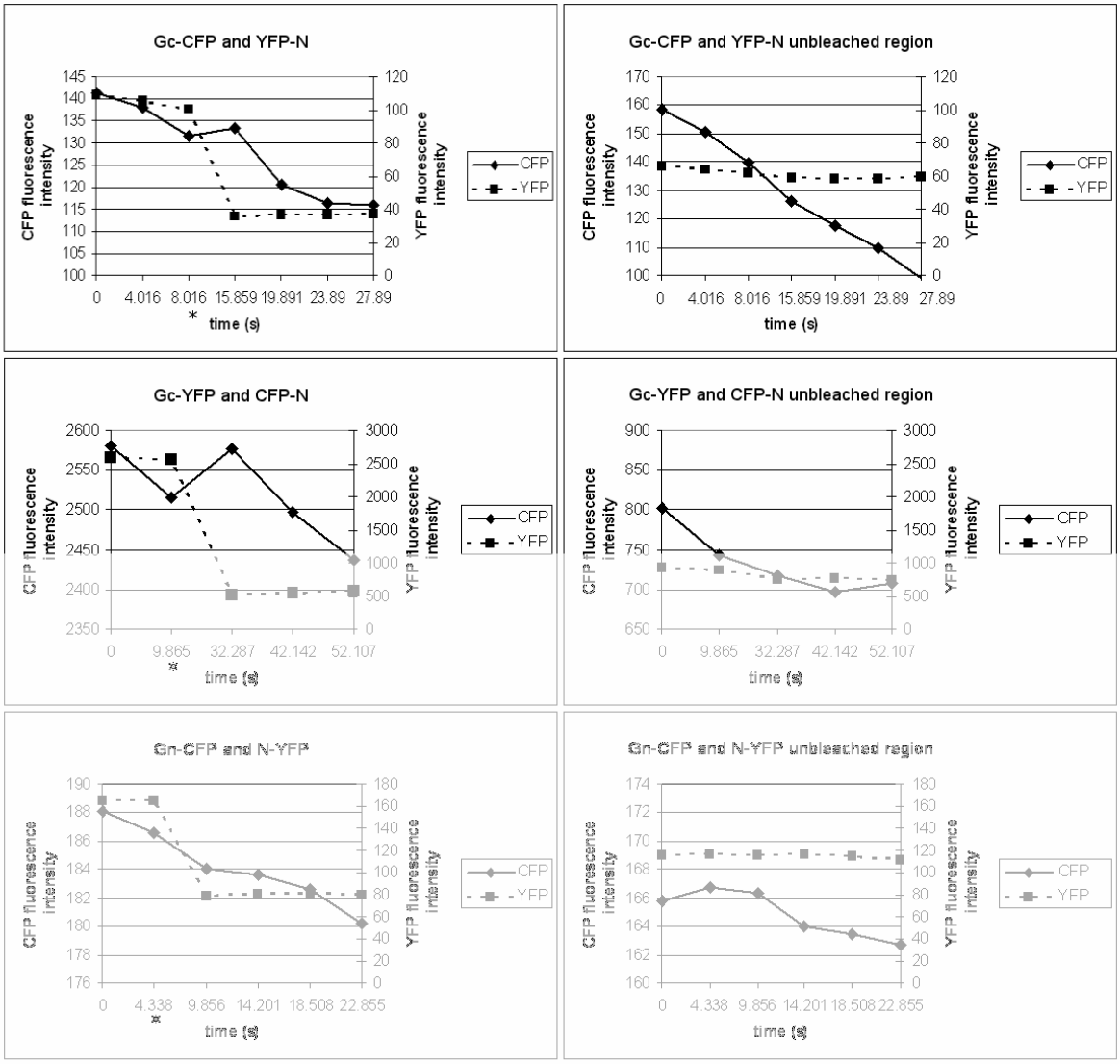


Figure 6. FRET between Gc-YFP and CFP-N as demonstrated using acceptor photobleaching. Cells were transfected with constructs coding for Gc-YFP and CFP-N (a, b), Gc-CFP and YFP-N (c, d) or Gn-CFP and N-YFP (e, f), and the CFP and YFP intensities (in arbitrary units) measured during the experiment. After the bleaching pulse (indicated by an asterisk), YFP intensities drop in the bleached region. A simultaneous increase in CFP intensity is observed only in bleached regions of cells transfected with Gc and N (a, c), but not with Gn and N (e) or in control regions in the same cell not subjected to photobleaching (b, d, f). A decrease in CFP fluorescence during the experiment is routinely observed in bleached as well as non-bleached regions of the cells.

Discussion

In order to shed more light on the interactions involved in the membrane acquisition of TSWV RNPs, CFP- and YFP- fusion constructs of N were co-expressed with Gc (G1) and Gn (G2) fusion constructs and cells were analysed for the in vivo occurrence of protein interactions. Using acceptor photobleaching and FLIM, interactions between N and Gc have been observed, but not between N and Gn. These results are the first experimental evidence for any bunyavirus indicating that the envelopment of RNPs by Golgi-membranes may require, or even be triggered by, the interaction of Gc and N. For other members of the Bunyaviridae, e.g. Uukuniemi virus, speculations have been made on interaction

between Gn and N based on the argument that the cytoplasmic tail of Gc, consisting of 5 amino acids only, would be too short for possible interactions with RNPs (Andersson *et al.*, 1997a). However, in the light of a functional homology of all bunyavirus Gc proteins (Cortez *et al.*, 2002) it seems likely that RNP envelopment involving Gc-N interactions described here applies to all bunyaviruses. The role of Gc during envelopment could then be similar to that of the matrix proteins of other ss(-) RNA viruses. Computer assisted binary and multiple sequence alignments have not revealed any significant sequence homologies between TSWV Gc ED and those of other bunyaviral Gc ED or matrix proteins of other ss(-)RNA viruses (data not shown). On the other hand, some structural similarity can be seen between the Uukuniemi Gc ED (KVKKS), a stretch in the TSWV Gc ED (amino acids 713-717; KSRSK) and the RNA binding domain of influenza A virus matrix protein (M1) that has been implied in its playing a role in the interaction with RNPs (amino acids 101-105; RKLKR) (Liu and Ye, 2002 and 2004). A similar stretch of positively charged amino acids can be observed in the ED of Gc proteins from representative members the Bunyaviridae, with the exception of Bunyamwera virus, where the domain may possibly arise from a discontinuous stretch of amino acids after folding.

The interaction observed for N and Gc raises the question of how this would fit into a model for TSWV particle assembly. Whereas earlier studies on transient co-expression of Gn and Gc in BHK21 cells indicated that Gc requires an interaction with Gn to become translocated to and retained in the Golgi system, data from this study showed an interaction between N and Gc, but not between N and Gn. During the co-expression and interaction of N and Gc a partial co-localisation was observed in perinuclear regions other than the Golgi, as verified by the Golgi marker GT, and similar to the unidentified perinuclear localisation observed for N during single expressions. Moreover, and strikingly, during co-expression of N and the glycoprotein precursor, N and Gc (and Gn) partially colocalised in the Golgi region. This altogether suggests that N-Gc interaction already takes place independently from Gn, with Gc likely anchored in the ER, after which the N protein sequesters Gc to a non-Golgi perinuclear region prior to becoming translocated to the Golgi region as a result from interaction between Gn and Gc. Experimental indications for this hypothesis have not been obtained so far.

Earlier transient expression studies have shown that the TSWV glycoproteins exhibit a similar trafficking behaviour to those of its animal-infecting counterparts (Kikkert *et al.*, 2001). Recently TSWV N was shown to localise at a perinuclear location, as similarly observed for several bunyaviruses (Andersson *et al.*, 2004b; Ravkov and Compans, 2001; Reichelt *et al.*, 2004), but the perinuclear localisation could not be easily explained within the context of virus assembly. For several bunyaviruses a co-localisation of N or RNPs with interferon-induced MxA in the perinuclear region was observed, suggesting MxA / GTPase-assisted sequestering and subsequent inactivation of viral N / RNP structures in the perinuclear region. Similarities with perinuclear aggregations formed by cells in response to misfolded protein have led to the suggestion that these aggregates could represent aggresomes (Andersson *et al.*, 2004b; Kochs *et al.*, 2002).

However, in view of the present findings regarding the perinuclear interaction between TSWV N and Gc, this hypothesis may be disputed as the perinuclear localisation and accumulation could well present a specific stage during particle assembly. The latter is being supported by observations for several large cytoplasmic DNA viruses where structural proteins were observed to concentrate in perinuclear foci called viral factories (Heath *et al.*, 2001; Jouvenet *et al.*, 2004; Rietdorf *et al.*, 2001). In these cases, viral factories and aggresomes have been shown to be related structures as they are both localised in the vicinity of the microtubule organising centre (MTOC) and require an active microtubular network for assembly. For TSWV, the perinuclear accumulation of N was also shown to depend on an intact microtubule network (chapter 3). Although the perinuclear localisation of TSWV N has not been confirmed to coincide with the MTOC yet, it is very well possible that TSWV and other bunyaviruses exploit the aggresome pathway to concentrate structural proteins at (intermediate) virus assembly sites as postulated for large cytoplasmic DNA viruses (Heath *et al.*, 2001).
Further support for the involvement of the aggresome pathway in virus assembly comes from the work on Mason-Pfizer monkey retrovirus (M-PMV), where M-PMV Gag protein localises to a pericentriolar domain identified as the MTOC in a microtubule and dynactin dependent manner. Here, assembly into immature capsids takes place. Upon co-expression with Env, exit of M-MPV Gag from the pericentriolar domain and transport to the site of virus assembly, i.e. the plasma membrane, is observed to take place (involving vesicular trafficking) (Sfakianos and Hunter, 2003; Sfakianos *et al.*, 2003). This last step shows an analogy with the rescue of TSWV N-Gc by Gn to the Golgi where particle envelopment takes place. If the aggresome pathway indeed applies to TSWV and the other bunyaviruses and the perinuclear accumulation and interaction of N and Gc represent a specific stage during particle assembly an intriguing question remains as to when, where and in what order interactions between the structural proteins occur in order to form new infectious enveloped virus particles.
Based on the data and arguments presented here, a model is postulated in which N or RNPs in a microtubule and actin dependent manner translocate to a perinuclear localisation (in mammalian cells) that may represent the MTOC. During this step, Gc, anchored at one end in the ER, would interact with the RNPs and mediate Golgi localisation through interaction with Gn. Subsequently, membrane acquisition takes place by wrapping as described earlier. Further studies are underway to analyse the interactions of TSWV Gn and Gc.

Acknowledgements
We are grateful to Dr. R. Y. Tsien for kindly providing the trans-Golgi marker GT-EYFP. This work was financially supported by the Netherlands organisation for Scientific Research, section Earth and Life Sciences (NWO/ALW) and the EU (grant HPRN-CT-2002-00262); the equipment was purchased owing to an NWO-Groot investment grant.

Chapter 5

The cytoplasmic domain of tomato spotted wilt virus Gn glycoprotein is required for Golgi retention and interaction with Gc

Abstract

Envelopment of tomato spotted wilt virus nucleocapsids takes place at the Golgi stacks of an infected cell. This is also the place where the two membrane glycoproteins Gn and Gc accumulate upon co-expression. The required Golgi retention signal has previously been demonstrated to reside within the Gn protein (Kikkert, M., Verschoor, A., Kormelink, R., Rottier, P. and Goldbach., R., 2001. J. Virol. 75:1004-1012) and has now been further defined using a series of progressive C-terminally truncated Gn proteins. The Golgi retention signal could be mapped to a stretch of 10 amino acids located on the cytoplasmic tail of Gn located 20 residues downstream the transmembrane domain. In addition, the intracellular distribution of chimeric proteins of Gc and Gn as well as chimeras of the glycoprotein of vesicular stomatitis virus (VSV-G) and Gn were studied and this demonstrated the requirement of the Gn transmembrane domain (TMD) for Golgi targeting. Since truncated Gn constructs lacking the C-terminal 20 amino acids that localised to the Golgi were no longer able to rescue Gc, the requirement of this domain for interaction with Gc could be demonstrated.

Marjolein Snippe, Leonie Smeenk, Rob Goldbach and Richard Kormelink
To be submitted

Introduction

Tomato spotted wilt virus (TSWV) is the type species of the genus *Tospovirus* within the family *Bunyaviridae*. Like all members of the *Bunyaviridae*, TSWV consists of enveloped, spherical particles containing a tri-partite RNA genome. The two glycoproteins that are present in the lipid envelope are encoded as a single precursor protein that is co-translationally cleaved into the N-terminal G2, from here on referred to as Gn, and C-terminal G1, from here on referred to as Gc. During viral infection of a plant cell, the two glycoproteins eventually accumulate in the Golgi complex (Kikkert *et al.*, 1999). Golgi stacks containing the two glycoproteins then wrap around viral ribonucleoprotein (RNP) complexes consisting of viral RNA, associated with nucleocapsid (N) protein and the putative viral RNA-dependent RNA polymerase to form doubly enveloped virus particles. These are thought to fuse with each other and with ER-derived membranes, resulting in the formation of large intracellular vesicles containing singly enveloped virus particles (Kikkert *et al.*, 1999). Heterologous expression studies using the mammalian Semliki Forest virus – BHK21 cell system have shown that upon co-expression of Gn and Gc, both proteins translocate to the Golgi complex. Upon separate expression though, only Gn is able to (partially) translocate to the Golgi complex, whereas Gc is retained in the ER, suggesting that Gc is rescued to the Golgi complex via heterodimerisation with Gn (Kikkert *et al.*, 2001). The same has been found for most bunyaviruses, the exception being the members of the genus *Hantavirus*, whose glycoproteins can exit the ER only when both are expressed in the same cell, after which they accumulate in the Golgi (Ruusala *et al.*, 1992; Shi and Elliott, 2002; Spiropoulou *et al.*, 2003). For a number of bunyaviruses the Golgi retention signal has been mapped to the cytoplasmic tail and / or the transmembrane domain (TMD) of the Gn protein (Andersson and Pettersson, 1998; Gerrard and Nichol, 2002; Matsuoka *et al.*, 1996; Shi *et al.*, 2004). Only for Crimean-Congo hemorrhagic fever nairovirus has the Golgi retention signal been mapped to the ectodomain of Gn (Bertolotti-Ciarlet *et al.*, 2005). Requirements for Golgi retention thus seem to differ among the different genera. In order to investigate the requirements for Golgi retention of the glycoproteins of TSWV, belonging to the only plant-infecting genus, we expressed a series of C-terminal deletion mutants of Gn in mammalian cells and analysed these for their intracellular behaviour. These experiments were complemented by studies on chimeric Gc, VSV-G and YFP constructs, respectively, harbouring C-terminal portions of Gn to map the Golgi retention signal and analyse the involvement of the Gn transmembrane domain.

Materials and methods

Cell culture

Baby hamster kidney (BHK21) cells were maintained at 37°C with 5% CO_2 in Glasgow MEM culture medium (Invitrogen) supplemented with 10%

foetal calf serum (FCS), 2.6 g/l tryptose phosphate broth (Sigma), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cell densities were kept at a maximum of 70 – 80% confluency.

Constructs

The pSFV1 vector (first described by Liljeström and Garoff, 1991), containing an *Nru*I linearisation site (designated pSFV1-N) was used for cloning and expression of the glycoproteins in BHK21 cells. For heterologous expression of glycoprotein mutants, the *Bam*HI site of the multiple cloning site was used to insert the genes. A schematic representation of the inserts is given in Figure 1; the sequences of the primers used for PCR amplification of the inserts are given in Table 1.

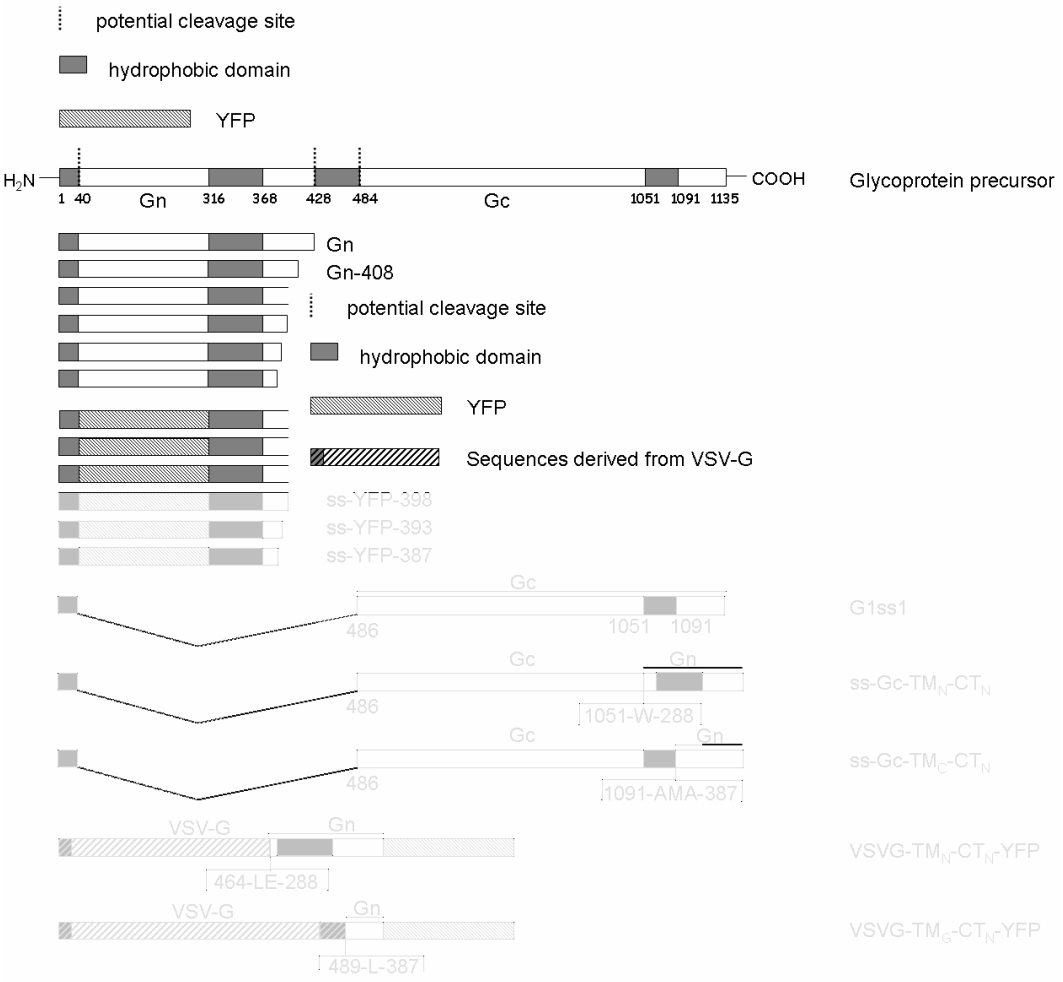


Figure 1. Constructs used in this study. Numbers refer to amino acid positions in the precursor protein. Numbers 464 and 489 in constructs VSVG-TMN-CTN-YFP and VSVG-TMG-CTN-YFP, respectively, refer to the amino acid positions in the VSV-G protein. Extra amino acids present at the junction sites are indicated in one-letter codes. For instance, in construct ss-Gc-TMC-CTN the last amino acid derived from Gc (amino acid 1091 of the precursor) is followed by the sequence Ala-Met-Ala and the Gn sequence starting from amino acid 387 of the precursor.
TMN, TMC and TMG refer to the TMDs derived from Gn, Gc and VSV-G, respectively. Likewise, CTN is the Gn cytoplasmic tail.

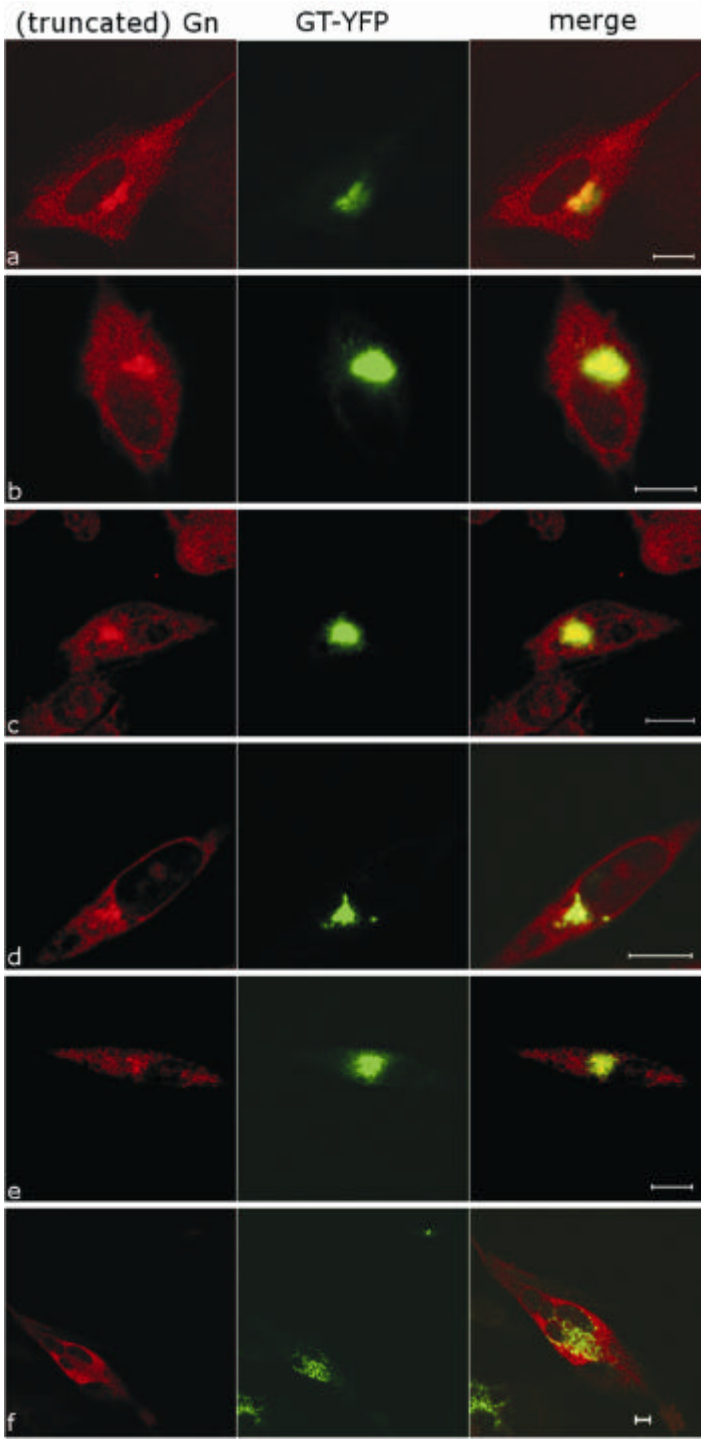
Constructs encoding full length Gn and Gc in the pSFV vector were made previously (Kikkert *et al.*, 2001; constructs pSFV-G2 and pSFV-G1b, respectively). The TSWV Gn and Gc genes were amplified from TSWV isolate BR-01 by PCR and fused at their carboxy termini to YFP as described previously (chapter 4). The resulting proteins are referred to as Gn-YFP and Gc-YFP, respectively.

