CHAPTER 7

Synthesis, Assembly, and Intracellular Transport of *Bunyaviridae* Membrane Proteins

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I. INTRODUCTION

One of the original criteria for classifying, in the early 1970s, a diverse collection of serologically related and unrelated arthropod-borne viruses into a new family, *Bunyaviridae*, was the site of maturation (budding) in a perinuclear region, later unambiguously shown to represent the Golgi complex (Murphy *et al.*, 1973; Kuismanen *et al.*, 1982). With only one documented exception (Anderson and Smith, 1987), all members of this large family studied so far have been found to mature in this intracellular organelle, raising the intriguing question of the molecular and cell biological mechanisms underlying this process.

Enveloped viruses acquire their lipoprotein coat by budding through one of the cellular membranes. Many viruses, such as alpha-, arena-, orthomyxo-, paramyxo-, rhabdo-, and retroviruses, mature at the plasma membrane. In these cases, virions are released after completion of the budding process directly into the extracellular space. In many other cases, the budding occurs

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at intracellular membranes representing waystations on the exocytic pathway that normally transports secretory and membrane proteins out to the plasma membrane. These include the endoplasmic reticulum (ER; rota- and flaviviruses), the newly identified intermediate compartment located between the ER and the Golgi complex (Saraste and Kuismanen, 1992; Hauri and Schweizer, 1992) (corona- and poxviruses; Sodeik *et al.*, 1993; Krijnse-Locker *et al.*, 1994), and the Golgi complex (bunya-, corona-, and rubellaviruses). In addition, herpesviruses have picked the rather exotic inner nuclear membrane as the site of the initial budding event (for reviews see Stephens and Compans, 1988; Pettersson, 1991; Matsuoka *et al.*, 1991; Griffiths and Rottier, 1992; Hobman, 1993). In these latter cases, mature virions are released either by cell lysis (rotaviruses), or after transport of the particles within vesicles to the plasma membrane. Virions are then released on fusion of these transport vesicles with the plasma membrane.

The details of the budding process of any virus are still poorly understood. For the last two decades, it has generally been assumed that viral nucleocapsids (cores) recognize regions on the cytoplasmic face of the membrane in the budding compartment, which have been modified by the accumulation of viral membrane glycoproteins (spikes or peplomers). A specific interaction between a core protein and the cytoplasmic tail of one of the spike proteins (equivalent to a receptor) is thought to trigger budding (Garoff and Simons, 1974; Whitt *et al.*, 1989; Suomalainen *et al.*, 1992). As more such interactions are recruited, the budding process progresses and host proteins are concomitantly excluded from the bud. In some viruses (e.g., rhabdo-, orthomyxo-, paramyxo-, retroviruses), a membrane (M) protein located between the lipid bilayer and the nucleocapsid may contribute to this interaction and play a role in the budding process (Rhee and Hunter, 1991). Such an M protein is missing from viruses such as the alpha-, flavi-, rubella-, bunya-, corona-, and arenaviruses. However, in some of these latter cases, a bulky cytoplasmic domain of one of the spike proteins may substitute for the M protein function. Immunolocalization of viral spike proteins has indicated that accumulation of one or several of these proteins appears to determine the site of budding. Thus, the membrane glycoproteins of viruses maturing at the plasma membrane are readily transported through the Golgi complex out to the cell surface. In contrast, one or several of the spike proteins of intracellularly maturing viruses, accumulate in the budding compartment and are not in most cases transported to the plasma membrane. It is thought that such proteins are retained in the budding compartment because of a retention motif or signal located somewhere in the protein. In general, viral spike proteins have been used as excellent models to dissect the exocytic pathway, and to map structural motifs in proteins conferring compartment-specific retention (Pettersson, 1991; Doms *et al.*, 1993).

Viral membrane proteins have also been useful in elucidating the early events taking place in the ER, such as core glycosylation, protein folding, and oligomerization (Doms *et al.*, 1993). The hemagglutinin (HA) of influenza

virus and the vesicular stomatitis virus (VSV) G protein have been particularly useful for studying these steps. The general picture that has emerged can be summarized as follows. The nascent polypeptide chain of spike proteins translocating through the ER membrane is cotranslationally core-glycosylated at Asn-X-Ser/Thr sites, and in some cases also proteolytically cleaved (e.g., the G1–G2 precursor of *Bunyaviridae* members). Folding of the nascent chain is also initiated cotranslationally and proceeds by disulfide-bridge formations catalyzed by protein disulfide isomerase (PDI). During folding, many proteins are found associated with the IgG heavy chain binding protein (BiP/grp78) and/or calnexin (Ou *et al.*, 1993), two chaperones that are believed to monitor and assist in the folding process, to prevent aggregation of incompletely folded proteins, and to prevent incompletely or misfolded proteins from leaving the ER. Other chaperones may also contribute to this process. Spike complexes, either homo-, or hetero-oligomers, are formed from subunit proteins in the ER. Oligomerization may start already while the subunits are still being folded, or may in other cases occur only after the individual subunits have been properly folded. Only when proteins have folded correctly, and assembled into oligomers, may they exit the ER and become transported via the intermediate compartment to the Golgi complex enclosed in transport vesicles. The process leading to mature, transport-competent complexes has been coined "quality control" (Hurtley and Helenius, 1989; Doms *et al.*, 1993). In the Golgi, N-linked glycans are further trimmed, leading to terminal glycosylation. Spike proteins destined to the plasma membrane may also undergo proteolytic cleavage at a late stage of the exocytic pathway.