Gn start	cccggatcc**ATG**AGAATTCTAAAACTACTAGAACTAGTGG
Gn stop	cccggatcc**tca**TTCCATGCTAGTCCACT
Gn 408 stop	cccggatccctca**tca**catatgGGGACACTCTGAAGAATG
Gn 403 stop	cccggatcc**tta**ATGCTCTTTTGAAGCTTT
Gn 398 stop	cccggatcc**tta**TTTGCTTTTGTTGCAAATGCAGAC
Gn 393 stop	cccggatcc**tta**AATGCAGACTTTAGTACA
Gn 387 stop	cccggatccctca**tca**catatgGTCACCACACAAGAGATTG
Gn ss up	gggcat**atg**AGCATCTGTGGCTCTGAAG
Gn tmd down	gggccatggAAATCAGCCAAAATACC
Gn ct down	gggccatggCCGAGTGTACTAAAGTCTGCATT
Gc lum up	gggccatggACTCTTAATATAATCCCAGAA
Gc tmd up	gggccatggCTCCTTTACAAATGGATGTCAG
Vsv start	cccggatcc**ATG**AAGTGCCTTTTGTAC
Vsv lum up	gggctcgagAGAGCTTTTCCAACTAC
Vsv tmd up	gggctcgagGCAAAGATGGATACC
Gn tmd down Xho	gggctcgagAAATCAGCCAAAATACC
Gn ct down Xho	gggctcgagTGTACTAAAGTCTGC
Yfp stop	cccggatccatatg**TTA**CTTGTACAGCTCGTCCATGCC

Table1. Primers used for the constructs in this work. Nucleotides in bold indicate start and stop codons; underlined nucleotides form the restriction sites used. Nucleotides identical to the template are capitalised.

A first series of (mutant) glycoprotein constructs, consisting of progressive deletion mutants of Gn, was obtained by PCR amplification using primer “Gn start” and Gn xxx stop” in which xxx refers to the last amino acid of Gn included in the construct (Table 1). All these constructs contain a translational stop codon at the end of the coding sequence, followed by a *Bam*HI restriction site.

For a second series of constructs, the ectodomain of Gn was replaced by a pH-insensitive YFP mutant (generously provided by G. van der Krogt, Molecular Biology department, Wageningen University), resulting in full length YFP flanked at its amino terminus by the signal sequence from Gn and at its carboxy terminus by the TMD and (truncations of the) cytoplasmic tail of Gn (Figure 1). The initial construct, termed ss-YFP-Gn, was produced by ligating the full length Gn gene into the *Bam*HI site of the pSK vector to be used as a template for a PCR using the Expand Long Template System (Roche) with a primer annealing to the signal sequence and extending upstream (designated Gn ss up), and one annealing at the start of the TMD and extending downstream (Gn tmd down). Both primers contained restriction sites that were used to subsequently insert PCR-amplified YFP.



Progressive deletion mutants of this construct were obtained by PCR amplification using the same primer sets as those used for the Gn deletion constructs. The resulting chimeric constructs are listed in Figure 1.

A third series of constructs was based on Gc in which the cytoplasmic tail, with or without the transmembrane domain, was exchanged with the corresponding part of Gn. A construct in which the N-terminal signal sequence of the precursor was linked in frame to the Gc coding sequence has been described previously (Kikkert *et al.*, 2001; construct pSFV-G1ss1). This construct was used in a PCR reaction with primers “Gn start” and “Gc lum up” and the PCR product was linked at its 3’ end to the C-terminal coding sequence of Gn, obtained from a PCR reaction on full length Gn

Figure 2. Expression patterns of C-terminally truncated TSWV Gn proteins in BHK cells. BHK21 cells were transfected with plasmids encoding Gn (wt) (a); Gn-408 (b); Gn-403 (c); Gn-398 (d); Gn-393 (e) or Gn-387 (f). In all cases, cells were cotransfected with a construct coding for the trans-Golgi marker galactosyl transferase fused to YFP (GT-YFP). Gn was detected by indirect immunofluorescence staining using rhodamin-Red (left panels). GT-YFP was detected directly using filters for GFP (green; middle panels). The panels on the right are merged images of the rhodamin and the YFP signals. For Gn truncations up to amino acid 398 (panels a-d), a clear accumulation in the Golgi region is visible. Panel e (construct Gn-393) shows Golgi accumulation to a limited extent. In panel f (construct Gn-387), the signal is reticular with no detectable Golgi accumulation, representing ER localisation. Bars indicate 10 µm.

using primers “Gn tmd down” and “Gn stop”. The resulting construct contained the signal sequence of the TSWV glycoprotein precursor linked to the lumenal domain of Gc and the transmembrane domain and cytoplasmic tail of Gn and was named ss-Gc-TM$_N$-CT$_N$. Likewise, a construct in which the TMD was derived from Gc instead of Gn was made combining two PCR fragments obtained by using primers “Gn start” and “Gc tmd up” on pSFV-G1ss as a template and “Gn ct down” and “Gn stop” on full length Gn as a template. The resulting clone was named ss-Gc-TM$_C$-CT$_N$ (see Figure 1).
In analogy, similar constructs were made with VSV-G as a core in which VSV-G stands for the glycoprotein of vesicular stomatitis virus. Due to the fact that all available antisera were raised against the C-terminal domain of VSV-G, chimeric constructs lacking the C-terminal domain were visualised by a C-terminal fusion to YFP. Previously it had been shown that a C-terminal fluorophore fusion does not influence the localisation of either Gn (chapter 4) or VSV-G (Dalton and Rose, 2001). A VSV-G construct, referred to as VSVG-TM$_N$-CT$_N$-YFP, in which the transmembrane domain and cytoplasmic tail were swapped for the ones from TSWV Gn was made by a three point ligation using two PCR fragments. These were made using primers “Vsv-start” and “Vsv lum up” on VSV-G and “Gn tmd down Xho” and “Yfp stop” on Gn-YFP. A next VSV-G construct, denoted VSVG-TM$_G$-CT$_N$-YFP, in which only the cytoplasmic tails of the two proteins were swapped, was made using primers “Vsv-start” and “Vsv tmd up” on VSV-G and “Gn ct down” and “Yfp stop” on Gn-YFP, followed by a three point ligation (Figure 1).
Full length VSV-G fused at its C-terminus to GFP (here referred to as VSVG-GFP) was kindly provided by Dr. F. van Kuppeveld and was constructed as described elsewhere (Toomre *et al*., 1999).
All constructs were expressed as described previously (chapter 4). In brief, capped RNA was synthesised *in vitro* using the SP6 mMessage mMachine (Ambion) and electroporated into BHK21 cells using the GenePulser II (BioRad) by applying two square wave pulses of 650 V and a duration of 5 ms with an interval of 5 seconds.
The Golgi marker Galactosyl Transferase-YFP (GT-YFP) was kindly provided by Dr. R. Tsien.

Indirect fluorescence analysis
Cells attached to coverslips were fixed in 96% ethanol for 20 minutes, washed with PBS and blocked with 5% BSA in PBS for 1 hour. Polyclonal antiserum was diluted in 1% BSA in PBS and incubated on the cells for 1 hour at room temperature. The cells were washed with PBS and incubated with secondary antibody conjugated to FITC (fluorescein isothiocyanate, Molecular probes) or Rhodamin-Red (Nordic) for 1 hour at room temperature. Cells were washed with PBS and examined in a Zeiss LSM510 confocal laser scanning microscope. Cross-talk free imaging was assured by using a multi-tracking mode.

Results

Intracellular localisation of truncated Gn proteins

Wild type TSWV Gn (residues 1 to 428 from the glycoprotein precursor; see Figure 1), expressed in mammalian cells using the Semliki Forest virus (SFV) expression system, had previously been demonstrated to localise to the Golgi complex where it is retained (Kikkert *et al*., 2001), indicating the presence of a Golgi retention signal in this protein. To map the domain responsible for Golgi retention of TSWV Gn, a series of C-terminal progressive deletion mutants (Figure 1; materials and methods) were made and expressed in baby hamster kidney (BHK21) cells using the SFV expression system. The intracellular localisation of the proteins was determined by indirect immunofluorescence analysis. These analyses show that truncated Gn proteins containing at least 30 amino acids of the C-terminal cytoplasmic domain (construct Gn-398) are still capable of becoming transported to and retained at the Golgi complex (Figure 2a-d). For construct Gn-393, containing 25 amino acids of the cytoplasmic tail, Golgi localisation was observed in approximately 60% of the cells. In the remaining 40% a reticular distribution was seen indicative of localisation in the endoplasmic reticulum (ER). Constructs containing less than 25 amino acids of the cytoplasmic domain were unable to exit the ER (Figure 2f).

Intracellular localisation of chimeric YFP-Gn proteins

To support the observation that the Golgi retention signal is located on the first 30 residues of the cytoplasmic tail, and to exclude the role of the lumenal domain in Golgi localisation, the lumenal domain of wild type as well as truncated Gn proteins was replaced by a pH-insensitive form of the yellow fluorescent protein YFP (Figure 1; materials and methods). Expression analyses of these constructs in BHK21 cells showed Golgi localisation for the full length construct (Figure 3a) and for the truncated proteins, provided these contained at least 30 amino acids of the cytoplasmic tail (Figure 3b-d). These results thus confirm the localisation profiles of the truncated wild type Gn proteins. Similarly as observed for Gn-393, construct ss-YFP-393, containing only 25 amino acids of the cytoplasmic tail, showed a partial Golgi and ER localisation. Constructs derived from ss-YFP-Gn containing less than 25 amino acids of the cytoplasmic tail were unable to exit the ER (Figure 3f). All together these data suggest that the cytoplasmic tail of TSWV Gn contains a Golgi retention signal and the lumenal domain is dispensable for this.

Intracellular localisation of chimeric Gc-Gn proteins

The results described above demonstrate that for Golgi retention of Gn, the first 30 residues of the cytoplasmic tail are essential. To substantiate this observation and simultaneously analyse the requirement of the Gn transmembrane domain for Golgi retention, constructs coding for chimeric Gc-Gn proteins were made and cloned into the SFV vector, expressed in

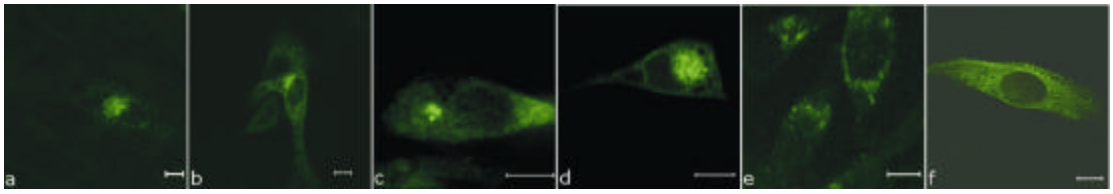


Figure 3. Expression patterns of chimeric YFP-Gn proteins in BHK cells. BHK21 cells were transfected with plasmids encoding ss-YFP-Gn (a); ss-YFP-408 (b); ss-YFP-403 (c); ss-YFP-398 (d); ss-YFP-393 (e) or ss-YFP-387 (f). The proteins were detected by direct fluorescence. For Gn truncations up to amino acid 398 (panels a-d), a clear accumulation in a perinuclear region is observed. This localisation was confirmed to be the Golgi by co-localisation with Golgi marker p58 (not shown). For construct ss-YFP-393 (panel e) Golgi staining was visible but less clear. Construct ss-YFP-387 displayed no Golgi accumulation but showed a reticular (ER) distribution. Bars indicate 10 µm.

BHK21 cells and detected by indirect immunofluorescence. The constructs were based on the core of Gc in which the cytoplasmic tail with or without the TMD was exchanged for the analogous part of Gn. The transmembrane domain was derived from Gn in construct ss-Gc-TM$_N$-CT$_N$ and from Gc in construct ss-Gc-TM$_C$-CT$_N$ (Figure 1).
Upon expression, ss-Gc-TM$_N$-CT$_N$ localised primarily to the Golgi complex (Figure 4a). In contrast, ss-Gc-TM$_C$-CT$_N$ was unable to exit the ER and showed a reticular distribution in all cells examined (Figure 4b). This protein displayed a punctuate pattern routinely observed upon expression of wild type TSWV Gc in mammalian cells (Figure 4c). These results clearly suggest that next to a domain on the cytoplasmic tail, the Gn TMD is required for Golgi localisation.

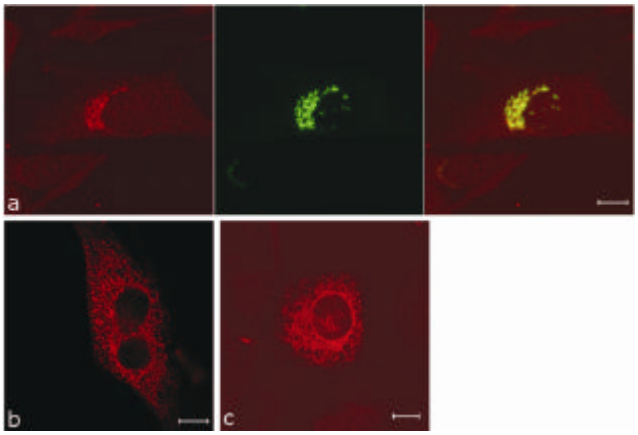


Figure 4. Expression patterns of chimeric Gc-Gn proteins in BHK cells. BHK21 cells were transfected with plasmids encoding ss-Gc-TMN-CTN (a); ss-Gc-TMC-CTN (b) or Gc (wt) (c). In panel a, a cotransfection with trans-Golgi marker GT-YFP is shown (left panel: ss-Gc-TMN-CTN; middle: GT-YFP; right: merge). Construct ss-Gc-TMN-CTN (panel a) localised predominantly to the Golgi area whereas construct ss-Gc-TMC-CTN (panel b) showed a clear reticular expression pattern resembling that of wild type Gc (panel c). Bars indicate 10 µm.

Intracellular localisation of chimeric VSV-G-Gn proteins

To further support the involvement of the TMD and cytoplasmic domain of Gn in Golgi retention, chimeric proteins were expressed consisting of VSV-G harbouring the cytoplasmic tail with or without the trasmembrane

domain of TSWV Gn as schematically depicted in Figure 1. The VSV-G protein is a well characterised protein that normally localises to the plasma membrane of mammalian cells. To facilitate detection of VSV-G constructs lacking its own cytoplasmic tail, YFP was fused to the carboxy terminal end (materials and methods; Figure 1). For comparison with wild type VSV-G, VSVG-GFP was used.

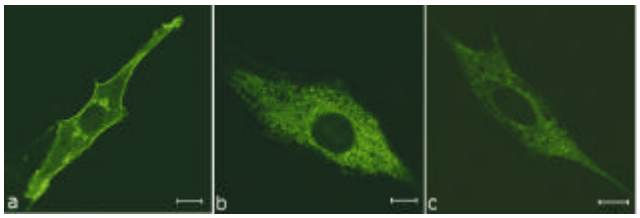


Figure 5. Expression of chimeric VSVG-Gn proteins in BHK cells. BHK21 cells were transfected with plasmids encoding VSVG-GFP (a); VSVG-TMN-CTN (b) or VSVG-TMG-CTN (c). Proteins were detected directly using GFP filter settings. Whereas VSVG-GFP accumulates in the plasma membrane (a), both Gn chimeras show a predominant reticular staining, although VSVG-TMG-CTN could occasionally been seen to accumulate in the Golgi (not shown). Bars indicate 10 µm.

Whereas VSVG-GFP, as expected, showed accumulation primarily at the cell membrane and minor accumulation in the Golgi complex (Figure 5a) as previously reported for both wild type VSV-G and its GFP fusion (Dalton and Rose, 2001; Rose and Bergman, 1982), chimeric VSVG-Gn-YFP proteins did not reach the plasma membrane at all. Instead, VSVG-TM$_N$-CT$_N$-YFP was observed in a very clear reticular pattern (Figure 5b). Cells expressing VSVG-TM$_G$-CT$_N$-YFP (Figure 5c) revealed the same picture but in approximately 15% of the cells some Golgi localisation was observed (not shown).

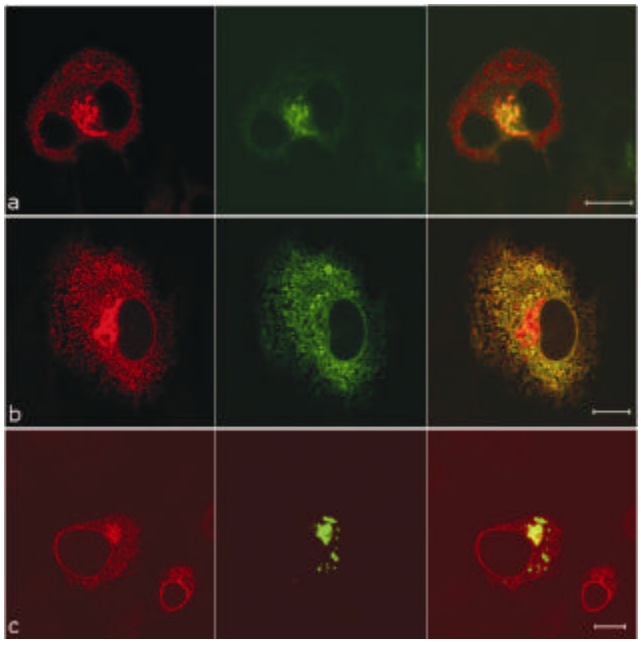


Figure 6. Rescuing of Gc to the Golgi by wild type, truncated or chimeric Gn protein. BHK21 cells were co-transfected with plasmids encoding Gn (wt) and Gc-YFP (a); Gn-408 and Gc-YFP (b) or ss-YFP-Gn and Gc (wt) (c). Proteins fused to YFP were detected directly and are shown in green (middle panels); proteins Gn, Gc and Gn-408 are not fused to a fluoro-phore and were detected by indirect immunofluorescence using rhodamin-Red (left panels). The panels on the right are merged panels of the rhodamin and YFP signals. Both full length Gn (panel a, left) and ss-YFP-Gn (panel c, middle) are capable of rescuing Gc to the Golgi. Construct Gn-408 (panel b, left), lacking the last 20 amino acids of the Gn cytoplasmic tail, although localising to the Golgi, is unable to rescue Gc-YFP as is apparent from the reticular pattern of this protein (panel b. middle). Bars indicate 10 µm.

Rescuing of TSWV Gc from ER to Golgi

To investigate if C-terminally truncated Gn proteins were still able to rescue Gc from the ER to the Golgi complex and to analyse the involvement of the lumenal domain of Gn herein, truncated Gn proteins or chimeric ss-YFP-Gn were co-expressed with Gc-YFP and Gc, respectively, and analysed for Gc localisation. Whereas wild type Gn efficiently rescued Gc-YFP to the Golgi complex (Figure 6a), deletion of 20 amino acids at the C-terminus of Gn completely abolished the rescuing capacity (Figure 6b). Surprisingly, this truncation (Gn-408) did not interfere with the capacity of Gn to localise to the Golgi but Gc expressed in the same cell was unable to leave the ER. Moreover, when wild type Gc was co-expressed with ss-YFP-Gn, Gc was found to colocalise with ss-YFP-Gn in the Golgi (Figure 6c), altogether suggesting that for the rescuing of Gc and its interaction with Gn the presence of the carboxy-terminal half of the cytoplasmic domain of Gn is essential and that the lumenal domain is not involved in rescuing or interaction.

Discussion

The results presented here demonstrate that the Golgi retention signal of the TSWV glycoproteins, required for TSWV envelope acquisition during plant infection, is located in the cytoplasmic tail of Gn in a domain of 10 amino acids starting 25 residues downstream the TMD. Research performed on related animal bunyaviruses has mostly led to similar conclusions, providing support for the idea that signals involved in intracellular targeting are conserved among plant, mammalian and insect cells. An exception is formed by members of the *Hantavirus* genus, whose glycoproteins are only seen to translocate to the Golgi apparatus if the two proteins are coexpressed in the same cell (Ruusala *et al*., 1992; Shi and Elliott, 2002). So far, no consensus sequence has been found for the Golgi retention signal of the different bunyaviruses but for several members a short stretch of amino acids downstream the TMD is required. For TSWV Gn the first 25 to 30 amino acids of the cytoplasmic tail, together with the Gn transmembrane domain, were shown to be essential for Golgi localisation as demonstrated by data from truncated proteins together with data from Gc chimeras harbouring these sequences. Removal of these sequences does not lead to translocation of the protein to the plasma membrane, as would be expected for proteins following the secretory pathway, but rather to retention in the ER, suggesting this domain does not function as a pure Golgi retention signal, but additionally is involved in facilitating ER exit.

Similar results were reported for RVFV Gn, where both the first 28 residues of the 73 amino acid cytoplasmic tail and the TMD were necessary and sufficient for Golgi localisation of either Gn or chimeric GFP. Removal of more than 58 C-terminal amino acids resulted in ER arrest (Gerrard and Nichol, 2002). For Punta Toro phlebovirus the first 10

amino acids of the Gn cytoplasmic tail, preceded by its transmembrane domain, are sufficient for Golgi retention (Matsuoka *et al.*, 1994). When this truncation was enlarged, leaving only one or two amino acids downstream the TMD, or when residues of this domain were mutated, Gn was transported to the plasma membrane, indicating the first 10 amino acids of the cytoplasmic tail are not required for ER exit, but essential for Golgi retention (Matsuoka *et al.*, 1994, 1996). For TSWV Gn, the cytoplasmic tail is not only required for Golgi retention, but also prevents ER arrest. Whether this ER arrest is due to the presence of an ER retention signal remains to be investigated.

In contrast to TSWV and Punta Toro virus, Uukuniemi phlebovirus Gn protein only requires the cytoplasmic tail for Golgi retention. Its cytoplasmic tail is able to translocate soluble GFP to the Golgi complex and retain it there. In addition, the ectodomain and transmembrane domain of CD4 fused to the cytoplasmic tail of Uuk Gn accumulated in the Golgi instead of reaching its default destination, the plasma membrane. On top of this, even a 30 amino acid fragment of the cytoplasmic tail of Uuk Gn, starting from 10 residues downstream the transmembrane domain, was still targeted to the Golgi apparatus (Andersson and Pettersson, 1998; Andersson *et al.*, 1997b). For Bunyamwera orthobunyavirus, the Golgi retention signal solely resides in the transmembrane domain, rather than in the cytoplasmic tail (Shi *et al.*, 2004). For Crimean-Congo hemorrhagic fever nairovirus, the situation is markedly different, as domains for both Gn-Gc interaction and Gn Golgi localisation have been mapped to the lumenal domain (Bertolotti-Ciarlet *et al.*, 2005).

Altogether, the mechanism of Golgi retention of the bunyavirus glycoproteins is still unclear and seems different for members of different genera, although variations in the definition of the transmembrane domain may also account for some of the discrepancies observed. For many bunyaviruses the cytoplasmic domain seems to be involved in ER exit and / or Golgi retention, something which is not uncommon for viral glycoproteins (Bültmann *et al.*, 2001; Ortiz de Zarate *et al.*, 2004; Rose and Bergmann, 1983; Thomas and Roth, 1994). Due to the absence of any primary sequence homology it is unclear if specific amino acid sequences are responsible for the observed Golgi retention.

Both VSV-G chimeras were unable to exit the ER, suggesting the context of the Gn TMD and cytoplasmic tail is important for Golgi trafficking and further. Only the protein containing the TMD of VSV-G (VSVG-TM$_G$-CT$_N$-YFP) was sometimes observed to accumulate in the Golgi. These observations contrast the observations for the chimeric Gc proteins, in which Golgi localisation was observed only for ss-Gc-TM$_N$-CT$_N$, containing the TMD of Gn. It should be noted here that an important difference between the two 'core' proteins (Gc and VSV-G) exist in their intracellular localisation: VSV-G normally translocates to the plasma membrane, i.e. exits the ER and passes through the Golgi whereas Gc remains in the ER. Thus, translocation from ER to Golgi could be mediated

partially by the TMD of VSV-G while for the final step (Golgi to plasma membrane) signals on the cytoplasmic tail are known to play a crucial role (Rose and Bergmann, 1983). Alternatively, exchanging the TMD and / or cytoplasmic tail with Gn may have hindered correct folding of the lumenal domain of VSV-G thereby abolishing ER exit.

Chimeric Gn containing the lumenal domain of Gc (ss-Gc-TMC-CTN) showed a punctuate pattern typical for TSWC Gc that shows some resemblance to ER exit sites (Hammond and Glick, 2000; Nishimura *et al.*, 1999). ER exit sites are specific sites on the ER where COP-II proteins collect their cargo for Golgi transport. A DxE motif (Asp-x-Glu, where x is any amino acid) has been demonstrated to function as a COP-II recruitment signal in a number of viral and non-viral proteins (Epping and Moye-Rowley, 2002; Mossessova *et al.*, 2003; Nishimura *et al.*, 1999; Votsmeier and Gallwitz, 2001). Sequence analysis revealed a DxE motif on the cytoplasmic tail of TSWV Gc, which may account for concentration of Gc at ER-exit sites. Although addition of this motif to the ER resident a subunit of the T-cell receptor a is sufficient for efficient ER export, for the yeast ABC transporter Yor1p additional signals are necessary to allow its exit from ER and to eventually reach the plasma membrane (Nishimura *et al.*, 1999; Epping and Moye-Rowley, 2002). A similar explanation seems to apply for TSWV Gc where such sequences may be provided *in trans* by Gn.

Next to Golgi retention, the TSWV Gn cytoplasmic tail, and more specifically the ultimate carboxy-terminal 20 amino acids, seem involved in heterodimer formation with Gc, since truncated Gn (Gn-408) still localising to the Golgi complex was no longer capable of rescuing Gc. Furthermore, the chimeric protein ss-YFP-Gn, containing the Gn signal sequence, trans membrane domain and cytoplasmic domain of Gn (but in which the lumenal domain has been replaced by YFP), was also able to efficiently rescue Gc to the Golgi. Similar functions have been suggested for Bunyamwera Gn. Although in a first report (Shi *et al.*, 2004), Golgi rescuing of Gc protein by Gn was only abolished when the entire Gn cytoplasmic tail and the transmembrane domain were deleted, more recently it was demonstrated that interaction between Bunyamwera virus Gn and Gc occurs through interaction of the cytoplasmic tails of the two proteins (Elliott, pers. comm.). For La Crosse orthobunyavirus, the interaction between Gn and Gc has also been suggested to involve the transmembrane domains and / or cytoplasmic tails (Pekosz and González-Scarano, 1996). On the other hand, for Punta Toro virus heterodimer formation between the two glycoproteins does not require the C-terminal domain of Gc (Chen and Compans, 1991). Further indications for the involvement of the C-terminal domains of the two TSWV proteins in heterodimer formation lie in the observation that upon co-expression of Gc-YFP and Gn-CFP (or in case of the inverse combination, i.e. Gc-CFP and Gn-YFP), the two fluorophores were never detected in the same cell (unpublished results). This could have been caused by Gc-Gn interactions

interfering with proper folding of the nearby fused fluorophores (i.e. at the carboxy termini of the glycoproteins), thus abolishing fluorescence. The recently developed method of bimolecular fluorescence complementation could provide a useful alternative to overcome this problem while studying protein interactions.

Acknowledgements
We thank Dr. R. Y. Tsien for kindly providing the trans-Golgi marker GT-EYFP, G. N. M. van der Krogt for the pH-insensitive form of YFP and Dr. A. G. P. Oomens for the plasmid encoding VSV-G. We are grateful to Dr. F. J. M. van Kuppeveld for providing the plasmid encoding VSVG-GFP and for fruitful discussion. We thank Dr. R. M. Elliott for sharing his unpublished results. This work was financially supported by the Netherlands organisation for Scientific Research, section Earth and Life Sciences (NWO/ALW) and by EU- RTN grant HPRN-CT-2002-00262.

Chapter 6

The Gc glycoprotein of tomato spotted wilt virus, a plant virus, induces cell fusion of mammalian cells

Abstract
Bunyaviral glycoproteins induce cell fusion in animal cell culture, which reflects the requirement for induction of viral and host membrane fusion prior to RNP release in the cytoplasm of a newly infected cell. Here, we report that the Gc glycoprotein of TSWV, a virus replicating not only in plants but also in its insect vector thrips, is capable of inducing cell fusion of mammalian cells. This finding is discussed in the context of fusion capacities of animal-infecting relatives of TSWV.

Marjolein Snippe, Rob Goldbach and Richard Kormelink
To be submitted

Introduction

Tomato spotted wilt virus (TSWV) is the type species of the genus *Tospovirus* within the family of *Bunyaviridae*. Tospoviruses are the only viruses in this family using plants as a host, whereas the other members infect mammals. Typical for bunyaviruses are the spherical, enveloped virus particles containing a segmented single strand RNA genome of negative or ambisense polarity. The viral envelope contains two types of viral glycoproteins, denoted Gn and Gc, where n and c refer to the N- and C-terminal coding position in the common precursor (Elliott, 1996). The glycoproteins are thought to play an important role in virus assembly where the actual envelope acquisition is thought to be triggered by the interaction of (one of the) glycoproteins with newly formed ribonucleoproteins (RNPs). Moreover, the glycoproteins act as viral attachment proteins to enable receptor-mediated endocytosis into mammalian cells. For TSWV, the glycoproteins have been hypothesised to act as viral attachment proteins during receptor mediated entry of thrips midgut epithelium cells (Bandla *et al*., 1998; Kikkert *et al*., 1998). For all bunyaviruses, irrespective of their host, Gn and Gc are assumed to be functionally equivalent, which is being supported by cell biological studies as well as sequence alignments where tospovirus and orthobunyavirus Gc proteins share a significant homology in a small stretch of amino acids (Figure 1; Cortez *et al*, 2002; Kormelink *et al*., 1992a; Lappin *et al*., 1994).

Figure 1. Alignment of Bunyavirus Gc proteins. The conserved region of Tospovirus and Orthobunyavirus Gc proteins was aligned with the corresponding region from the representatives of the other genera (*Hantavirus*, *Phlebovirus* and *Nairovirus*, respectively). Sequences start at the following amino acids, counted in the precursor protein: tomato spotted wilt virus (accession number JQ1928): 683; impatiens necrotic spot virus (B42544): 660; gloxinia virus (AAC15466): 664; groundnut bud necrosis virus (NP_619703): 664; groundnut ringspot virus (AAU10600): 685; Bunyamwera virus (P04505): 1025; La Crosse virus (P09612): 1033; germiston virus (P12430): 1029; Cache Valley virus (AAF33115): 1026; Tahyna virus (AAF71257): 1032; Hantaan virus (GNVUH7): 727; Rift Valley fever virus (VGVURF): 799; Crimean Congo hemorrhagic fever virus (NP_950235): 1157. The boxed area is the predicted fusion loop found by Garry and Garry (2004).

Upon feeding of thrips in their larval L1 and L2 stages on TSWV infected plants, virus particles enter the midgut epithelium cells and after replication disseminate to the other thrips organs, specifically to the surrounding muscle cells and the salivary glands. In the latter organ, virus particles are observed that finally become secreted into the salivary gland ducts (de Assis Filho *et al*., 2002; Nagata *et al*., 1999, 2002; Ullman *et al*., 1992; Wijkamp *et al*., 1993). The virus is being spread to other plants through feeding of viruliferous thrips on healthy plants.

TSWV infection of thrips cells is thought to involve receptor-mediated uptake. The function of viral attachment protein has been postulated to be carried out by Gn (Kikkert *et al.*, 1998; Whitfield *et al.*, 2004), although other experimental data seem to point to the involvement of Gc in this role as well (Bandla *et al.*, 1998; Medeiros *et al.*, 2000). The function of viral fusion protein has not been assigned so far to either of the two glycoproteins, although these proteins are the most likely candidates for this process. In this work, we have investigated the involvement of Gn and / or Gc in membrane fusion and we show that the function of fusion protein is performed by Gc.

Materials and methods

Cell culture

Baby hamster kidney cells (BHK21) were maintained at 37°C with 5% C2 in Glasgow MEM culture medium (Invitrogen) supplemented with 10% foetal calf serum (Invitrogen), 2.6 g/l tryptose phosphate broth (Sigma), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cell densities were kept at a maximum of 70-80% confluency.

Constructs

Both TSWV glycoproteins were cloned separately into the Semliki Forest virus expression system (pSFV) as described by Kikkert *et al.* (2001; construct G1b) and depicted in Figure 2. In brief, the constructs were amplified from a cDNA clone of TSWV isolate BR01 using specific primers containing a start codon at the 5' end and a translational stop codon at the 3' end, flanked by *Bam*HI sites for cloning into pSFV.

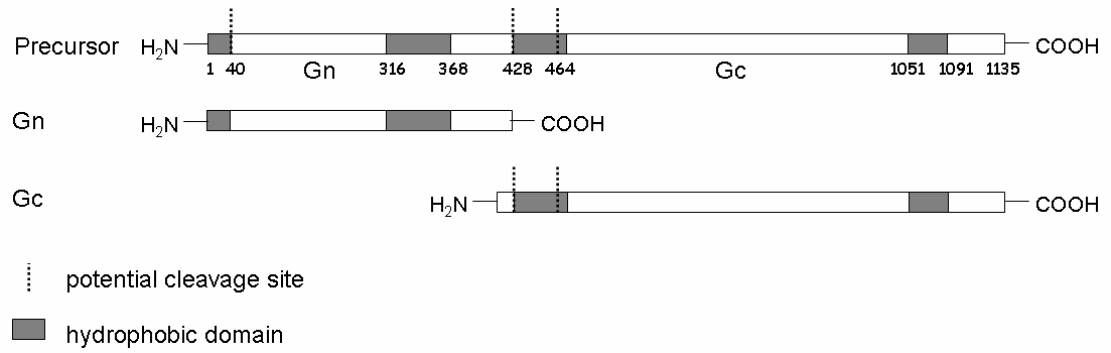


Figure 2. Schematic representation of the TSWV glycoprotein precursor and the constructs used in this chapter. Numbers refer to the amino acid sequence.

Semliki Forest virus expression system

The constructs were linearised with restriction enzyme *Nru*I, purified with a proteinase K treatment followed by phenol extraction and transcribed *in vitro* using the SP6 mMessage mMachine (Ambion). The RNA was used for transfection by electroporation in an electroporation buffer (2 mM HEPES, 15 mM potassium phosphate buffer pH 7.2, 250 mM mannitol, 1 mM $MgCL_2$) applying 2 pulses of 650 V and a duration of 5 ms with an interval of 5 seconds as described earlier (chapter 4).

Low pH treatment
Transfected cells were incubated for 24 hours and incubated in PBS at pH 5.8, 6.5 or 7.2 for 5-10 minutes. After this, the PBS was removed, fresh medium was added to the cells and the cells were incubated for another 16-40 hours.

Indirect fluorescence analysis
Cells were harvested 24 to 48 hours post transfection and subjected to indirect fluorescence analysis as described earlier (chapter 4) and cells were analysed using a Laborlux microscope (Leica) on which a digital camera was mounted (Coolsnap; Photometrics).

Results and discussion
To investigate the involvement of a fusion capacity of Gn, Gc or both in final RNP release into the cytoplasm, the proteins were expressed separately in mammalian cells. Upon expression of Gc in BHK21 cells, large syncytia were frequently observed (Figure 3a). Upon expression of Gn, sometimes small syncytia were observed at a very low frequency (Figure 3b). Syncytia formation induced by Gc was not affected upon co-expression with TSWV N (data not shown). No syncytia were observed upon co-expression of Gn and Gc.

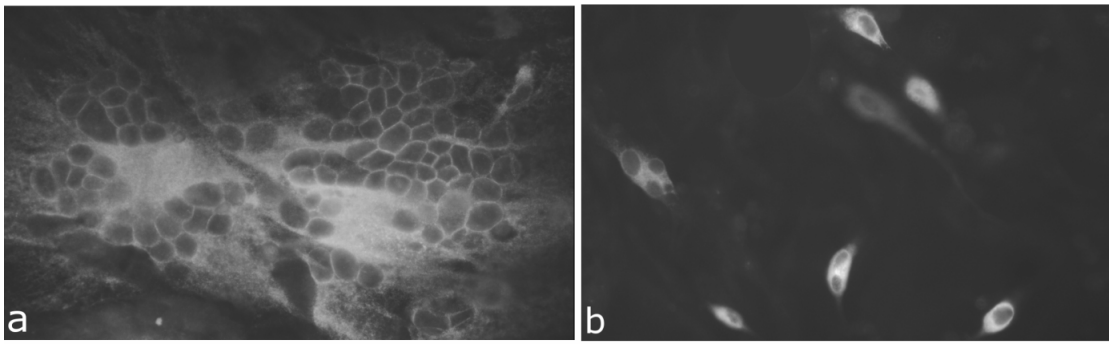


Figure 3. BHK21 cells expressing TSWV Gc (panel a) or Gn (panel b). Cells were transfected with constructs coding for Gc or Gn, incubated for 20-48 hours, fixed and subjected to immunodetection. Upon expression of Gc, large syncytia were frequently observed. Cells expressing Gn did less frequently form syncytia but in this case, the syncytia were much smaller and less abundant. Nuclei were confirmed by DAPI staining (data not shown).

As Gc has recently been reported to be cleaved at low pH, suggesting pH-dependent fusion could be involved in TSWV endocytosis (Whitfield *et al.* 2005), we tested this hypothesis as well. However, lowering the pH did not lead to an increase in syncytia formation (data not shown).
These findings are in accordance with theoretical models which predict that the Gc proteins of the *Bunyaviridae*, including TSWV Gc, function as type II fusion proteins (Garry and Garry, 2004). Strikingly, the region in TSWV Gc that is proposed to function as the fusion loop on the basis of computational modelling is the exact same region that shows a significant protein sequence similarity with orthobunyavirus Gc as earlier reported (Kormelink *et al.*, 1992a; Cortez *et al.*, 2002; Figure 1).

For several viruses belonging to the closely related *Hantavirus* and *Orthobunyavirus* genera, the glycoproteins induce cell fusion of mammalian cells, a capacity that is required for the release of RNPs into the cytoplasm after fusion of the viral and endosomal membranes. Cells infected with La Crosse orthobunyavirus (LAC), as well as cells expressing only La Crosse glycoproteins Gn and Gc, display cell fusion at pH 6 or lower. This effect is not seen upon expression of LAC Gc in the absence of Gn (Jacoby *et al.*, 1993; Bupp *et al.*, 1996). However, Gc is likely to be involved in this process as LAC Gc undergoes pH-dependent conformational changes (Pekosz and González-Scarano, 1996). Likewise, mammalian cells infected with California Encephalitis orthobunyavirus fuse with neighbouring cells at mildly acidic pH values, which is inhibited in the presence of monoclonal antibodies directed against Gc (Hacker and Hardy, 1997). Similar pH-dependent cell fusion is observed upon infection of cell cultures with different hantaviruses or after transient expression of their corresponding glycoprotein precursor (McCaughey *et al.*, 1999; Ogino *et al.*, 2004).
In plant cells infected with TSWV, the presence of a cell wall impedes syncytia formation. However, in these cells the fusion of internal membranes is observed in the course of infection. During TSWV envelope acquisition, a Golgi stack containing the two glycoproteins wraps around the RNPs, which results in the formation of doubly enveloped virus particles (DEVs) (Kikkert *et al.*, 1999; Kitajima *et al.*, 1992). The DEVs fuse with ER-derived membranes and with each other resulting in the formation of large vesicles in the cell, containing an accumulation of singly enveloped virus particles (SEVs) (Kikkert *et al*, 1999). In this process, an important role for the glycoproteins can be envisaged; however, to what extent the two mechanisms (i.e. syncytia formation and intracellular membrane fusion) are related remains to be determined.

Acknowledgements
This work was financially supported by the Netherlands organisation for Scientific Research, section Earth and Life Sciences (NWO/ALW) and the EU (grant HPRN-CT-2002-00262).

Chapter 7

General discussion

Introduction

At the onset of the research described in this thesis, a tentative model for tomato spotted wilt virus (TSWV) assembly had been suggested based on previous research using infected plant tissue or protoplasts (Kikkert *et al.*, 1999; Kitajima *et al.*, 1992). Observations in infected protoplasts had pointed to the occurrence of a phenomenon termed "wrapping", in which Golgi stacks, modified by the two viral glycoproteins present, curve around RNPs, thus leading to the formation of doubly enveloped virus particles (DEVs). Fusion of DEVs with each other and/or with ER membranes was proposed to result in the accumulation of singly enveloped virus particles (SEVs) within larger intracellular vesicles. These SEVs are considered the mature form of the virus and accumulate in the cell until uptake by thrips takes place. Mammalian bunyaviruses in contrast exit the cell by the budding of Golgi-derived vesicles through the plasma membrane, a mechanism that is not employed by tospoviruses, obviously due to the presence of a rigid cell wall in plant cells. The determinants responsible for this different behaviour are assumed to lie largely in the properties of the viral glycoproteins. To gain more insight in the process of assembly of progeny virus particles, in this thesis the behaviour (intracellular trafficking and protein-protein interactions) of the TSWV structural proteins was studied in living cells, either separately or in different combinations.

To this end the viral proteins were expressed in mammalian cells and studied using fluorescence microscopy, making use of FRET and FLIM techniques in particular. For the investigation of interactions *in vivo*, FRET and FLIM were chosen because of the non-invasive nature of these techniques that allows for detection of protein-protein interactions that can be followed in time as well as pinpointed to specific regions within the context of a living cell. For these studies mammalian cells were chosen over plant cell systems in view of a couple of advantages. Firstly, these cells lack the disadvantages of autofluorescence caused by the presence of chloroplasts, which often hampers fluorescence measurements. Secondly, for mammalian cell systems a good choice of (viral) gene expression systems has become available to (co-)express specific proteins without the complicating background of a full TSWV infection. Given our previous results we chose the Semliki forest virus expression system (Kikkert *et al.*, 2001). Indeed, because of the evolutionary relationship of TSWV with mammalian viruses in the *Bunyaviridae* family, mammalian cells have been used in our previous research and found to render similar localisation patterns as those found in infected plant cells (Kikkert *et al.*, 2001). This is not too surprising, as for this virus species virions should be formed both in thrips and in plant cells, meaning the signals required for (co)localisation as well as those involved in virion formation must function in different cellular environments. Employing a mammalian cell system, moreover, also allows for easy comparison of the findings for TSWV with what is known for the other bunyaviruses.

Model

Based on the results described in the preceding chapters of this thesis and on literature data, a model for virion assembly in plant cells is proposed (Figure 1). The different steps proposed in this model will be discussed separately in more detail in the next paragraphs. Briefly, the model proposes that after virus entry and uncoating in the cell, transcription of the viral genome by the L protein present in virions takes place, followed by production of the viral proteins and replication of the viral genome. N is readily produced and together with viral RNA and L protein forms RNPs in the cytosol (Figure 1, step 1).

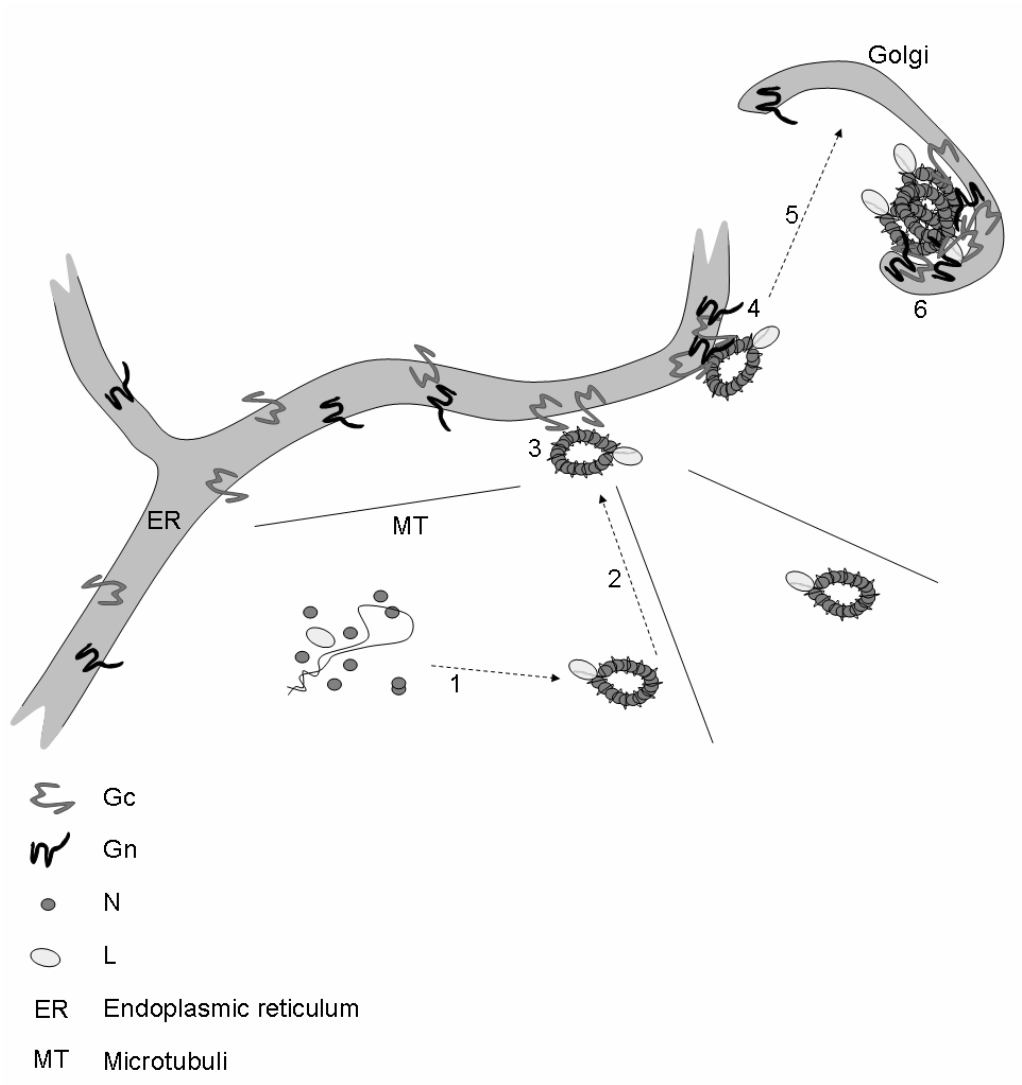


Figure 1. Hypothetical model of TSWV assembly in a plant cell extrapolated from observations in mammalian cells and infected plant cells. Viral RNA, nucleocapsid protein (N) and L protein assemble into RNPs in the cytoplasm (1). RNPs are transported to distinct subcellular domains by microtubuli (and actin filaments) (2). RNPs interact with the cytoplasmic tail of Gc (3). The two glycoproteins Gn and Gc interact (4) and the complex translocates to the Golgi (5). Wrapping of a Golgi stack around the RNPs results in the formation of doubly enveloped virus particles (6).

Subsequently, RNPs are transported to a specific site that may have an analogy to the microtubule organising centre (MTOC) of mammalian cells, through interaction of N with components of the cytoskeleton (step 2). Here, interaction of the RNPs

with Gc occurs by specific interaction of N and the cytoplasmic tail of Gc (step 3). Furthermore, an interaction between the Gc tail and (viral) RNA may also be involved in this association of Gc to RNPs. Meanwhile, the largest portion of each Gc molecule remains localised in the ER lumen where it is processed to reach its mature conformation. After maturation of Gc, interaction of Gc and Gn takes place (step 4) and the RNP-Gc-Gn complex translocates to the Golgi apparatus (step 5) where envelopment takes place (step 6). After the formation of DEVs, followed by SEV accumulation in intracellular vesicles, the virus is taken up by the thrips and enters the midgut epithelial cells by attachment of Gn to a receptor followed by fusion of the viral envelope with the plasma membrane, induced by Gc.

Virus entry

After uptake of the virions by thrips, TSWV, in contrast to other bunyaviruses, has not been seen in endosomes, but rather seems to release its RNPs directly by fusion of the viral membrane to that of the thrips midgut cells. For the process of membrane fusion and entry, Gn has been postulated as the viral attachment protein, attachment possibly occurring via the RGD motif present at its N-terminus (Kikkert *et al.*, 1998; Whitfield *et al.*, 2004). It was previously not known what causes the subsequent fusion of the two membranes, although the glycoproteins had been assumed to be responsible. It is not known if virus entry involves endosomal uptake, or if RNPs are directly released into the cytoplasm, but either way requires fusion of viral and host membranes at some stage during the entry process. In chapter 6, fusion of mammalian cells induced by Gc, and not by Gn, is described. Our results strongly suggest that Gc on its own, i.e. in the absence of Gn, is capable of inducing membrane fusion. This fusion is not dependent on or enhanced by low pH, which is striking in light of cleavage of Gc occurring in a pH-dependent fashion (Whitfield *et al.*, 2005 and this thesis, chapter 2), and when looking at of other bunyaviruses, notably the orthobunyaviruses La Crosse virus and California encephalitis virus and several hantaviruses, that are dependent on low pH for membrane fusion (Bupp *et al.*, 1996; Hacker and Hardy, 1997; Jacoby *et al.*, 1993; McCaughey *et al.*, 1999; Ogino *et al.*, 2004). These seemingly conflicting observations could result from the use of mammalian cells for TSWV or other experimental artefacts; alternatively, they could indicate a genuine difference in the mechanism of induction of membrane fusion by TSWV Gc as compared to animal-infecting bunyaviruses. In the latter case, the pH-induced cleavage may have a different function, possibly related to a specific RNP function since (partially) cleaved forms of Gc can be found in RNP preparations, but not in purified virions, as described in chapter 2. After completing one or several rounds of infection in the thrips, the virus is injected into the plant cell during feeding of the thrips. The process of membrane shedding once the virus has been delivered to the cell remains as yet an enigma.

RNP formation

Following infection of a host plant cell, progeny viral RNA can first be detected 16 hours after infection. Around the same time point, N is first detected (Kikkert *et al.*, 1997) and upon expression readily forms homodimers or –multimers

throughout the cell (chapter 3). Homotypic interactions are seen throughout the cell and not only in the aggregates that are so typical for cells expressing the N protein and eventually accumulate in large aggregates at sites possibly analogous to the microtubule organising centre (MTOC). The interactions take place independent of the presence or absence of viral RNA and may even be required for the interaction with viral RNA as for Hantaan virus specific affinity exists between trimeric N and viral RNA that is abolished in case of monomeric and dimeric N (Mir and Panganiban, 2004). For TSWV N, stable dimer bands, but no stable trimer bands are seen on a polyacrylamide gel (chapter 2) whereas for Bunyamwera virus the building blocks are N monomers rather than dimers or trimers (Elliott, pers. comm.), pointing to a possible genus-specific mechanism. It is likely that in the case of TSWV, mono- or dimeric (or perhaps trimeric) N associates with viral RNA in the cytoplasm from early time points during the infection. Association of L to the N-RNA complexes may be expected to take place, rendering RNP complexes in the cytoplasm.

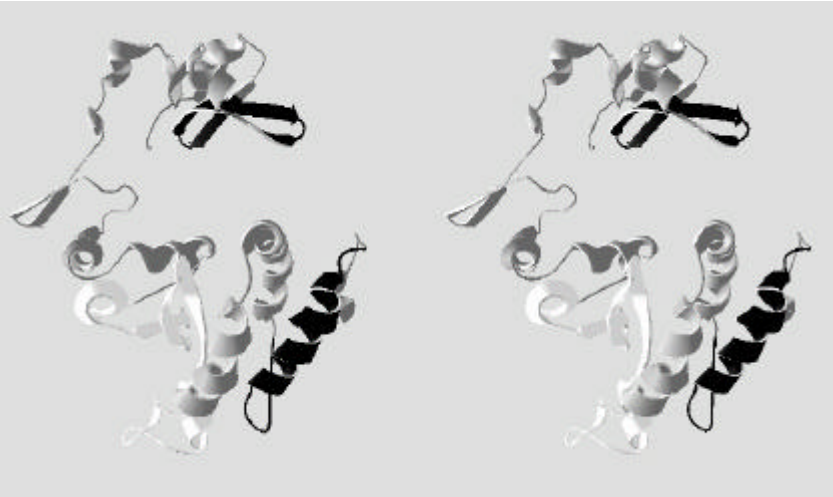


Figure 2. TSWV nucleocapsid protein (folding predicted by HMMSTR / Rosetta at http://www.bioinfo.rpi.edu). On the right bottom side of the molecule, the C-terminal part, consisting of three helices separated by loops. Regions in black play a role in homotypic interaction (Kainz *et al.*, 2004; Uhrig *et al.*, 1999); Regions in dark grey and also those in black are involved in sequence-aspecific RNA binding (Richmond *et al.*, 1998).(Convergent stereo image.)

Computer models of Tula hantavirus, supported by experimental data, indicated that Tula virus N has a curved shape and that trimerisation occurs through intertwining of the C-terminal alpha-helices, thereby establishing a shared hydrophobic space (Kaukinen *et al.*, 2004). The curved structure shows some similarity to that of influenza A virus and rabies virus N proteins (Martín-Benito *et al.*, 2001; Schoehn *et al.*, 2001). Interaction of the N-terminal helices contributes to the formation of stable Tula virus N trimers. Computer modelling of TSWV N also renders a curved protein with a helix – loop – helix structure at the C-terminal part of the protein (Figure 2), which has already been shown to be involved in homotypic interaction (Kainz *et al.*, 2004; Uhrig *et al.*, 1999).

The RNA, when encapsidated by N, is in the case of Tula virus suggested to be located in the 'hole' which is formed in the middle part of the trimers (Kaukinen *et al*., 2004). On the other hand, since the curved shape of N as found by computer-assisted modelling shows some similarities to that of influenza virus NP, the structure of influenza RNPs should be kept in mind as well. In these RNPs, the RNA is proposed to be wrapped around the NP molecules, thus exposing part of the RNA (Portela and Digard, 2002). This model, schematically represented in figure 3, is supported for TSWV by the fact that viral genomic RNA is present in RNPs in a dense, probably coiled or supercoiled form (Kellmann *et al*., 2001) that

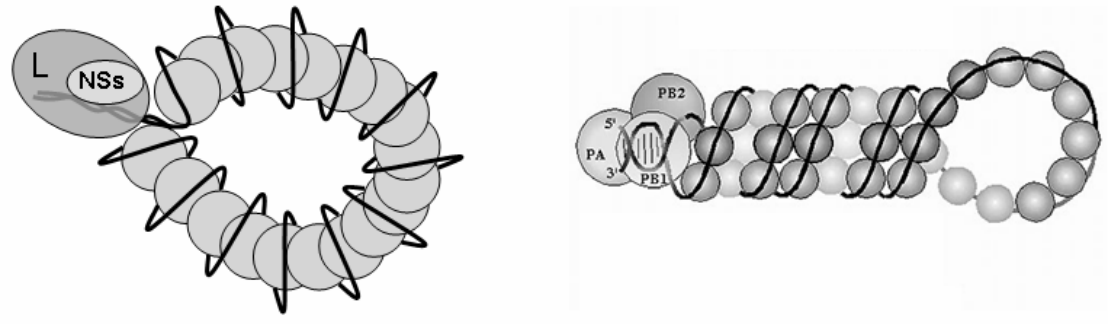


Figure 3. Model for TSWV RNP (left) and influenza A virus RNP (right). The influenza cartoon was kindly provided by Dr. Paul Digard, Department of Pathology, University of Cambridge, United Kingdom.

is difficult to envisage in the relatively small intermolecular space of multimeric N, but would be logical if the RNA is coiled around N. If this is the case for TSWV, the RNA would be accessible for Gc and could thus play a role in the interaction of Gc with RNPs, as is hypothesised to be the case for the interaction of the influenza A virus matrix protein with viral RNPs (Ye *et al*., 1999). Moreover, the viral RNA would be accessible for interaction with the movement protein NSm which has the capability to bind RNA, albeit with low affinity, in addition to interacting with the N protein (Soellick *et al.*, 2000)

No data are as yet available on the regions of N involved in the specific recognition of and interaction with viral RNA. Since there are no clear conserved domains for all tospoviruses, prediction of such regions is difficult.

Virion envelopment

While some of the RNPs associate with NSm for cell-to-cell movement (Kormelink *et al*., 1994), another portion of the RNP complexes is destined for envelopment and the formation of mature virions. These RNPs associate with the glycoproteins to induce the wrapping of a Golgi stack as a first step in the process of membrane acquisition. Whereas previously, educated guesses and speculations had led to the assumption this association involved an interaction between RNPs and Gn (Kikkert and Kormelink, unpublished data), this thesis demonstrates, rather surprisingly, that N interacts with Gc rather than Gn (chapter 4). These results are furthermore supported by the observation in chapter 2 that even after centrifugation over a cesium sulphate gradient, RNPs consistently contain some Gc, whereas no Gn can be detected. The interaction between N and (most likely) the cytoplasmic tail of

Gc may be enhanced by the presence of viral RNA, analogous to the interaction between influenza A virus matrix protein M1 and RNPs (Ye *et al.*, 1999). This is being supported by the observation that, like influenza A virus M1, Gc proteins from several different bunyaviruses display a stretch of positively charged amino acids in their cytoplasmic domains, even within the very short Uukuniemi virus Gc tail. Additional (primary or secondary) sequence similarities between the cytoplasmic tail of Gc and other matrix proteins have not been found so far.
The interaction of RNPs with Gc takes place in the cytoplasm as well as at the site where N is seen to accumulate, which could be the MTOC or a similarly located structure. For translocation of N to this site, a functioning cytoskeleton is essential (chapter 3). The possibility that this accumulation is due to a cellular defence mechanism (as earlier suggested for several bunyaviruses), rather than a specific viral pathway, can not be excluded on the basis of this research (Andersson *et al.*, 2004a; Frese *et al.*, 1996; Kochs *et al.*, 2002). However, the indication that TSWV N interacts with the cytoskeleton as shown in chapter 3, suggests this may be a cellular transport mechanism exploited by the virus to its benefit. Furthermore, a direct interaction has been shown to exist between Black Creek Canal hantavirus N protein with actin filaments (Ravkov *et al.*, 1998). Evidence from work on Tula virus N suggests that transport to this perinuclear site moreover requires that Tula virus N be capable of trimer formation (Kaukinen *et al.*, 2004). An accumulation of aggregates is also seen upon expression of TSWV N in plant cells, but in this case the aggregates are distributed more dispersed and not located at one clear perinuclear position (D. Ribeiro, pers. comm.). Although the possibility of an artefact caused by expression in a heterologous cell system cannot be excluded, this difference may on the other hand well be caused by a different cellular architecture of plant versus mammalian cells. After all, in plant cells several organelles have different intracellular distributions. The Golgi, for instance, is positioned around the MTOC and both have a perinuclear localisation in mammalian cells, whereas in plant cells the Golgi is fractionated and the function of the MTOC is carried out by multiple nucleation sites on the nuclear envelope and the plasma membrane (Brandizzi *et al.*, 2004; Erhardt *et al.*, 2002; Lambert, 1993). This raises the possibility that RNPs are either assembled or concentrated in both cell systems at an MTOC-like site (this term is used since in a non-dividing plant cell, no clear MTOC has been defined), from which subsequent transport to the Golgi takes place.
Meanwhile, the main portion of each Gc molecule still remains in the ER with only the carboxy-terminal tail in a suitable position to interact with the RNPs. The mechanism of ER retention is unclear, as no ER retention or retrieval motif, such as KDEL, is present in Gc. However, an HDEL sequence is present in many orthobunyavirus Gc proteins in the region that is very conserved between the *Tospovirus* and *Orthobunyavirus* genera. It is tempting to speculate that the TSWV sequence HTDFYST present in the place of the KHDEL sequence of many orthobunyaviruses (see chapter 6, Figure 1) may play a role in ER retention (or retrieval), although this sequence is not highly conserved among the tospoviruses. Alternatively, Gc may be retained in the ER through an interaction with one or more ER-resident chaperones because of improper folding in the absence of Gn. It

has been demonstrated for Uukuniemi virus that Gc reaches its mature conformation slowly and only associates with Gn after maturation, while during maturation both immature proteins are transiently associated to calnexin and calreticulin (Persson and Pettersson, 1991; Veijola and Pettersson, 1999). In our model, the ER-localised portion of Gc folds during or even after translocation to the perinuclear site. After acquiring its mature conformation, Gc is ready to interact with Gn. The results described in chapter 5 suggest this interaction involves the cytosolic tails of the glycoproteins, although other domains may be involved as well. This is in agreement with preliminary findings by Elliott and co-workers that interaction between the two Bunyamwera virus glycoproteins occurs through the cytoplasmic tails of the two proteins (Elliott, pers. comm.) but it contrasts reports that for Punta Toro virus the C-terminal domain of Gc is not required for heterodimer formation (Chen and Compans, 1991). Thus, different domains may be involved for the different genera, all leading to the formation of glycoprotein heterodimers. Further indications for the involvement of the C-terminal domains of the two proteins in heterodimer formation lie in the observation that fluorescence from the two glycoprotein-fluorophore fusion proteins is never observed in the same cell (chapter 5). This could indicate that interaction interferes with proper folding of the fluorophore and thus abolishes fluorescence. Further experiments with fusion and wild type constructs should clarify this issue.

After interaction the whole complex (Gn-Gc-RNP) translocates to the Golgi where membrane acquisition takes place. Golgi translocation and retention is mediated by the Golgi retention motif on the carboxy terminus of Gn, as is shown in chapter 5. The crucial amino acids do not represent any known Golgi retention motif and furthermore act in concert with the trans-membrane domain of Gn, since this is also required for Golgi targeting.

During membrane acquisition, DEVs are formed as an intermediate that may display the cytoplasmic portions of the two viral glycoproteins on the cytosolic side of the double membrane. The subsequent fusion, in plant cells, of DEVs with ER-derived membranes could conceivably occur by the **K**H**K**WT motif on the cytoplasmic tail of Gn exposed after interaction with Gc as has been suggested earlier (Kikkert, 1999). If the hydrophobic domain between the Gn cytoplasmic tail and Gc is cleaved off of Gn, the double lysine motif could become located at the extreme carboxy terminus of Gn. It then constitutes a double lysine motif that is involved in ER retrieval when present at the cytoplasmic carboxy terminus of type I membrane proteins (Jackson *et al.*, 1993; Letourneur *et al.*, 1994). On the other hand, there are indications that Gc has a profound effect on the plant internal membrane system, pointing to a possible role for Gc in the mechanism of internal membrane fusion (D. Ribeiro, pers. comm.). It is interesting in this context to note the presence of such a retrieval signal in all phlebovirus Gc proteins (Gerrard and Nichol, 2002) as well as some hantaviruses, while many orthobunyaviruses contain a known ER retrieval motif (HDEL) or a similar motif (HDEH) in a region with significant similarity to TSWV Gc. It is also tempting to speculate that the membrane fusion properties of Gc (described in chapter 6) are somehow involved in the fusion of DEVs to each other and / or to ER-derived membranes in the

formation of SEVs in infected plant cells. However, the proposed fusion domain resides in the lumen of the Golgi or ER and would thus not be in a suitable position to mediate fusion (Figure 4). Proteins that mediate membrane fusion in plant cells mostly fall in one of the two groups of so-called SNARE proteins, i.e. the T- (for 'target') and V- (for 'vesicle') snares. All these proteins are displayed on the cytoplasmic side of the membrane (Alberts *et al.*, 1994; Pratelli *et al.*, 2004; Ungar and Hughson, 2003).

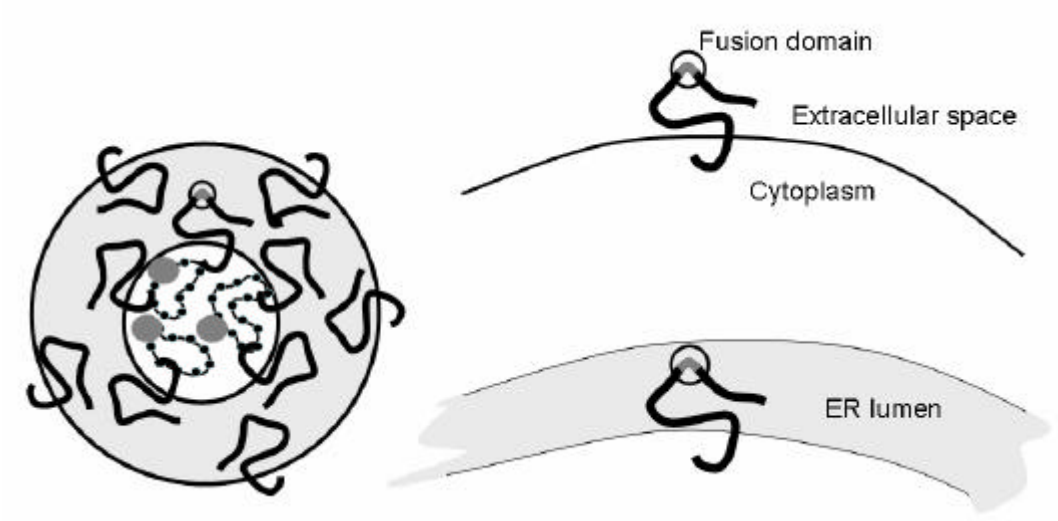


Figure 4. Location of the proposed Gc fusion domain (encircled) in Gc present in a DEV (left), on the plasma membrane (top right) and in the ER (bottom right).

In thrips cells, although no stages of budding of TSWV RNPs into the Golgi have ever been observed, budding likely results in the formation of more common secretory vesicles that translocate to and fuse with the cell membrane, releasing the mature virus particles.

Concluding remarks

The interaction between different viral factors with viral and host factors is a fascinating area attracting ever more attention of researchers for different purposes (combating viral diseases in humans; protection of cattle and crops against viruses; and biotechnological production using virus expression systems, etcetera). This thesis sheds a light on different aspects of the process of TSWV particle assembly and demonstrates the potential of *in vivo* techniques such as FRET-based microscopy to elucidate the complicated networks involved in the interactions between virus and host cell that determine the eventual outcome of the infection (death of the cell, removal of the virus or a balance in the form of a latent infection). This opens the door to further investigation, not only of interactions between different viral components, continuing the research that is the main topic of this work, but also of other intriguing aspects of viral infection such as the interplay between virus and host cell, enabling the virus to hijack the cellular machinery for its own benefit. For TSWV, this will eventually help to elucidate the mechanisms that enable the virus to infect both plant and insect cells, following different morphogenesis pathways in the two cell types.

Summary

Members of the Bunyaviridae have spherical, enveloped virus particles that acquire their lipid membrane at the Golgi complex. For the animal-infecting bunyaviruses, virus assembly involves budding of ribonucleoprotein particles (RNPs) into vacuolised lumen of the Golgi complex, after which the enveloped particles are secreted. The maturation of tomato spotted wilt virus (TSWV), a bunyavirus infecting plants, is different in that virions acquire their membrane by wrapping of a Golgi stack around RNPs after which the enveloped particles eventually accumulate in large vesicles in the plant cell. TSWV also multiplies in its insect vector thrips, and here particles are secreted from salivary gland cells into the gland ducts. The latter seems a logic requirement to allow virus passage to healthy host plants.
To further study the process of TSWV particle assembly, the interactions between the structural N, Gn and Gc proteins in mature virus particles, as well as their intracellular behaviour in vivo have been the main target of this Ph. D. thesis.

After an introductory chapter on bunyavirus particle assembly (chapter 1), the protein composition of purified TSWV RNPs and enveloped particles was studied in chapter 2. In enveloped virus preparations, the three major structural proteins, i.e. the nucleocapsid protein (N) and the two envelope glycoproteins Gn and Gc, were detected in monomeric as well as oligomeric forms. Glycoprotein Gc but not Gn was observed tightly bound to RNPs, suggesting Gc is involved in RNP envelopment. Analysis of cytoplasmic RNPs and mature virus particles for other viral proteins revealed, surprisingly, the presence of the so-called nonstructural protein NSs. Whereas mature virus particles contained only traces of NSs, RNP preparations clearly contained larger amounts of this protein, which could be related to an earlier reported difference in transcriptional/replicational activity between both.

To study the process of virus assembly in more detail, fluorescence microscopy methods were employed for the in vivo detection of protein interactions, rendering information concerning the intracellular localisation simultaneously (chapter 3). For this a system was set up in mammalian cells and as a first protein to be studied the cytosolic N protein was selected. This protein was already known to form homo-oligomeric structures in vitro. Using fusions of N with either yellow or cyan fluorescent protein (YFP and CFP, respectively), pairs were created to function as a donor (CFP) and acceptor (YFP) fluorophore for fluorescence resonance energy transfer (FRET). Using acceptor photobleaching and fluorescence lifetime imaging microscopy (FLIM) to measure FRET, N was observed to form homodimers and -multimers throughout the cytoplasm before eventually accumulating in a non-Golgi, perinuclear region on a microtubuli- and actin-dependent manner.

In a similar way, potential in vivo interactions between N and the viral glycoproteins were investigated (chapter 4). While no interaction between N and Gn was observed, these studies demonstrated interaction between N and Gc, in

agreement with the earlier observation (chapter 2) that some Gc remains tightly bound to purified RNPs. The interactions between N and Gc localised to the non-Golgi perinuclear area, similar to transiently expressed N. These data provided further support for the idea that interactions between N and Gc are involved in envelopment of the viral RNPs. While studying the possible formation of heterodimers of Gn and Gc, it appeared that with the constructs used, FRET could not be applied for this purpose, as fluorescence from the two fluorophore fusion proteins was never observed in the same cell. This could be due to the fact that interaction between the two glycoproteins interfered with proper folding of (one of) the fluorophores, resulting in greatly reduced and possibly undetectable fluorescence.

Another intriguing question – relevant to a broader cell biological field as well – concerns the signal responsible for Golgi localisation of the two glycoproteins during infection. Previous work had shown that Gn carries a Golgi retention signal and is able to rescue Gc from the ER to the Golgi, suggesting a heterodimerisation of Gn and Gc. For a number of other bunyaviruses, the Golgi retention signal had been mapped to the C-terminal transmembrane domain (TMD) and / or cytoplasmic tail of Gn. Using C-terminal deletion mutants of TSWV Gn and chimeric Gc and vesicular stomatitis virus glycoprotein (VSV-G) constructs (chapter 5), it was shown that both the TMD and the first 30 amino acids of the 60 residues-sized cytoplasmic tail of Gn are necessary for Golgi localisation. The lumenal domain was shown not to be required for Golgi localisation, nor was its presence required for rescuing of Gc. By deletion mapping the 20 most C-terminal residues of the cytoplasmic tail were shown to be crucial for interaction with Gc.

Large cells containing multiple nuclei were frequently observed when Gc was expressed. This phenomenon was further investigated in chapter 6, and was shown probably to result from a cell fusion activity of this glycoprotein. Cell fusion is not likely to occur during the plant infection cycle, but may play a role in the infection cycle or the process of virus entry within the thrips vector. The fusion was not pH-dependent and not observed with Gn.

Chapter 7 discusses the major findings of this Ph. D. research in a broader perspective, and presents a model for TSWV particle assembly in which all observations have been accomodated.

Samenvatting

Het tomatenbronsvlekkenvirus (tomato spotted wilt virus: TSWV) behoort tot de *Bunyaviridae*. Alle bunyavirussen kenmerken zich door een driedelig RNA genoom en een lipidemembraan. Het genoom is binnen de membraan georganiseerd in zogenaamde ribonucleotideproteïnen (RNPs), een hecht complex van genomisch RNA en nucleocapside (N) eiwitten. In de lipidemembraan bevinden zich 2 typen glycoproteinen, Gn en Gc genaamd. Binnen de *Bunyaviridae* vertegenwoordigt TSWV het enige genus van plantenvirussen, die behalve in planten ook repliceren in tripsen, insecten die de virussen overdragen van plant op plant.

De dier-infecterende bunyavirussen verkrijgen hun lipidemembraan (meestal afkomstig van het Golgi-apparaat van de gastheercel) door middel van "budding". Na budding in het Golgi-apparaat worden de door een dubbele lipidemembraan omgeven virale RNPs naar de celmembraan getransporteerd en daar uitgescheiden, waarbij de deeltjes een enkele membraan overhouden. Voor TSWV verloopt het proces iets anders. In de geïnfecteerde plantencel worden de RNPs als het ware ingepakt door een cistern van het Golgi-apparaat zonder daarmee het Golgi-apparaat binnen te komen en uiteindelijk hopen de virusdeeltjes zich binnen de cel op in grote compartimenten. In de trips wordt het virus wel uitgescheiden van geïnfecteerde cellen in de speekselklierbuizen, hetgeen een logisch vereiste lijkt voor de verspreiding van het virus naar een volgende gastheerplant.

Om een beter inzicht te krijgen in het proces dat uiteindelijk resulteert in nieuwe virusdeeltjes zijn in dit proefschrift de interacties onderzocht tussen de structurele eiwitten van TSWV (het nucleocapside-eiwit N en de glycoproteïnen Gn en Gc).

Hierbij diende de informatie zoals die beschikbaar was voor dier-infecterende bunyavirussen als vertrekpunt (hoofdstuk 1). In hoofdstuk 2 is allereerst de eiwitsamenstelling van RNPs en virusdeeltjes onderzocht. De structurele eiwitten N, Gn en Gc werden aangetoond zowel in monomere als in oligomere vorm. Glycoproteïne Gc, maar niet Gn, bleek een sterke binding te hebben met RNPs, en dit wijst op de betrokkenheid van Gc bij het proces waarop RNPs hun lipidemembraan verkrijgen. De analyses van RNPs en complete virusdeeltjes toonden verder aan dat in beide het zogenaamde niet-structurele virale eiwit NSs aanwezig was. Terwijl hele virusdeeltjes slechts kleine hoeveelheden NSs bevatten, was aanzienlijk meer van dit eiwit aanwezig in RNP zuiveringen. Dit zou mogelijk verband kunnen houden met eerder gerapporteerde verschillen in transcriptie- en replicatieactiviteiten van virusdeeltjes versus RNPs.

Om onderdelen van het virusassemblageproces in meer detail te begrijpen zijn fluorescentiemicroscopische technieken gebruikt waarmee het mogelijk was eiwitten en eiwitinteracties *in vivo* te volgen en tegelijkertijd informatie te verkrijgen over de plaats in de cel waar de eiwitten en interacties zich bevinden. Hiervoor werd een experimentele procedure opgezet waarin in eerste instantie het N-eiwit tot expressie werd gebracht in zoogdiercellen. Van dit eiwit was reeds bekend dat het in staat is homo-oligomeren te vormen *in vitro*. Door middel van fusies van N met de fluorescente eiwitten YFP (yellow fluorescent protein: geel fluorescerend eiwit) en CFP (cyan fluorescent protein: cyaanblauw fluorescerend eiwit) werden eiwitparen gecreëerd om als donor en acceptor te fungeren bij FRET (fluorescence resonance energy transfer: energie die wordt overgedragen van de donor naar de acceptor). Door gebruik te maken van twee verschillende technieken om de energieoverdracht te meten, namelijk het inactiveren van de acceptor met een laserpuls ("acceptor

photobleaching") en het meten van de levensduur van de fluorescentie ("fluorescence lifetime imaging": FLIM) kon aangetoond worden dat N homodimeren en –multimeren vormde door het gehele cytoplasma voordat het zich uiteindelijk ophoopte in een gebied nabij de kern, gebruikmakend van het cytoskelet. Dit gebied overlapte niet of slechts ten dele met het Golgi-apparaat. Deze resultaten suggereren dat ook bij afwezigheid van viraal RNA de eerste interacties optreden (dimerisatie van N) voor de vorming van RNPs.

Op soortgelijke wijze werden de potentiële *in vivo* interacties tussen N en de twee glycoproteïnen onderzocht (hoofdstuk 4). Deze interacties zijn waarschijnlijk cruciaal voor het verwerven van de membraan door de RNPs. Deze studies leverden geen aanwijzingen op voor het optreden van interacties tussen N en Gn, maar wel voor interacties tussen N en Gc. Dit was in overeenstemming met eerdere waarnemingen dat aan gezuiverde RNPs kleine maar significante hoeveelheden Gc gebonden zijn (hoofdstuk 2). De interacties tussen N en Gc werden voornamelijk gevonden in een gebied nabij de kern dat lijkt overeen te komen met het gebied waar ook N zich ophoopt. Deze gegevens ondersteunden de gedachte dat interacties tussen N en Gc een rol spelen bij het verkrijgen van de virale membraan door de RNPs. Voor het bestuderen van mogelijke interacties tussen Gn en Gc bleek de gebruikte techniek minder geschikt, waardoor alleen indirecte aanwijzingen voor het optreden van onderlinge interacties werden verkregen.

Voor de vorming van virusdeeltjes speelt, behalve de interacties tussen de betrokken virale eiwitten, de intracellulaire localisatie van de eiwitten een belangrijke rol. Een intrigerende vraag, die ook relevant is in een bredere celbiologische context, betreft dan ook het signaal dat er voor zorgt dat de beide virale glycoproteïnen tijdens de infectie accumuleren in het Golgi-apparaat. Uit eerder onderzoek was naar voren gekomen dat Gn een zogenaamd Golgi-retentiesignaal bevat en in staat is ook Gc mee te voeren naar het Golgi-apparaat. In hoofdstuk 5 zijn deletiemutanten van het Gn eiwit alsmede chimere constructen, enerzijds bestaande uit een deel van Gn en anderzijds uit een deel van Gc of VSV-G (dit is een glycoproteïne afkomstig van het niet verwante vesicular stomatitis virus), gebruikt om het Golgi-retentiesignaal nauwkeuriger binnen Gn te karteren. De resultaten van deze experimenten wezen uit dat zowel het transmembraandomein als de eerste 30 aminozuren van het 60 aminozuren tellende cytoplasmatische deel van Gn nodig zijn voor Golgi-localisatie. Het deel van Gn dat zich in het lumen van het Golgi-apparaat bevindt bleek niet nodig voor Golgi-localisatie, noch voor het meevoeren van Gc. De 20 aminozuren op de uiterste C-terminus van Gn bleken onmisbaar voor interactie met Gc.

Zodra Gc in zoogdiercellen tot expressie werd gebracht, werden vaak abnormaal grote cellen met een veelvoud aan kernen waargenomen. Dit fenomeen werd in hoofdstuk 6 nader onderzocht en bleek waarschijnlijk het gevolg te zijn van het vermogen van Gc om celfusie te bewerkstelligen. Vermoedelijk is deze eigenschap niet functioneel tijdens de infectie van een plant, maar mogelijk wel tijdens het infectieproces van de trips. De celfusie was niet pH-afhankelijk en werd niet waargenomen bij Gn.

In hoofdstuk 7 zijn de belangrijkste bevindingen uit dit proefschrift in een breder perspectief geplaatst en wordt een model gepresenteerd dat de verschillende waarnemingen samenbrengt die leiden tot de vorming van nieuwe virusdeeltjes.

References

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. D. (1994). Molecular biology of the cell. Third edition. Garland Publishing, inc., New York.

Alfadhi, A., Love, Z., Arvidson, B., Seeds, J., Willey, J. and Barklis, E. (2001). Hantavirus nucleocapsid oligomerization. J. Virol. **75**: 2019-2023.

Anderson, G. W. Jr. and Smith, J. F. (1987). Immunoelectron microscopy of Rift valley fever viral morphogenesis in primary rat hepatocytes. Virology **161**: 91-100.

Andersson, A. M., Melin, L., Persson, R., Raschperger, E., Wikstrom, L. and Petterson, R. F. (1997a). Processing and membrane topology of the spike proteins G1 and G2 of Uukuniemi virus. J. Virol. **71**: 218-225.

Andersson, A. M., Melin, L., Bean, A. and Pettersson, R. F. (1997b). A retention signal necessary and sufficient for Golgi localization maps to the cytoplasmic tail of a *Bunyaviridae* (Uukuniemi virus) membrane glycoprotein. J. Virol. **71**: 4717-4727.

Andersson, A. M. and Pettersson, R. F. (1998). Targeting of a short peptide derived from the cytoplasmic tail of the G1 membrane glycoprotein of Uukuniemi virus (*Bunyaviridae*) to the Golgi complex. J. Virol. **72**: 9585-9596.

Andersson, I., Bladh, L., Mousavi-Jazi, M., Magnusson, K.-E., Lundkvist, A., Haller, O. and Mirazimi, A. (2004a). Human MxA protein inhibits the replication of Crimean-Congo hemorrhagic fever virus. J. Virol. **78**: 4323-4329.

Andersson, I., Simon, M., Lundkvist, A., Nilsson, M., Holmstrom, A., Elgh, F. and Mirazimi, A. (2004b). Role of actin filaments in targeting of Crimean Congo hemorrhagic fever virus nucleocapsid protein to perinuclear regions of mammalian cells. J. Med. Virol. **72**: 83-93.

Antic, D., Wright, K. E. and Kang, C. Y. (1992). Maturation of Hantaan virus glycoproteins G1 and G2. Virology **189**: 324-328.

Arai, R. Nakagawa, H., Tsumoto, K., Mahoney, W., Kumagai, I., Ueda, H. and Nagamune, T. (2001). Demonstration of a homogeneous noncompetitive immunoassay based on bioluminescence resonance energy transfer. Anal. Biochem. **289**: 77-81.

Bandla, M. D., Campbell, L. R., Ullman, D. E. and Sherwood, J. L. (1998). Interaction of tomato spotted wilt tospovirus (TSWV) glycoproteins with a thrips midgut protein, a potential cellular receptor for TSWV. Phytopathology **88**: 98-104.

Bar, P. M., Bassham, D. C. and Raikhel, N. V. (1996). Transport of proteins in eukaryotic cells: More questions ahead. Plant Mol. Biol. **32**: 223-249.

Bark, S. J. and Hahn, K. M. (2000). Fluorescent indicators of peptide cleavage in the trafficking compartments of living cells: Peptides site-specifically labeled with two dyes. Meth. Orlando. **20**: 429-435.

Bastiaens, P. I. H. and Jovin, T. M. (1996). Microspectroscopic imaging tracks the intracellular processing of a signal transduction protein: Fluorescent-labeled protein kinase C beta-I. Proc. Natl. Acad. Sci. USA **93**: 8407-8412.

Beaton, A. R. and Krug, R. M. (1984). Synthesis of the templates for influenza virion RNA replication *in vitro*. Proc. Natl. Acad. Sci. USA **81**: 4682-4686.

Beaton, A. R. and Krug, R. M. (1986). Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5'capped end. Proc. Natl. Acad. Sci. USA **83**: 6282-6286.

Bellocq, C. and Kolakofsky, D. (1987). Translational requirement for LaCrosse virus S messenger RNA synthesis: A possible mechanism. J. Virol. **61**: 3960-3967.

Bellocq, C., Raju, R., Patterson, J. and Kolakofsky, D. (1986). Translational requirement of LaCrosse virus small messenger RNA synthesis *in vitro* studies. J. Virol. **61**: 87-95.

Bendtsen, J. D., Nielsen, H., von Heijne, G. and Brunak, S. (2004). Improved prediction of signal peptides: signalP 3.0. J. Mol. Biol. **340**: 783-795.

Berland, K. M., So, P. T. C., Chen, Y., Mantulin, W. W. and Gratton, E. (1996). Scanning tow-photon fluctuation correlation spectroscopy: Particle counting measurements for detection of molecular aggregation. Biophys. J. **71**: 410-420.

Berney, C. and Danuser, G. (2003). FRET or no FRET: A quantitative comparison. Biophys. J. **84**: 3992-4010.

Bertolotti-Ciarlet, A., Smith, J., Strecker, K., Paragas, J., Altamura, L. A., McFalls, J. M., Frias-Stäheli, N., García-Sastre, A., Schmaljohn, C. S. and Doms, R. W. (2005). Cellular localization and antigenic characterization of Crimean-Congo hemorrhagic fever virus glycoproteins. J. Virol. **79**: 6152-6161.

Bishop, D. H. L., Gay, M. E. and Matsuoko, Y. (1983). Non-viral heterogeneous sequences are present at the 5'ends of one species of snowshoe hare bunyavirus S complementary RNA. Nucl. Acids. Res. **11**: 6409-6418.

Blakqori, G., Kochs, G., Haller, O. and Weber, F. (2003). Functional L polymerase of La Crosse virus allows in vivo reconstitution of recombinant nucleocapsids. J. Gen. Virol. **84**: 1207-1214.

Bonnet, G., Krichevsky, O. and Libchaber, A. (1998). Kinetics of conformational fluctuations in DNA hairpin loops. Proc. Natl. Acad. Sci. USA **95**: 8602-8606.

Booth, T. F., Gould, E. A. and Nuttall, P. A. (1991). Structure and morphogenesis of Dugbe virus *Bunyaviridae* Nairovirus studied by immunogold electron microscopy of ultrathin cryosections. Virus Res. **21**: 199-212.

Borst, J. W., Hink, M., van v. Hoek, A. and Visser, A. J. W. G. (2003). Multiphoton microspectroscopy in living plant cells. Proc. SPIE. **4963**: 231-238.

Bosch, B. J., de Haan, C. A. M. and Rottier, P. J. M. (2004). Coronavirus spike glycoprotein, extended at the carboxi terminus with green fluorescent protein, is assembly competent. J. Virol. **78**: 7369-7378.

Bouloy, M. and Hannoun, C. (1976). Studies on Lumbovirus replication: I RNA-dependent RNA polymerase associated with virions. Virol. **69**: 258-264.

Bouloy, M., Janzen, C., Vialat, P., Khun, H., Pavlovic, J., Huerre, M. and Haller, O. (2001). Genetic evidence for an interferon-antagonistic function of Rift Valley fever virus nonstructural protein NSs. J. Virol. **75**: 1371-1377.

Bouloy, M., Krans-Ozden, S., Horodniceanu, F. and Hannoun, C. (1973/1974). 3 segment RNA genome of Lumbo virus bunyavirus. Intervirology **2**: 173-180.

Bouloy, M., Pardigon, N., Vialat, P., Gerbaud, S. and Girard, M. (1990). Characterization of the 5' and 3' ends of viral messenger RNA isolated from BHK21 cells infected with Germiston virus bunyavirus. Virology **175**: 50-58.

Braakman, I. and van Anken, E. (2000). Folding of viral envelope glycoproteins in the endoplasmic reticulum. Traffic **1**: 533-539.

Braam, J., Ulmanen, I. and Krug, R. M. (1983). Molecular model of a eukaryotic transcription complex functions and movements of Influenza P proteins during capped RNA primed transcription. Cell **34**: 609-618.

Brandizzi, F., Frangne, N., Marc-Martin, S., Hawes, C., Neuhaus, J.M and Paris, N. (2002). The destination for single-pass membrane proteins is influenced markedly by the length of the hydrophobic domain. Plant Cell **14**: 1077-1092.

Brandizzi, F., Irons, S. L. and Evans, D. E. (2004). The plant nuclear envelope: new prospects for a poorly understood structure. New Phytologist **163**: 227-246.

Brideau, A. D., Del Rio, T., Wolffe, E. J. and Enquist, L. W. (1999). Itracellular trafficking and localization of the pseudorabies virus Us9 type II envelope protein to host and viral membranes. J. Virol. **73**: 4372-4384.

Bridgen, A., Dalrymple, D.A., Weber, F. and Elliott, R.M. (2004). Inhibition of Dugbe Nairovirus replication by human MxA protein. Vir. Res. **99**: 47-50.

Bridgen, A., Weber, F., Fazakerley, J. K. and Elliott, R. M. (2001). Bunyamwera bunyavirus nonstructural protein NSs is a nonessential gene product that contributes to viral pathogenesis. Proc. Natl. Acad. Sci. USA **98**: 664-669.

Bucher, E., Sijen, T., de Haan, P., Goldbach, R. and Prins, M. (2003). Negative-strand tospoviruses and tenuiviruses carry a gene for a suppressor of gene silencing at analogous genomic positions. J. Virol. **77**: 1329-1336.

Bültmann, A., Muranyi, W., Seed, B. and Haas, J. (2001). Identification of two sequences in the cytoplasmic tail of the human immunodeficiency virus type I envelope glycoprotein that inhibit cell surface expression. J. Virol. **75**: 5263-5276.

Bupp, K., Stillmock, K. and González-Scarano, F. (1996). Analysis of the intracellular transport properties of recombinant La Crosse virus glycoproteins. Virology **220**: 485-490.

Carette, J. E., Stuiver, M., van Lent, J., Wellink, J. and van Kammen, A. (2000). Cowpea mosaic virus infection induces a massive proliferation of endoplasmic reticulum but not Golgi membranes and is dependent on de novo membrane synthesis. J. Virol. **74**: 6556-6563.

Caton, A. J. and Robertson, J. S. (1980). Structure of the host-derived sequences present at the 5'ends of Influenza virus mRNA. Nucl. Acids. Res. **8**: 2591-2603.

Chapman, E. J., Hilson, P. and German, T. L. (2003). Association of L protein and in vitro Tomato spotted wilt virus RNA-dependent RNA polymerase activity. Intervirol. **46**: 177-181.

Chen, S. Y. and Compans, R. W. (1991). Oligomerization, transport, and Golgi retention of Punta Toro virus glycoproteins. J. Virol. **65**: 5902-5909.

Chen, S. Y., Matsuoka, Y. and Compans, R. W. (1991). Golgi complex localization of the Punta Toro virus G2 protein requires its association with G1 protein. Virology **183**: 351-365.

Cole, N. B., Smith, C. L., Sciaky, N., Terasaki, M., Edidin, M. and Lippincott-Schwartz, J. (1996). Diffusional mobility of Golgi proteins in membranes of living cells. Science **273**: 797-801.

Collett, M. S. (1986). Messenger RNA of the M segment RNA of Rift Valley fever virus. Virology **151**: 151-156.

Collett, M. S., Purchio, A. F., Keegan, K., Frazier, S., Hays, W., Anderson, D. K., Parker, M. D., Schmaljohn, S., Schmidt, J. and Dalrymple, J. M. (1985). Complete nucleotide sequence of the M RNA segment of Rift Valley fever virus. Virology **144**: 228-245.

Cortez, I., Aires, A., Pereira, A. M., Goldbach, R., Peters, D. and Kormelink, R. (2002). Genetic organisation of Iris yellow spot virus M RNA: indications for functional homology between the G(C) glycoproteins of tospoviruses and animal-infecting bunyaviruses. Arch. Virol. **147**:2313-2325

Dalton, K. P. and Rose, J. K. (2001). Vesicular stomatitis virus glycoprotein containing the entire green fluorescent protein on its cytoplasmic domain is incorporated efficiently into virus particles. Virology **279**: 414-421.

de Assis Filho, F. M., Naidu, R. A., Deom, C. M. and Sherwood, J. L. (2002). Dynamics of Tomato spotted wilt virus replication in the alimentary canal of two thrips species. Phytopathology **92**: 729-733.

de Ávila, A. C., Huguenot, C., Resende, R. de O., Kitajima, E. W., Goldbach, R. W. and Peters, D. (1990). Serological differentiation of 20 isolates of tomato spotted wilt virus. J. Gen. Virol. **71**: 2801-2807.

de Haan, P., Kormelink, R., Resende, D., van Poelwijk, F., Peters, D. and Goldbach, R. (1991). Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. J. Gen. Virol. **72**:2207-2216.

de Haan, P., Wagemakers, L., Peters, D. and Goldbach, R. (1989). Molecular cloning and terminal sequence determination of the S and M RNA species of Tomato spotted wilt virus. J. Gen. Virol. **70**: 3469-3474.

de Haan, P., Wagemakers, L., Peters, D. and Goldbach, R. (1990). The S RNA segment of Tomato spotted wilt virus has an ambisense character. J. Gen. Virol. **71**: 1001-1008.

Dhar, R., Chanock, R.M. and Lai, C.-J. (1980). Nonviral oligonucleotides at the 5'terminus of cytoplasmic influenza viral mRNAs deduced from cloned complete genomic sequences. Cell **21**:495-500.

DiBonito, P., Nicoletti, L., Mochi, S., Accardi, L., Marchi, A. and Giorgi, C. (1999). Immunological characterization of Toscana virus proteins. Arch. Virol. **144**: 1947-1960.

Doyle, C., Sambrook, J. and Gethign, M.-J. (1986). Analysis of progressive deletions of the transmembrane and cytoplasmic domains of Influenza hemagglutinin. J. Cell Biol. **103**: 1193-1204.

Duijsings, D., Kormelink, R. and Goldbach, R. (1999). Alfalfa mosaic virus RNAs serve as cap donors for tomato spotted wilt virus transcription during coinfection of Nicotiana benthamiana. J. Virol. **73**: 5172-5175.

Duijsings, D., Kormelink, R. and Goldbach, R. (2001). *In vivo* analysis of the TSWV cap-snatching mechanism: ingle basse complementarity and primer length requirements. EMBO J. **20**: 2545-2552.

Dunn, E. F., Pritlove, D. C., Jin, H. and Elliott, R. M. (1995). Transcription of a recombinant Bunyavirus RNA template by transiently expressed Bunyavirus proteins. Virology **211**: 133-143.

Eigen, M. and Rigler, R. (1994). Sorting single molecules: Application to diagnostics and evolutionary biotechnology. Proc. Natl. Acad. Sci. USA **91**: 5740-5747.

Elliott, R. M. (1990). Molecular biology of the *Bunyaviridae*. J. Gen. Virol. **71**: 501-22.

Elliott, R. M. (1996). The *Bunyaviridae* (New York: Plenum press).

Epping, E. A. and Moye-Rowley, W. S. (2002). Identification of interdependent signals required for anterograde traffic of the ATP-binding cassette transporter protein Yor1p. J. Biol. Chemistry **277**: 34860-34869.

Erhardt, M., Stoppin-Mellet, V., Campagne, S., Canaday, J., Mutterer, J., Fabian, T., Sauter, M., Muller, T., Peter, C., Lambert, A.-M. and Schmit, A.-C. (2002). The plant Spc98p homologue colocalizes with ?-tubulin at microtubule nucleation sites and is required for microtubule nucleation. J. Cell Sci. **115**: 2423-2431.

Erickson, M. G., Moon, D. L. and Yue, D. T. (2003). DsRed as a potential FRET partner with CFP and GFP. Biophys. J. **85**: 599-611.

Eshita, Y., Ericson, B., Romanowski, V. and Bishop, D. H. L. (1985). Analyses of the messenger RNA transcription processes of Snowshoe hare bunyavirus small and medium-sized RNA species. J. Virol. **55**: 681-689.

Fabian, M. R. and White, K. A. (2004). 5'-3' RNA-RNA interaction facilitates cap- and poly(A) tail-independent translation of Tomato bushy stunt virus mRNA: a potential common mechanism for *Tombusviridae*. J. Biol. Chem. **279**: 28862-28872.

Filho, F.M. de Assis, Naidu, R.A., Deom, C.M. and Sherwood, J.L. (2002). Dynamics of tomato spotted wilt virus replication in the alimentary canal of two thrips species. Phytopath. **92**: 729-733.

Fischer, W. and Spies, J. (1987). Identification of a mammalian glutaminyl cyclase converting glutaminyl into pyroglutaminyl peptides. Proc. Natl. Acad. Sci. USA **84**: 3628-3632.

Flick, R., Elgh, F., Hobom, G. and Pettersson, R.F. (2002). Mutational analysis of the Uukuniemi virus (Bunyaviridae) promoter reveals two regions of functional importance. J. Virol. **76**: 10849-10860.

Flick, R., Flick, K., Feldmann, H. and Elgh, F. (2003a). Reverse genetics for Crimean-Congo hemorrhagic fever virus. J. Virol. **77**: 5997-6006.

Flick, K., Hooper, J.W., Schmaljohn, C.S., Pettersson, R.F., Feldmann, H. and Flick, R. (2003b). Rescue of Hantaanvirus minigenomes. Virology **306**: 219-224.

Flick, K., Katz, A., Överby, A., Feldmann, H., Pettersson, R.F. and Flick, R. (2004). Functional analysis of the noncoding regions of the Uukuniemi virus (*Bunyaviridae*) RNA segments. J. Virol. **78**: 11726-11738.

Flick, R. and Petterson, R. F. (2001). Reverse genetics system for Uukuniemi virus (*Bunyaviridae*): RNA polymerase I-catalyzed expression of chimeric viral RNAs. J. Virol. **75**: 1643-1655.

Flohr, F., Schneider-Schaulies, S., Haller, O. and Kochs, G. (1999). The central interactive region of human MxA GTPase is involved in GTPase activation and interaction with viral target structures. FEBS Letters **364**: 24-28.

Francki, R. I. B., Milne, R. G. and Hatta, T. (1985). Tomato spotted wilt virus group. In: Atlas of Plant Viruses, vol.1, Boca Raton, CRC Press: 101-110.

Frese, M., Kochs, G., Feldmann, H., Hertkorn, C. and Haller, O. (1996). Inhibition of Bunyaviruses, Phleboviruses and Hantaviruses by human MxA protein. J. Virol. **70**: 915-923.

Gadella, T. W. J. Jr. (1999). Fluorescence lifetime imaging microscopy (FLIM): Instrumentation and applications. In: , Mason, W. T. (ed.). Fluorescent and luminescent probes, 2nd edition, Academic Press: 467-479.

Gadella, T. W. J., van der Krogt, G. N. M. and Bisseling, T. (1999). GFP-based FRET microscopy in living plant cells. Trends Plant Sci. **4**:287-291.

Gahmberg, N., Kuismanen, E., Keränen, S. and Pettersson, R. F. (1986). Uukuniemi virus glycoproteins accumulate in and cause morphological changes of the Golgi complex in the absence of virus maturation. J. Virol. **57**: 899-906.

Gallie, D. R. (1998). A tale of two termini: A functional interaction between termini of an mRNA is a prerequisite for efficient translation initiation. Gene Amsterdam **216**: 1-11.

Gallie, D. R. and Kobayashi, M. (1994). The role of the 3'-untranslated region of non-polyadenylated plant viral mRNAs in regulating translational efficiency. Gene Amsterdam **142**: 159-165.

Galperin, E., Verkusha, V. V. and Sorkin, A. (2004). Three-chromophore FRET microscopy to analyze multiprotein interactions in living cells. Nat. Methods **1**: 209-217.

Garcin, D. and Kolakofsky, D. (1990). A novel mechanism for the initiation of Tacaribe Arenavirus genome replication. J. of Virol. **64**: 6196-6203.

Garry, C. E. and Garry, R. F. (2004). Proteomics computational analyses suggest that the carboxyl terminal glycoproteins of Bunyaviruses are class II viral fusion protein (beta-penetrenes). Theor Biol Med Model **1**: 10.

Gentsch, J. R., Bishop, D. H. L. and Obijeski, J. F. (1977). The virus particle nucleic acids and proteins of four bunyaviruses. J. Gen. Virol. **34**: 257-268.

Gerbaud, S., Pardigon, N., Vialat, P. and Bouloy, M. (1987). The S segment of the Germiston bunyavirus genome: Coding strategy and transcription. In: Mahy, B. and Kolakofsky, D. (eds.). The Biology of negative strand viruses, Elsevier: 191-198.

Gerrard, S. R. and Nichol, S. T. (2002). Characterization of the Golgi retention motif of Rift Valley fever virus Gn glycoprotein. J. Virol. **76**: 12200-12210.

Giordano, L., Jovin, T. M., Masahiro, I. and Jares-Erijman, E. (2002). Diheteroarylethenes as thermally stable photoswichable acceptors in photochromic fluorescence resonance energy transfer (pcFRET). J. Am. Chem. Soc. **124**: 7481-7489.

Goldbach, R. W. and Peters, D. (1994). Possible causes of the emergence of tospovirus diseases. Semin. Virol. **5**: 113-120.

Goldbach, R. and Peters, D. (1996). Molecular and biological aspects of tospoviruses. In: The *Bunyaviridae* . R. M. Elliott, ed. (New York: Plenum Press), pp. 129-157.

Gordon, G. W., Berry, G. Liang, X. H., Levine, B. and Herman, B. (1998). Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. Biophys. J. **74**: 2702-2713.

Grinberg, A. V., Hu, C. D. and Kerppola, T. K. (2004). Visualization of Myc/Max/Mad family dimers and the competition for dimerization in living cells. Mol. Cell. Biol. **24**: 4294-4308.

Gutiérrez-Escolano, A. L., Brito, Z. U., del Angel, R. M. and Jiang, X. (2000). Interaction of cellular proteins with the 5' end of Norwalk virus genomic RNA. J. Virol. **74**: 8558-8562.

Hacker, J.K. and Hardy, J.L. (1997). Adsorptive endocytosis of California encephalitis virus into mosquito and mammalian cells: a role for G1. Virol **235**: 40-47.

Hacker, J.K., Volkman, L.E. and Hardy, J.L. (1995). Requirement for the G1 protein of California encephalitis virus in infection *in vitro* and *in vivo*. Virol **206**: 945-953.

Haller, O., Janzen, C., Vialat, P., Huerre, M., Pavlovic, J. and Bouloy, M. (2000). High virulence of attenuated Rift valley fever virus in mice lacking a type I interferon system. J. Clin. Virol. **18**: 192.

Hammond, A. T. and Glick, B. S. (2000). Dynamics of transitional endoplasmic reticulum sites in vertebrate cells. Mol. Biol. Cell **11**: 3013-3030.

Hassani-Mehraban, A., Saaijer, J., Peters, D., Goldbach, R. and Kormelink, R. (2005). A new tomato-infecting tospovirus from Iran. Phytopathology 852-858.

Heath, C. M., Windsor, M. and Wileman, T. (2001). Aggresomes Resemble Sites Specialized for Virus Assembly. J. Cell Biol. **153**: 449–455.

Hess, S.-T., Huang, S. H., Heikal, A. A. and Webb, W. W. (2002). Biological and chemical applications of fluorescence correlation spectroscopy: A review. Biochem. **41**: 697-705.

Hink, M. A., Bisseling, T., and Visser, A. J. W. G. (2002). Imaging protein-protein interactions in living cells. Plant Mol. Biol. **50**: 871-883.

Hu, C. D., Chinekov, Y. and Kerppola, T. K. (2002). Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular bluorescence complementation. Mol. Cell **9**: 789-798.

Hu, C. D. and Kerppola, T. K. (2003). Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. Nat. Biotech **21**: 539-545.

Huiet, L., Feldstein, P. A., Tsai, J. H. and Falk, B. W. (1993). The maize stripe virus major noncapsid protein messenger RNA transcripts contain heterogeneous leader sequences at their 5' termini. Virol. **197**: 808-812.

Ie, T. S. (1971). Electron microscopy of developmental stages of Tomato spotted wilt virus in plant cells. Virology **43**: 468-479.

Ihara, T., Matsuura, Y., and Bishop, D.H.L. (1985a). Analyses of the mRNA transcription processes of Punta Toro phlebovirus (*Bunyaviridae*). Virol. **147**: 317-325.

Ihara, T., Smith, J., Dalrymple, J. M. and Bishop, D. H. L. (1985b). Complete sequences of the glycoproteins and M RNA of Punta Toro phlebovirus compared to those of Rift Valley fever virus. Virology **144**: 246-259.

Immink, R. G. H., Gadella, T. W. J. Jr., Ferrario, S., Busscher, M. and Angenent, G. C. (2002). Analysis of MADS box protein-protein interactions in living plant cells. Proc. Natl. Acad. Sci.USA **99**: 2416-2421.

Imreh, G., Maksel, D., de Monvel, J. B., Branden, L. and Hallberg, E. (2003). ER retention may play a role in sorting of the nuclear pore membrane protein POM121. Exp. Cell Res. **284**: 173-184.

Jackson, M. R., Nilsson, T. and Peterson, P. (1993). Retrieval of transmembrane proteins to the endoplasmic reticulum. J. Cell Biol. **121**: 317-333.

Jacoby, D. R., Cooke, C., Prabakaran, I., Boland, J., Nathanson, N. and González-Scarano, F. (1993). Expression of the La Crosse M segment proteins in a recombinant vaccinia expression system mediates pH-dependent cellular fusion. Virology **193**: 993-996.

Jäntti, J., Hildén, P., Rönkä, H., Mäkiranta, V., Keränen, S. and Kuismanen, E. (1997). Immunocytochemical analysis of Uukuniemi virus budding compartments: role of the intermediate compartment and the Golgi stack in virus maturation. J. Virol. **71**: 1162-1172.

Jin, H. and Elliott, R. M. (1991). Expression of functional Bunyamwera virus L protein by recombinant vaccinia viruses. J. Virol. **65**: 4182-4189.

Jin, H. and Elliott, R. M. (1993). Characterization of Bunyamwera virus S RNA that is transcribed and replicated by the L protein expressed from recombinant vaccinia virus. J. Virol. **67**: 1396-1404.

Johnston, J. A., Ward, C. L. and Kopito, R. R. (1998). Aggresomes: A cellular response to misfolded proteins. J. Cell Biol. **143**: 1883-1898.

Jouvenet, N., Monaghan, P., Way, M. and Wileman, T. (2004). Transport of African swine fever virus from assembly sites to the plasma membrane is dependent on microtubules and conventional kinesin. J. Virol. **78**:7990-8001.

Kainz, M., Hilson, P., Sweeney, L., DeRose, E. and German, T. L. (2004). Interaction between Tomato spotted wilt virus N protein monomers involves nonelectrostatic forces governed by multiple distinct regions in the primary structure. Phytopathology **94**: 759-765.

Kanerva, M., Melen, K., Vaheri, A. and Julkunen, I. (1996). Inhibition of puumula and tula hantaviruses in Vero cells by MxA protein. Virol. **224**: 55-62.

Katiliene, Z., Katilius, E. and Woodbury, N. W. (2003). Single molecule detection of DNA looping by NgoMIV restriction endonuclease. Biophys. J. **84**: 4053-4061.

Kaukinen, P., Koistinen, V., Vapalahti, O., Vaheri, A. and Plyusnin, A. (2001). Interaction between molecules of Hantavirus nucleocapsid protein. J. Gen. Virol. **82**: 1845-1853.

Kaukinen, P., Kumar, V., Tulimaki, K., Engelhardt, P., Vaheri, A. and Plyusnin, A. (2004). Oligomerization of hantavirus N protein: C-terminal alpha-helices interact to form a shared hydrophobic space. J. Virol. **78**: 13669-13677

Kaukinen, P., Vaheri, A. and Plyusnin, A. (2003). Mapping the regions involved in homotypic interactions of Tula hantavirus N protein. J. Virol. **77**: 10910-10916.

Kellman, J.-W., Liebisch, P., Schmitz, K.-P. and Piechulla, B. (2001). Visual representation by atomic force microscopy (AFM) of tomato spotted wiltvirus ribonucleoproteins. Biol. Chem. **382**: 1559-1562.

Kermode, A. R. (1996). Mechanisms of intracellular protein transport and targetting in plant cells. Crit. Rev. Plant Sci **15**: 285-423.

Kikkert, M. (1999). Role of the envelope glycoproteins in the infection cycle of Tomato spotted wilt virus. PhD thesis, Wageningen Agricultural University, The Netherlands.

Kikkert, M., Meurs, C., van de Wetering, F., Kormelink, R. and Goldbach, R. (1998). Binding of Tomato spotted wilt virus to a 94 kDa thrips protein. Phytopathology **88**: 63-69.

Kikkert, M., van Lent, J., Storms, M., Bodegom, P., Kormelink, R. and Goldbach, R. (1999). Tomato spotted wilt virus particle morphogenesis in plant cells. J. Virol. **73**: 2288-2297.

Kikkert, M., van Poelwijk, F., Storms, M., Bloksma, H., Karsies, W., Kormelink, R. and Goldbach, R. (1997). A protoplast system for studying tomato spotted wilt virus infection. J. Gen. Virol. **78**: 1755-1763.

Kikkert, M., Verschoor, A., Kormelink, R., Rottier, P. and Goldbach, R. (2001). Tomato spotted wilt virus glycoproteins exhibit trafficking and localization signals that are functional in mammalian cells. J. Virol. **75**: 1004-1012.

Kinjo, M. and Rigler, R. (1995). Ultrasensitive hybridization analysis using fluorescence correlation spectroscopy. Nucleic Acids Res. **23**: 1795-1799.

Kitajima, E. W., de Ávila, A. C., Resende, R. de O., Goldbach, R. W. and Peters, D. (1992). Comparative cytological and immunogold labelling studies on different isolates of Tomato spotted wilt virus. J. Submicrosc. Cytol. Pathol. **24**: 1-14.

Kochs, G., Janzen, C., Hohenberg, H. and Haller, O. (2002). Antivirally active MxA protein sequesters La Crosse virus nucleocapsid protein into perinuclear complexes. Proc. Natl. Acad. Sci. USA **99**: 3153-3158.

Kohl, A., Clayton, R. F., Weber, F., Bridgen, A., Randall, R. E. and Elliott, R. M. (2003). Bunyamwera virus nonstructural protein NSs counteracts interferon regulatory factor 3-mediated induction of early cell death. J. Virol. **77**: 7999-8008.

Köhler, R. H., Cao, J., Zipfel, W. R., Webb, W. W. and Hanson, M. R. (1997). Exchange of protein molecules through connections between higher plant plastids. Science **276**: 2039-2042.

Kormelink, R. (1994). Structure and expression of the Tomato spotted wilt virus genome, a plant-infecting Bunyavirus. PhD thesis, Wageningen Agricultural, The Netherlands.

Kormelink, R., De Haan, P., Meurs, C., Peters, D. and Goldbach, R. (1992a). The nucleotide sequence of the M RNA segment of Tomato spotted wilt virus: A bunyavirus with two ambisense RNA segments. [published erratum appears in J. Gen. Virol. 1993 Apr;74(Pt 4):790] J. Gen. Virol. **73**: 2795-2804.

Kormelink, R., de Haan, P., Peters, D. and Goldbach, G. (1992b). Viral RNA synthesis in Tomato spotted wilt virus-infected *Nicotiana rustica* plants. J. Gen. Virol. **73**: 687-693.

Kormelink, R., Kitajima, E. W., de Haan, P., Zuidema, D., Peters, D. and Goldbach, R. (1991). The nonstructural protein (NSs encoded by the ambisense S RNA of Tomato spotted wilt virus is associated with fibrous structures in infected plant cells. Virology **181**: 459-468.

Kormelink, R., Storms, M., van Lent, J., Peters, D. and Goldbach, R. (1994). Expression and subcellular location of the NSm protein of Tomato spotted wilt virus (TSWV), a putative viral movement protein. Virology **200**: 56-65.

Kormelink, R., Van Poelwijk, F., Peters, D. and Goldbach, R. (1992c). Non-viral heterogeneous sequences at the 5' ends of tomato spotted wilt virus (TSWV) mRNAs. J. Gen. Virol. **73**: 2125-2128.

Kuismanen, E., Bang, B., Hurme, M. and Pettersson, R. F. (1984). Uukuniemi virus maturation: immunofluorescence microscopy with monoclonal glycoprotein-specific antibodies. J. Virol. **51**: 137-146.

Kuismanen, E., Hedman, K., Saraste, J. and Pettersson, R. F. (1982). Uukuniemi virus maturation: accumulation of virus particles and viral antigens in the Golgi complex. Mol. Cell Biol. **2**: 1444-1458.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685.

Lambert, A.-M. (1993). Microtubule-organizing centers in higher plants. Curr. Op. Cell Biol. **5**: 116-122.

Lappin, D. F., Nakitare, G. W., Palfreyman, J. W. and Elliott, R. M. (1994). Localization of Bunyamwera bunyavirus G1 glycoprotein to the Golgi requires association with G2 but not with NSm. J. Gen. Virol. **75**: 3441-3451.

Larson, D. R., Ma, Y. M., Vogt, V. M. and Webb, W. W. (2003). Direct measurement of Gag-Gag interaction during retrovirus assembly with FRET and fluorescence correlation spectroscopy. J. Cell Biol. **162**: 1233-1244.

Law, M. D., Speck, J. and Moyer, J. W. (1992). The M RNA of Impatiens necrotic spot Tospovirus (*Bunyaviridae*) has an ambisense genomic organization. Virology **188**: 732-741.

Lawson, R. H., Dienelt, M. M. and Hsu, H. T. (1996). Ultrastructural comparisons of defective, partially defective, and nondefective isolates of Impatiens necrotic spot virus. Phytopathology **86**: 650-661.

Lee, B.-H., Yoshimatsu, K., Maeda, A., Ochiai, K., Morimatsu, M., Araki, K., Ogino, M., Morikawa, S. and Arikawa, J. (2003). Association of the nucleocapsid protein of the Seoul and Hantaan hantaviruses with small ubiquitin-like modifier-1-related molecules. Vir. Res. **98**: 83-91.

Lees, J. F., Pringle, C. R. and Elliott, R. M. (1986). Nucleotide sequence of the Bunyamwera virus M RNA segment: conservation of structural features in the Bunyavirus glycoprotein gene product. Virology **148**: 1-14.

Lenz, O., ter Meulen, J., Feldmann, H., Klenk, H. D. and Garten, W. (2000). Identification of a novel consensus sequence at the cleavage site of the lassa virus glycoprotein. J. Virol. **74**: 11418-11421.

Lenz, O., ter Meulen, J., Klenk, H. D., Seidah, N. G. and Garten, W. (2001). The Lassa virus glycoprotein precursor GP-C is proteolytically processed by subtilase SKI-1/S1P. Proc. Natl. Acad. Sci. USA **98**: 12701-12705.

Leonard, S., Viel, C., Beauchemin, C., Daigneault, N., Fortin, M. G. and Laliberte, J. F. (2004). Interaction of VPg-pro of Turnip mosaic virus with the translation initiation factor 4E and the poly(A)-binding protein *in planta*. J. Gen. Virol. **85**: 1055-1063.

Letourneur, F., Gaynor, E. C., Hennecke, S., Démollière, C., Duden, R., Emr, S. D., Riezman, H. and Cosson, P. (1994). Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. Cell **79**: 1199-1207.

Li, X. D., Makela, T. P., Guo, D., Soliymani, R., Koistinen, V., Vapalahti, O., Vaheri, A., Lankinen, H. (2002). Hantavirus nucleocapsid protein interacts with the Fas-mediated apoptosis enhancer Daxx. J. Gen. Virol. **83**: 759–766.

Lidke, D. S., Nagy, P., Barisas, B. G., Heintzmann, R., Post, J. N., Lidke, K. A., Clayton, A. H. A., Arndt-Jovin, D. J. and Jovin, T. M. (2003). Imaging molecular interactions in cells by dynamic and static fluorescence anisotropy (rFLIM and rFRET). Biochem. Soc. Trans. **31**: 1020-1027.

Liljeström, P. and Garoff, H. (1991). A new generation of animal cell expression vectors based on the Semliki forest virus replicon. Bio/technology **9**:1356-1361.

Liu, J. and Lu, Y. (2002). FRET study of a trifluorophore-labeled DNAzyme. J. Am. Chem. Soc. **124**: 15208-15216.

Liu, T. and Ye, Z. (2002). Restriction of viral replication by mutation of the influenza virus matrix protein. J. Virol. **76**:13055-13061.

Liu, T. and Ye, Z. (2004). Introduction of a temperature-sensitive phenotype into influenza A/WSN/33 virus by altering the basic amino acid domain of influenza virus matrix protein. J. Virol. **78**:9585-9591.

Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G. and Tsien, R. Y. (1998). Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. Proc. Natl. Acad. Sci. USA. **95**:6803-6808.

Löber, C., Anheier, B., Lindow, S., Klenk, H. D. and Feldmann, H. (2001). The Hantaan virus glycoprotein precursor is cleaved at the conserved pentapeptide WAASA. Virology **289**: 224-229.

Lopez, N., Muller, R., Prehaud, C. and Bouloy, M. (1995). The L protein of Rift valley fever virus can rescue viral ribonucleoproteins and transcribe synthetic genome-like RNA molecules. J. Virol. **69**: 3972-3979.

Ludwig, G. V., Christensen, B. M., Yuill, T. M. and Schultz, K. T. (1989). Enzyme processing of LaCrosse virus glycoprotein G1: a bunyavirus-vector infection model. Virology **171**: 108-113.

Ludwig, G. V., Israel, B. A., Christensen, B. M., Yuill, T. M. and Schultz, K. T. (1991). Role of La Crosse virus glycoproteins G1 in attachment of virus to host cells. Virol **181**: 564-571.

Lyons, M. and Heyduk, J. (1973). Aspects of the developmental morphology of California encephalitis virus in cultured vertebrate and arthropod cells and in mouse brain. Virology **54**: 37-52.

Maiss, E., Ivanova, L., Breyel, E. and Adam, G. (1991). Cloning and sequencing of the S RNA from a Bulgarian isolate of tomato spotted wilt virus. J. Gen. Virol. **72**: 461-464.

Martín-Benito, J., Area, E., Ortega, J., Llorca, O., Valpuesta, J. M., Carrascosa, J. L. and Ortín, J. (2001). Three-dimesional reconstruction of a recombinant influenza virus ribonucleoprotein particle. EMBO Rep. **2**: 313-317.

Matsuda, D. and Dreher, T. W. (2004). The tRNA-like structure of Turnip yellow mosaic virus RNA is a 3'-translational enhancer. Virology **321**: 36-46.

Matsuoka, Y., Ihara, T., Bishop, D. H. L. and Compans, R. W. (1988). Intracellular accumulation of punta toro glycoproteins expressed from cloned cDNAs. Virol. **167**: 251-260.

Matsuoka, Y., Chen, S. Y. and Compans, R. W. (1994). A signal for Golgi retention in the Bunyavirus G1 glycoprotein. J. Biol. Chem. **269**: 22565-22573.

Matsuoka, Y., Chen, S. Y., Holland, C. E. and Compans, R. W. (1996). Molecular determinants of Golgi retention in the Punta Toro virus G1 protein. Arch. Biochem. Biophys. **336**: 184-189.

McCaughey, C., Shi, X., Elliott, R. M., Wyatt, D. E., O'Neill, H. J. and Coyle, P. V. (1999). Low pH-induced cytopathic effect – a survey of seven hantavirus strains. J Virol Meth **81**: 193-197.

Medeiros, R. B., Figueiredo, J., Resende, R. de O. and Ávila, A. C. (2005). Expression of a viral polymerase–bound host factor turns human cell lines permissive to a plant- and insect infecting virus. Proc. Natl. Acad. Sci. USA **102**: 1175-1180.

Medeiros, R. B., Resende, R. de O. and de Ávila, A. C. (2004). The plant virus *Tomato Spotted Wilt Tospovirus* activates the immune system of its main insect vector, *Frankliniella occidentalis*. J. Virol. **78**: 4976–4982.

Medeiros, R. B., Ullman, D. E., Sherwood, J. L. and German, T. L. (2000). Immunoprecipitation of a 50-kDa protein: a candidate receptor component for tomato spotted wilt tospovirus (*Bunyaviridae*) in its main vector, *Frankliniella occidentalis*. Vir. Res. **67**: 109-118.

Meulewaeter, F., van Lipzig, R., Gultayev, A. P., Pleij, C. W., van Damme, D., Cornelissen, M. and van Eldik, G. (2004). Conservation of RNA structures enables TNV and BYDV 5' and 3' elements to cooperate synergistically in cap-independent translation. Nucl. Acids. Res. **32**: 1721-1730

Milne, R. G. (1970). An electron microscopic study of Tomato spotted wilt virus in sections of infected cells and in negative stain preparations. J. Gen. Virol. **6**: 267-276.

Mir, M. and Panganiban, A. (2004). Trimeric hantavirus nucleocapsid protein binds specifically to the viral RNA panhandle. J. Virol. **78**: 8281-8288.

Misumi, Y., Sohda, M., Tashiro, A., Sato, H. and Ikehara, Y. (2001). An essential cytoplasmic domain for the Golgi localization of coiled-coil proteins with a COOH-terminal membrane anchor. J. Biol. Chem. **276**: 6867-6873.

Mohamed, N. A. (1981). Isolation and characterisation of subviral structures from Tomato spotted wilt virus. J. Gen. Virol. **53**: 197-206.

Mohamed, N. A., Randles, J. W. and Francki, R. I. B. (1973). Protein composition of Tomato spotted wilt virus. Virology **56**: 12-21.

Mossessova, E., Bickford, L. C. and Goldberg, J. (2003). SNARE selectivity of the COPII coat. Cell **114**: 483-495.

Mozdzanowski, J., Bongers, J. and Anumula, K. (1997). High-yield deblocking of amino termini of recombinant immunoglobulins with pyroglutamate aminopeptidase. Anal. Biochem. **260**: 183-187.

Munro, S. (1998). Localization of proteins to the Golgi apparatus. Trends Cell Biol. **8:**11–15.

Murchie, A. I. H., Davis, B., Isel, C., Afshar, M., Drysdale, M. J., Bower, J., Potter, A. J., Starkey, I. D., Swarbrick, T. M., Mirza, S., Prescott, C. D., Vaglio, P., Aboul-ela, F. and Karn, J. (2003). Structure-based drug design targeting an inactive RNA

conformation: Exploiting the flexibility of HIV-1 TAR RNA. J. Mol. Biol. **336**: 625-638.

Murphy, F. A., Harrison, A. K. and Whitfield, S. G. (1973). Bunyaviridae: Morphologic and morphogenetic similarities of Bunyamwera serologic supergroup viruses and several other arthropod-borne viruses. Intervirol. **1**; 297-316.

Nagata, T., Inoue-Nagata, A. K., Prins, M., Goldbach, R. and Peters, D. (2000). Impeded thrips transmission f defective Tomato spotted wilt virus isolates. Phytop. **90**: 454-459.

Nagata, T., Inoue-Nagata, A. K., Smid, H. M., Goldbach, R. and Peters, D. (1999). Tissue tropism related to vector competence of Frankliniella occidentalis for tomato spotted wilt tospovirus. J Gen Virol **80**: 507-515.

Nagata, T., Inoue-Nagata, A. K., van Lent, J., Goldbach, R. and Peters, D. (2002). Factors determining vector competence and specificity for transmission of Tomato spotted wilt virus. J. Gen. Virol. **83**: 663-671.

Nagy, P., Vamosi, G., Bodnar, A., Lockett, S. J. and Szollosi, J. (1998). Intensity-based energy transfer measurements in digital imaging microscopy. Europ. Biophys. J. **27**: 377-389.

Naidu, R. A., Ingle, C. J., Deom, C. M. and Sherwood, J. L. (2004). The two membrane glycoproteins of Tomato spotted wilt virus show differences in lectin-binding properties and sensitivities to glycosidases. Virology **319**: 107-117.

Nakitare, G. W., and Elliot, R. M. (1993). Expression of the Bunyamwera virus M genome segment and intracellular localization of NSm. Virol. **195:**511–520.

Neeleman, L., Olsthoorn, R. C. L, Linthorst, H. J. M. and Bol, J. F. (2001). Translation of a nonpolyadenylated viral RNA is enhanced by binding of viral coat protein or polyadenylation of the RNA. Proc. Natl. Acad. Sci. USA **98**: 14286-14291.

Nehls, S., Snapp, E. L., Cole, N. B., Zaal, K. J. M., Kenworthy, A. K., Roberts, T. H., Ellenberg, J., Presley, J. F., Siggia, E. and Lippincott-schwartz, J. (2000). Dynamics and retention of misfolded proteins in native ER membranes. Nature Cell Biol. **2**: 288-295.

Nishimura, N., Bannykh, S., Slabough, S., Matteson, J., Altschuler, Y., Hahn, K. and Balch, W. E. (1999). A di-acidic (DXE) code directs concentration of cargo during export from the endoplasmic reticulum. J. Biol. Chem. **274**: 15937-15946.

Obijeski, J. F. and Murphy, F. A. (1977). *Bunyaviridae*: recent biochemical developments. J. Gen. Virol. **37**: 1-14

Objieski, J. F., Bishop, D. H. L., Palmer, E. L. and Murphy, F. A. (1976). Segmented genome and nucleocapsid of La Crosse virus. J. Gen. Virol. **20**: 664-675.

Ogino, M., Yoshimatsu, K., Ebihara, H., Araki, K., Lee, B.-H., Okumura, M. and Arikawa, J. (2004). Cell fusion activities of hantaan virus envelope glycoproteins. J Virol **78**: 10776-10782.

Ortiz de Zarate, I. B., Kaelin, K. and Rozenberg, F. (2004). Effects of mutations in the cytoplasmic domain of herpes simplex virus type I glycoprotein B on intracellular transport and infectivity. J. Virol. **78**: 1540-1551.

Patterson, J. L. and Kolakofsky, D. (1984). Characterization of La Crosse virus small-genome transcripts. J. of Virol. **49**: 680-685.

Patterson, J. L., Holloway, B. and Kolakofsky, D. (1984). La Crosse virions contain a primer-stimulated RNA polymerase and a methylated cap-dependent endonuclease J. of Virol. **52**: 215-222.

Patton, J. T., Davis, N. L. and Wertz, G. W. (1984). Nucleocapsid protein alone satisfies the requirement for protein synthesis during RNA replication of Vesicular stomatitis virus. J. Virol. **49**: 303-309.

Pekosz, A., Griot, C., Nathanson, N. and González-Scarano, F. (1995). Tropism of bunyaviruses: Evidence for a G1 glycoprotein-mediated entry pathway common to the California serogroup. Virology **214**: 339-348.

Pekosz, A. and González-Scarano, F. (1996). The extracellular domain of La Crosse virus G1 forms oligomers and undergoes pH-dependent conformational changes. Virology **225**: 243-247.

Pekosz, A., Griot, C., Nathanson, N. and González-Scarano, F. (1995). Tropism of bunyaviruses: Evidence for a G1 glycoprotein-mediated entry pathway common to the California serogroup. Virol. **214**: 339-348.

Persson, R. and Pettersson, R. F. (1991). Formation and intracellular transport of a heterodimeric viral spike protein complex. J. Cell Biol. **112**: 257-266.

Peters, D. (1998). An updates list of plant species susceptible to tospoviruses. In: Peters, D. and Goldbach, R (eds.). Recent progress in tospovirus and thrips research, Wageningen, The Netherlands: 107-110.

Petterson, R. and Kääriäinen, L. (1973). The ribonucleic acids of Uukuniemi virus, a non-cubal tick-borne arbovirus. Virology **56**: 608-619.

Plotch, S. J., Bouloy, M., Ulmanen, I. and Krug, R. M. (1981). A unique cap 7 methyl guanosine 5' tri phosphoryl 5' -2 -O methyl nucleoside dependent Influenza virion endo nuclease cleaves capped RNA to generate the primers that initiate viral RNA transcription. Cell **23**: 847 -858.

Poch, O., Sauvaget, I., Delarue, M. and Tordo, N. (1989). Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. EMBO J. **8**: 3867 -3874.

Pollok, B. A. and Heim, R. (1999). Using GFP in FRET-based applications. Trends Cell Biol. **9**: 57 -60.

Portela, A. and Digard, P. (2002). The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. J. Gen. Virol. **83**: 723 - 734.

Pouwels, J. (2004). Functional analysis of the Cowpea mosaic virus movement protein. PhD thesis, Wageningen University, The Netherlands.

Pramanik, A., Thyberg, P. and Rigler, R. (2000). Molecular interactions of peptides with phospholipid vesicle membranes as studied by fluorescence correlation spectroscopy. Chem. Phys. Lipids **104**: 35 -47.

Pratelli, R., Sutter, J. -U. and Blatt, M. R. (2004). A new catch in the SNARE. Trends Plant Sci. **9**: 187 -195.

Prehaud, C., Lopez, N., Blok, M. J., Obry, V. and Bouloy, M. (1997). Analysis of the 3' terminal sequence recognized by the Rift Valley fever virus transcription complex in its ambisense S segment. Virology **227**: 189 -197.

Raju, R. and Kolakofsky, D. (1986a). Inhibitors of protein synthesis inhibit both LaCrosse virus S messenger and S genome synthesis *in vivo*. Virus Res. **5**: 1 -10.

Raju, R. and Kolakofsky, D. (1986b). Translational requirement of LaCrosse virus small messenger RNA synthesis *in vivo* studies. J.Virol. **61**:96 -103.

Raju, R., Raju, L., Hacker, D., Garcin, D., Compans, R. and Kolakofsky, D. (1990). Nontemplated bases at the 5' ends of Tacaribe virus messenger RNA. Virology **174**: 53 -59.

Ramirez, B. -C., Garcin, D., Calvert, L. A., Kolakofsky, D. and Haenni, A. -L. (1995). Capped nonviral sequences at the 5' end of the mRNAs of rice hoja blanca virus RNA4. J. Virol. **69**: 1951 -1954

Ranki, M. and Pettersson, R. F. (1975). Uukuniemi virus contains an RNA polymerase. J. of Virol. **16**:1420 -1425.

Rauer, B., Neumann, E., Widegren, J. and Rigler, R. (1996). Fluorescence correlation spectrometry of the interaction kinetics of tetramethylrhodamine alpha-bungarotoxin with *Torpedo californica* acetylcholine receptor. Biophys. Chem. **58**: 3-12.

Ravkov, E. V. and Compans, R. W. (2001). Hantavirus nucleocapsid protein is expressed as a membrane-associated protein in the perinuclear region. J. Virol. **75**: 1808-1815.

Ravkov, E. V., Nichol, S. T. and Compans, R. W. (1997). Polarized entry and release in epithelial cells of Black Creek Canal virus, a new world hantavirus. J. Virol. **71**: 1147-1154.

Ravkov, E. V., Nichol, S. T., Peters, C. J. and Compans, R. W. (1998). Role of actin microfilaments in Black Creek Canal virus morphogenesis. J. Virol. **72**: 2865-2870.

Reichelt, M., Stertz, S., Krijnse-Locker, J., Haller, O. and Kochs, G. (2004). Missorting of LaCrosse virus nucleocapsid protein by the interferon-induced MxA GTPase involves smooth ER membranes. Traffic **5**: 772-784.

Rhoades, E., Gussakovsky, E. and Haran, G. (2003). Watching proteins fold one molecule at a time. Proc. Natl. Acad. Sci. USA **100**: 3197-3202.

Richmond, K. E., Chenault, K., Sherwood, J. L. and German, T. L. (1998). Characterization of the nucleic acid binding properties of tomato spotted wilt virus nucleocapsid protein. Virology **248**: 6-11.

Rietdorf, J., Ploubidou, A., Reckmann, I.,Holmström, A., Frischknecht, F.,Zettl, M., Zimmermann, T. and Way, M. (2001). Kinesin-dependent movement on microtubules precedes actin-based motility of vaccinia virus. Nature Cell Biology **3**:992-1000.

Rönkä, H., Hildén, P., von Bonsdorff, C. H. and Kuismanen, E. (1995). Homodimeric association of the spike glycoproteins G1 and G2 of Uukuniemi virus. Virology **211**: 241-250.

Rönnholm, R. (1992). Localization to the Golgi complex of Uukuniemi virus glycoproteins G1 and G2 expressed from cloned cDNAs. J. Virol. **66**: 4525-4531.

Rönnholm, R. and Pettersson, R. F. (1987). Complete nucleotide sequence of the M RNA segment of Uukuniemi virus encoding the membrane glycoproteins G1 and G2. Virology **160**: 191-202.

Rose, J. K. and Bergmann, J. E. (1982). Expression from cloned cDNA of cell-surface secreted forms of the glycoprotein of vesicular stomatitis virus in eucaryotic cells. Cell **30**: 753-762.

Rose, J. K. and Bergmann, J. E. (1983). Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. Cell **34**: 513-524.

Rupp, R. A. W., Snider, L. and Weintraub, H. (1994). Xenopus embryos regulate the nuclear localization of XmyoD. Genes Dev. **8**:1311-1323.

Ruusala, A., Persson, R., Schmaljohn, C. S. and Pettersson, R. F. (1992). Coexpression of the membrane glycoproteins G1 and G2 of Hantaan virus is required for targeting to the Golgi complex. Virology **186**: 53-64.

Rwambo, P. M., Shaw, M. K., Rurangirwa, F. R. and deMartini, J. C. (1996). Ultrastructural studies on the replication and morphogenesis of Nairobi sheep disease virus, a Nairovirus. Arch. Virol. **141**: 1479-1492.

Salanueva, I. J., Novoa, R. R., Cabezas, P., Lopez-Iglesias, C., Carrascosa, J. L., Elliott, R. M. and Risco, C. (2003). Polymorphism and structural maturation of Bunyamwera virus in golgi and post-golgi compartments. J. Virol. **77**: 1368-1381.

Sanchez, A. J., Vincent, M. J. and Nichol, S. T. (2002). Characterization of the glycoproteins of Crimean-Congo hemorrhagic fever virus. J. Virol. **76**: 7263-7275.

Sato, M., Ozawa, T., Inukai, K., Asano, T. and Umezawa, Y. (2002). Fluorescent indicators for imaging protein phosphorylation in single living cells. Nat. Biotechnol. **20**: 287 - 294.

Schmaljohn, C. and Dalrymple, J.M. (1983). Analysis of Hantaan virus RNA: Evidence for a new genus of Bunyaviridae. Virol. **131**: 482-491.

Schmaljohn, C. S., Schmaljohn, A. L. and Dalrymple, J. M. (1987). Hantaan virus M RNA coding strategy, nucleotide sequence and gene order. Virology **157**: 31-39.

Schoehn, G., Iseni, F., Mavrakis, M., Blondel, D. and Ruigrok, R. W. H. (2001). Structure of recombinant rabies virus nucleoprotein-RNA complex and identification of the phosphoprotein binding site. J. Virol. **75**: 490-498.

Schweizer, A., Fransen, J. A. M., Bachi, T., Ginsel, L. and Hauri, H. P. (1988). Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubulo-vesicular compartment at the cis-side of the Golgi apparatus. J. Cell Biol. **107:**1643-1654.

Sciaky, N., Presley, J., Smith, C. L., Zaal, K. J. M., Cole, N., Moreira, J. E., Terasaki, M., Siggia, E. and Lippincott Schwartz, J. (1997). Golgi tubule traffic and the effects of Brefeldin A visualized in living cells. J. Cell Biol. **139**: 1137-1155.

Sekar, R. B. and Periasamy, A. (2003). Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. J. Cell Biol. **160**: 629-633.

Severson, W., Partin, L., Schmaljohn, C. and Jonsson, C. B. (1999). Characterization of the Hantaan virus nucleocapsid protein-ribonucleic acid interaction. J. Biol. Chem. **274**: 33732-33739.

Severson, W. E., Xu, X. and Jonsson, C. B. (2001). Cis-acting signals in encapsidation of Hantaan virus S-segment viral genomic RNA by its N protein. J. Virol. **75**: 2646-2652.

Sfakianos, J. N. and Hunter, E. (2003). M-PMV capsid transport is mediated by Env/Gag interactions at the pericentriolar recycling endosome. Traffic **4**:671-680.

Sfakianos, J. N., LaCasse, R. A. and Hunter, E. (2003). The M-PMV cytoplasmic targeting-retention signal directs nascent Gag polypeptides to a pericentriolar region of the cell. Traffic **4**:660-670.

Shi, X. H. and Elliott, R. M. (2002). Golgi localization of Hantaan virus glycoproteins requires coexpression of G1 and G2. Virology **300**: 31-38.

Shi, X.H. and Elliott, R.M. (2004). Analysis of N-linked glycosylation of Hantaan virus glycoproteins and the role of oligosaccharide side chains in protein folding and intracellular trafficking. J. Virol. **78**: 5414-5422.

Shi, X.H., Lappin, D.F. and Elliott, R.M. (2004). Mapping the Golgi targeting and retention signal of Bunyamwera virus glycoproteins. J. Virol. **78**: 10793-10802.

Shimi, T., Koujin, T., Segura-Totten, M., Wilson, K. L., Haraguchi, T. and Hiraoka, Y. (2004). Dynamic interaction between BAF and emerin revealed by FRAP, FLIP, and FRET analyses in living HeLa cells. J. Stuct. Biol. **147**: 31-41.

Shimizu, T., Toriyama, S., Takahashi, M., Akutsu, K. and Yoneyama, K. (1996). Non-viral sequences at the 5' termini of mRNAs derived from virus-sense and virus-complementary sequences of the ambisense RNA segments of rice stripe Tenuivirus. J. Gen. Virol. **77**: 541-546.

Shvartsman, D. E., Kotler, M., Tall, R. D., Roth, M. G. and Henis, Y. (2003). Differently anchored influenza hemagglutinin mutants display distinct interaction dynamics with mutual rafts. J. Cell Biol. **163**: 879-888.

Silva, M. S. (2004). Mechanisms underlying Cowpea mosaic virus systemic infection. PhD thesis, Wageningen University, The Netherlands.

Silva, M. S., Wellink, J., Goldbach, R. W. and van Lent, J. W. M. (2002). Phloem loading and unloading of Cowpea mosaic virus in *Vigna unguiculata*. J. Gen. Virol. **83**: 1493-1504.

Simons, J. F., Persson, R. and Pettersson, R. F. (1992). Association of the nonstructural protein NSs of Uukuniemi virus with the 40S ribosomal subunit. J. Virol. **66**: 4233-4241.

Simons, J. F. and Pettersson, R. F. (1991). Host-derived 5' ends and overlapping complementary 3' ends of the two messenger RNAs transcribed from the ambisense S segment of Uukuniemi virus. J. Virol. **65**: 4741-4748.

Smith, J. F. and Pifat, D. Y. (1982). Morphogenesis of sandfly fever viruses *Bunyaviridae* family. Virology **121** 61-81.

Soellick, T. R., Uhrig, J. F., Bucher, G. L., Kellmann, J. W. and Schreier, P. H. (2000). The movement protein NSm of Tomato spotted wilt tospovirus (TSWV): RNA binding, interaction with the TSWV N protein, and identification of interacting plant proteins. Proc. Natl. Acad. Sci. USA **97**: 2373-2378.

Soldan, S. S., Plassmeyer, M. L., Matukonis, M. K. and González-Scarano, F. (2005). LaCrosse virus nonstructural protein NSs counteracts the effects of short interfering RNA. J. Virol. **79**: 234-244.

Spiropoulou, C. F., Goldsmith, C. S., Shoemaker,T. R., Peters,C. J. and Compans, R. W. (2003). Sin Nombre virus glycoprotein trafficking. Virol. **308**: 48-63.

Storms, M. M. H., Kormelink, R., Peters, D., van Lent, J. W. M. and Goldbach, R. W. (1995). The nonstructural NSm protein of Tomato spotted wilt virus induces tubular structures in plant and insect cells. Virology **214**: 485-493.

Storms, M. M. H., van der Schoot, C., Prins, M., Kormelink, R., van Lent, J. W. M. and Goldbach, R. W. (1998). A comparison of two methods of microinjection for assessing altered plasmodesmal gating in tissues expressing viral movement proteins. Plant J. **13**: 131-140.

Sundin, D. R., Beaty, B. J., Nathanson, N. and González-Scarano, F. (1987). A G1 glycoprotein epitope of La Crosse virus: a determinant of infection of Aedes triseriatus. Science **235**: 591-592.

Takeda, A., Sugiyama, K., Nagano, H., Mori, M., Kaido, M., Mise, K., Tsuda, S. and Okuno, T. (2002). Identification of a novel RNA silencing suppressor, NSs protein of Tomato spotted wilt virus. FEBS lett. **532**: 75-79.

Tas, P. W. L., Boerjan, M. L. and Peters, D. (1977). The structural proteins of Tomato spotted wilt virus. J. Gen. Virol. **36**: 267-279.

Thomas, D. and Roth, M. G. (1994). The basolateral targeting signal in the cytoplasmic domain of glycoprotein G from vesicular stomatitis virus resembles a variety of intracellular targeting motifs related by primary sequence but having diverse targeting activities. J. Biol. Chem. **269**: 15732-15739.

Toomre, D., Keller, P., White, J., Olivo, J.-C. and Simons, K. (1999). Dual-color visualization of trans-Golgi network to plasma membrane traffic along microtubules in living cells. J. Cell Sci. **112**: 21-33.

Tordo, N., de Haan, P., Goldbach, R. and Poch, O. (1992). Evolution of negative – stranded RNA genomes. Sem. Virol. **3**: 341-357.

Truong, K. and Ikura, M. (2001). The use of FRET imaging microscopy to detect protein-protein interactions and protein conformational changes in vivo. Curr. Opin. Struct. Biol. **11**: 573-578.

Tsuda, S., Fujisawa, I., Ohnishi, J., Hosokawa, D. and Tomaru, K. (1996). Localization of Tomato spotted wilt tospovirus in larvae and pupae of the insect vector *Thrips setosus*. Phytopathology **86**: 1199-1203.

Uhrig, J. F., Soellick, T. R., Minke, C. J., Philipp, C., Kellmann, J. W. and Schreier, P. H. (1999). Homotypic interaction and multimerization of nucleocapsid protein of Tomato spotted wilt tospovirus: identification and characterization of two interacting domains. Proc. Natl. Acad. Sci. USA **96**: 55-60.

Ullman, D. E., Cho, J. J., Mau, R. F. L., Westcoc, D. M. and Custer, D. M. (1992). A midgut barrier to Tomato spotted wilt virus acquisition by adult Western flower thrips. Phytopathology **82**: 1333-1342.

Ullman, D. E., German, T. L., Sherwood, J. L., Westcot, D. M. and Cantone, F. A. (1993). Tospovirus replication in insect vector cells: Immunocytochemical evidence that the nonstructural protein encoded by the S RNA of Tomato spotted wilt tospovirus is present in thrips vector cells. Phytopathology **83**: 456-463.

Ullman, D. E., Westcot, D. M., Chenault, K. D., Sherwod, J. L., German, T. L., Bandla, M. D., Cantone, F. A. and Duer, H. L. (1995). Compartmentalization, intracellular transport, and autophagy of tomato spotted wilt tospovirus proteins in infected thrips cells. Phytopathology **85**: 644-654.

Ulmanen, I., Broni, B. A. and Krug, R. M. (1981). Role of 2 of the Influenza virus core P proteins in recognizing cap 1 structures on RNA and in initiating viral RNA transcription. Proc. Natl. Acad. Sci. USA **78**: 7355-7359.

Ungar, D. and Hughson, F. M. (2003). SNARE protein structure and function. Annu. Rev. Cell Dev. Biol. **19**: 493-517.

van den Hurk, J., Tas, P. W. L. and Peters, D. (1977). The RNA of Tomato spotted wilt virus. J. Gen. Virol. **36**: 81-91.

van Knippenberg, I. (2005). Analysis of Tomato spotted wilt virus genome transcription. PhD thesis, Wageningen University, The Netherlands.

van Knippenberg, I., Goldbach, R. and Kormelink, R. (2002). Purified Tomato spotted wilt virus particles support both genome replication and transcription *in vitro*. Virology **303**: 278-286.

van Knippenberg, I., Goldbach, R. and Kormelink, R. (2004). *In vitro* transcription of TSWV is independent of translation. J. Gen. Virol. **85**: 1335-1338.

van Kuppeveld, F. J. M., Melchers, W. J. G., Willems, P. H. G. M. and Gadella, T. W. J. Jr. (2002). Homomultimerization of the coxsackievirus 2B protein in living cells visualized by fluorescence resonance energy transfer microscopy. J. Virol. **76**: 9446-9456.

van Poelwijk, F., Boye, K., Oosterling, R., Peters, D. and Goldbach, R. (1993). Detection of the L protein of Tomato spotted wilt virus. Virology **197**: 468-470.

van Poelwijk, F., Kolkman, J. and Goldbach, R. (1996). Sequence analysis of the 5' ends of Tomato spotted wilt virus N mRNAs. Arch. Virol. **141**: 177-184.

Veijola, J. and Petterson, R. F. (1999). Transient association of calnexin and calreticulin with newly synthesized G1 and G2 glycoproteins of Uukuniemi virus (family *Bunyaviridae*). J. Virol. **73**: 6123-6127.

Verkleij, F. N., de Vries, P. and Peters, D. (1982). Evidence that Tomato spotted wilt virus RNA is a positive strand. J. Gen. Virol. **58**: 329-338.

Verkley, F. N. and Peters, D. (1983). Characterization of a defective form of Tomato spotted wilt virus. J. Gen. Virol. **64**: 677-682.

Vialat, P. and Bouloy, M. (1992). Germiston virus transcriptase requires active 40S riboosomal subunits and utilizes capped cellular RNAs J. of Virol. **66**: 685-693.

Vincent, M. J.,Sanchez, A. J., Erickson, B. R., Basak, A., Chretien, M., Seidah, N. G. and Nichol, S. T. (2003). Crimean Congo Hemorrhagic fever virus glycoprotein proteolytic processing by subtilase SKI-1. J. Virol. **77**: 8640-8649.

Violin, J. D., Zhang, J., Tsien, R. Y. and Newton, A. C. (2003). A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. J. Cell Biol. **161**: 899-909.

Visser, A. J. W. G. and Hink, M. (1999). New perspectives of Fluorescence Correlation Spectroscopy. J. Fluorescence **9**: 81-87.

von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. Nucl. Acids. Res. **14**: 4683-4690.

Votsmeier, C. and Gallwitz, D. (2001). An acidic sequence of a putative yeast Golgi membrane protein binds COPII and facilitates ER export. EMBO J. **20**: 6742-6750.

Walter, M., Chaban, C., Schutze, K., Batistic, O., Weckermann, K., Nake, C., Blazevic, D., Grefen, C., Schumacher, K., Oecking, C., Harter, K. and Kudla, J.(2004). Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J. **40**: 428-438.

Watkins, C. A. and Jones, I. M. (1993). Association of the 40S ribosomal subunit with the NSs nonstructural protein of Punta Toro virus. Abstr. Int. Congres Virol., Glasgow, Scotland: 136.

Watrob, H. M., Pan, C. P. and Barkley, M. D. (2003). Two-step FRET as a structural tool. J. Am. Chem. Soc. **125**: 7336-7343.

Weber, F., Bridgen, A., Fazakerley, J. K., Stretenfeld, H., Kessler, N., Randall, R. E. and Elliott, R. M. (2002). Bunyamwera Bunyavirus nonstructural protein NSs counteracts the induction of alpha/beta interferon. J. Virol. **76**: 7949-7955.

Weber, F., Dunn, E. F., Bridgen, A. and Elliott, R. M. (2001). The Bunyamwera virus nonstructural protein NSs inhibits viral RNA synthesis in a minireplicon system. Virology **281**: 67-74.

White, J. and Stelzer, E. (1999). Photobleaching GFP reveals protein dynamics inside live cells. Trends Cell Biol. **9**: 61-65.

Whitfield, A. E., Ullman, D. E. and German, T. L. (2004). Expression and characterization of a soluble form of Tomato spotted wilt virus glycoprotein GN. J. Virol. **78**: 13197-13206.

Whitfield, A. E., Ullman, D. E. and German, T. (2005). Tomato spotted wilt virus glycoprotein Gc is cleaved at acidic pH. Vir. Res. **110**: 183-186.

Wijkamp, I. (1995). Virus-vector relationships in the transmission of tospoviruses. PhD thesis, Wageningen University, The Netherlands.

Wijkamp, I., van Lent, J., Kormelink, R., Goldbach, R. and Peters, D. (1993). Multiplication of Tomato spotted wilt virus in its insect vector, *Frankliniella occidentalis*. Phytopathology **83**: 986-991.

Xia, Z.P. and Liu, Y.H. (2001). Reliable and global measurement of fluorescence resonance energy transfer using fluorescence microscopes. Biophys. J. **81**: 2395-2402.

Xu, X., Gerard, A. L. V., Huang, B. C. B., Anderson, D. C., Payan, D. G. and Luo, L. (1998). Detection of programmed cell death using fluorescence energy transfer. Nucleic Acids Res. **26**: 2034-2035.

Xu, X., Severson, W., Villegas, N., Schmaljohn, C. and Jonsson, C. B. (2002). The RNA binding domain of the Hantaan virus N protein maps to a central, conserved region. J. Virol. **76**: 3301-3308.

Xu, Y., Piston, D. W. and Johnson, C. H. (1999). A bioluminescence resonance energy transfer (BRET) system: Application to interacting circadian clock proteins. Proc. Natl. Acad. Sci. USA **96**: 151-156.

Ye, Z., Liu, T., Offringa, D. P., McInnis, J. and Levandowski, R. A. (1999). Association of Influenza virus matrix protein with ribonucleoproteins. J. Virol. **73**: 7467-7473.

Yoshimatsu, K., Lee, B. H., Araki, K., Morimatsu, M., Ogino, M., Ebihara, H. and Arikawa, J. (2003). The multimerization of hantavirus nucleocapsid protein depends on type-specific epitopes. J. Virol. **77**: 943-952.

Yoshizaki, H., Ohba, Y., Kurokawa, K., Itoh, R. E., Nakamura, T., Mochizuki, N., Nagashima, K. and Matsuda, M. (2003). Activity of Rho-family GTPases during cell division as visualized with FRET-based probes. J. Cell Biol. **162**: 223-232.

Nawoord

Dit boekje is het resultaat van een tijd 'onderweg zijn', op weg naar nieuwe dingen, van de eerste onzekere stappen tot een steeds doelgerichter tocht. Onderweg waren behalve veel nieuwe ervaringen ook veel mensen die een eindje mee opliepen of op strategische punten klaarstonden met advies of een praatje, erg belangrijk om door te gaan als het even tegenzat, of om me een goede kant op te sturen waar ik zelf blindelings rechtdoor was gegaan. Natuurlijk bedoel ik dan in de eerste plaats mijn promotor en co-promotor: Rob, je kritische aanpak kreeg mij bij tijd en wijle boven op de kast; toch heeft deze niet alleen op onmisbare wijze bijgedragen tot de uitvoering van het onderzoek en het tot stand komen van dit werk, maar is hij zeker ook van invloed geweest op mijn kijk op onderzoek in ruimere zin. Richard, jouw dagelijkse motiverende aanwezigheid, zowel in de hoedanigheid van begeleider als in die van geïnteresseerde kamergenoot heeft mijn reis een geweldige ervaring gemaakt en gezorgd dat ik bijna iedere dag met plezier naar de vakgroep kwam. Meer dan eens nam het onderzoek dankzij jou een onverwachte zijweg die uit bleek te monden in een snellere danwel een interessante extra route. Jan, jouw hulp bij alles wat met microscopen te maken had, op willekeurige tijdstippen geboden na zinnige maar ook na nogal wat onzinnige vragen van mijn kant bleek steeds aan te moedigen en te motiveren. Ook Jan Willem zorgde er met zijn enthousiasme voor dat ik me nu met plezier en iets van deskundigheid kan bewegen in de wondere wereld van de fluorescentie-microscopie. Het lab was een plaats waar ik graag was. Dick, Henriek, Danny, Ingeborg, Theo, Daniela, Afshin, Simone, Cristiano, Etienne, Hans, Janneke, de baculo's verderop en nog wat verderop de whispo's: elke reis is een feest met jullie erbij! Davy, Leonie en Jeroen, tijdelijke reisgenoten, brachten afwisseling in het onderzoek en daarbuiten. Met wisselende resultaten op experimenteel vlak maar alles bij elkaar buitengewoon leerzaam, zeker voor mij. Buiten het lab lag een wereld aan discussies, kroeg en terras, rennen, bananen en andere dingen die mislukte proeven weer in perspectief zetten. Theo, Ingeborg, Marilia, Nina, Gorben, Mariëlle – wauw! Nog verderop bleek zelfs een wereld te bestaan waar men niet over virussen praatte: Dubbel & Dwars, de WSKOV, Cuiabá-groep, B2Z, zondagochtenden met Kas en Maaike, "Spaans": jullie zijn geweldig!
De laatste stapjes zette ik terwijl ik al in Londen zat and I want to say a very big **thank you** to George and Maddy, everyone in our office and the others at the department. A special thanks to Audrey for making me feel at home on this new and exciting journey in so little time. You are very special people and I feel very lucky to have met you all. And of course everyone from Parr's Priory: you must be just about as crazy as I am, always ready for some fun and nonsense when I least need it (Saturdays at 7 am). Karen en Daniela liepen het laatste stukje mee en hielpen met de praktische dingen die voor aankomst geregeld moeten zijn. Daniela hielp bovendien de lay-out van dit boekje terugvinden toen ik in een laatste fase toch wel een beetje zenuwachtig werd. Jan Willem, Anita en Karen hebben waarschijnlijk maar half een beeld van wat ik nu deed onderweg en daar zal dit boekje maar weinig aan veranderen. Maar jullie belangstellende aanwezigheid werd gewaardeerd! Bert, we hebben al gaande iets moois en waardevols opgebouwd en ik hoop dat we dat op een of andere manier kunnen vasthouden. Jullie hebben allemaal op jullie eigen manier een heel belangrijke bijdrage geleverd aan dit werk. Zonder jullie was ik niet hier gekomen.
Bedankt!

Curriculum vitae

Marjolein Snippe werd op 12 september 1976 geboren in Groningen. In 1994 behaalde zij haar VWO diploma aan het Praedinius Gymnasium in Groningen en na een jaar vrijwilligerswerk in Belo Horizonte, Brazilië, begon zij in 1995 aan de studie bioprocestechnologie aan wat toen nog was de Landbouwuniversiteit Wageningen. Tijdens een eerste afstudeeronderzoek werkte zij aan de structurele eiwitten van het garnalenvirus WSSV (white spot syndrome virus) bij de vakgroep Virologie van de Landbouwuniversiteit Wageningen, begeleid door ir. Mariëlle van Hulten, prof. Just Vlak en prof. Rob Goldbach. Een tweede afstudeeronderzoek betrof de immuunrespons van het doodshoofdaapje *Saimiri sciureus* op infectie met de malariaparasiet *Plasmodium falciparum*. Dit onderzoek werd uitgevoerd bij het Instituto Oswaldo Cruz in Rio de Janeiro onder begeleiding van Leonardo Carvalho en prof. Claudio Ribeiro. In 2001 studeerde zij af en begon met promotieonderzoek aan de vakgroep Virologie van (inmiddels) Wageningen Universiteit bij Dr. Richard Kormelink en prof. Rob Goldbach. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Sinds september 2005 is zij als post-doc werkzaam in de groep van dr. George Santis bij King's College London, waar zij werkt aan adenovirus-receptor interacties en hoe deze interacties processen zoals intracellulair transport en virusontmanteling beïnvloeden.

The work described in this thesis was performed at the Laboratory of Virology of Wageningen University and was financially supported by the Netherlands organisation for Scientific Research, section Earth and Life Sciences (NWO/ALW).

Education Statement of the Graduate School Experimental Plant Sciences

Start-up phase

First presentation of the project: unraveling of the dual enveloping pathways of tomato spotted wilt virus (march 2001); Writing a review or introduction chapter (january 2005)

Scientific exposure

EPS PhD student days (2001, 2003); EPS Theme 2 symposium (oral pres.) (june 2005); Dutch annual virology symposium (2001, 2002, 2004, 2005); Nederlandse kring voor plantenvirologie (november 2001); NWO-CW meeting Lunteren (november 2001); NWO-ALW meeting Lunteren (2002, 2003, 2005); Nederlandse en Duitse kring voor plantenvirologie (2001, 2005); EMBO workshop cell biology of virus infection (september 2001); Glyco XVI (august 2001); International Congress on Virology (july 2002); International conference on negative strand viruses (june 2003); European plant endomembrane group (august 2003); Focus on Microscopy (march 2005)

Presentations:

EMBO workshop cell biology of virus infection (poster) (september 2001); International Congress on Virology (poster) (july 2002); International conference on negative strand viruses (poster) (june 2003); European plant endomembrane group (poster + oral presentation) (august 2003); ALW Lunteren (oral presentation) (april 2005); Ned / Duitse kring voor plantenvirologie (oral presentation) (march 2005); Focus on Microscopy (poster) (march 2005)

IAB interview (2004)

In-depth studies

winterschool bioinformatics (december 2000); FEBS course microspectroscopy (october 2000)