The purpose of this chapter is to review the synthesis, assembly, and intracellular transport of the bunyavirus membrane glycoproteins G1 and G2 that constitute the structural unit of *Bunyaviridae* spikes. For a more detailed description of the structure and expression of the medium-sized (M) RNA segment encoding the membrane glycoproteins, the reader is refered to the chapters in this volume covering the molecular biology of the members of the individual genera.

II. MORPHOGENESIS AND ASSEMBLY

Despite considerable variation in the size of the structural proteins and the genomic RNA segments, the overall structure of the various members of the *Bunyaviridae* family is very similar. Virus particles measure about 90–100 nm in diameter (Pettersson and von Bonsdorff, 1987) and contain four proteins: two glycoproteins, G1 and G2, associated with the envelope and forming the spikes, and two internal proteins associated with the RNA genome, the major nucleocapsid protein, N, and the minor RNA-dependent RNA polymerase, L.

Early electron microscopic studies (Murphy *et al.*, 1968) showed that

virus particles mature intracellularly by budding into smooth vesicles in a perinuclear region of infected mouse brain cells and tissue culture cells (for references see Murphy *et al.*, 1973; Bishop and Shope, 1979; Pettersson *et al.*, 1988). Using organelle-specific markers, this site of accumulation of viral proteins and budding was later unambiguously shown to represent the Golgi complex in UUK (Kuismanen *et al.*, 1982; Gahmberg *et al.*, 1986b; Rönnholm, 1992), Bunyamwera (BUN) (Nakitare and Elliott, 1993), and Rift Valley fever (RVF) (Wasmoen *et al.*, 1988) virus-infected cells. The only animal *Bunyaviridae* member reported to bud at a site other than the Golgi is a strain of the phlebovirus RVF virus that was found to mature both intracellularly (in the Golgi) and at the plasma membrane in primary rat hepatocytes (Anderson and Smith, 1987). Tospoviruses, with tomato spotted wilt (TSW) virus as the prototype member, appear to have a more complex and as yet ill-defined mode of maturation. Particles seem to bud into the ER lumen or at Golgi membranes. Double enveloped particles are found in the cytoplasm and particles accumulate within ER-like membranes. In infected cells, envelope proteins have so far been localized by immunogold labeling only to virus particles, but not to any particular membranes (Kitajima *et al.*, 1992).

The morphogenesis and the maturation process have been studied at the EM or light microscopic levels for UUK (von Bonsdorff *et al.*, 1970; Kuismanen *et al.*, 1982, 1984, 1985; Gahmberg *et al.*, 1986a,b), RVF (Anderson and Smith, 1987), and sandfly fever (SF) viruses (Smith and Pifat, 1982), which are all members of the *Phlebovirus* genus (Francki *et al.*, 1991), as well as Dugbe virus (a nairovirus; Booth *et al.*, 1991).

Using immunofluorescence (Kuismanen *et al.*, 1982, 1984) and immuno-EM (Kuismanen *et al.*, 1985) both the glycoproteins and the nucleocapsid protein of UUK virus were found to accumulate in the Golgi area (Fig. 1). The helical nucleocapsids were found to line up underneath the membrane of distended Golgi vesicles. As G1 and G2 accumulated in the Golgi complex, progressively more nucleocapsids also entered the Golgi region. Little if any N protein was seen associated with the ER or the plasma membrane. Thus, a specific interaction between nucleocapsids and membranes containing the viral glycoproteins seems to exist only in the Golgi complex. Why no such interactions appear to occur already in the ER, which also contains high amounts of G1 and G2, is not clear, but it may relate to incorrect conformation or organization of the spikes, or to the topology or accessibility of the cytoplasmic tail of one of the glycoproteins that is likely to interact with the nucleocapsids. However, nucleocapsids can associate with the ER membrane in UUK-infected cells treated with tunicamycin, which inhibits N-glycosylation (Kuismanen *et al.*, 1984), or in cells infected with a temperature-sensitive mutant of UUK virus at the restrictive temperature (Gahmberg, 1984; Gahmberg *et al.*, 1986b). In these cases, exit from the ER of G1 and G2 is arrested. Whether budding into the ER lumen can occur under these conditions has not been studied. However, in the presence of brefeldin A, which

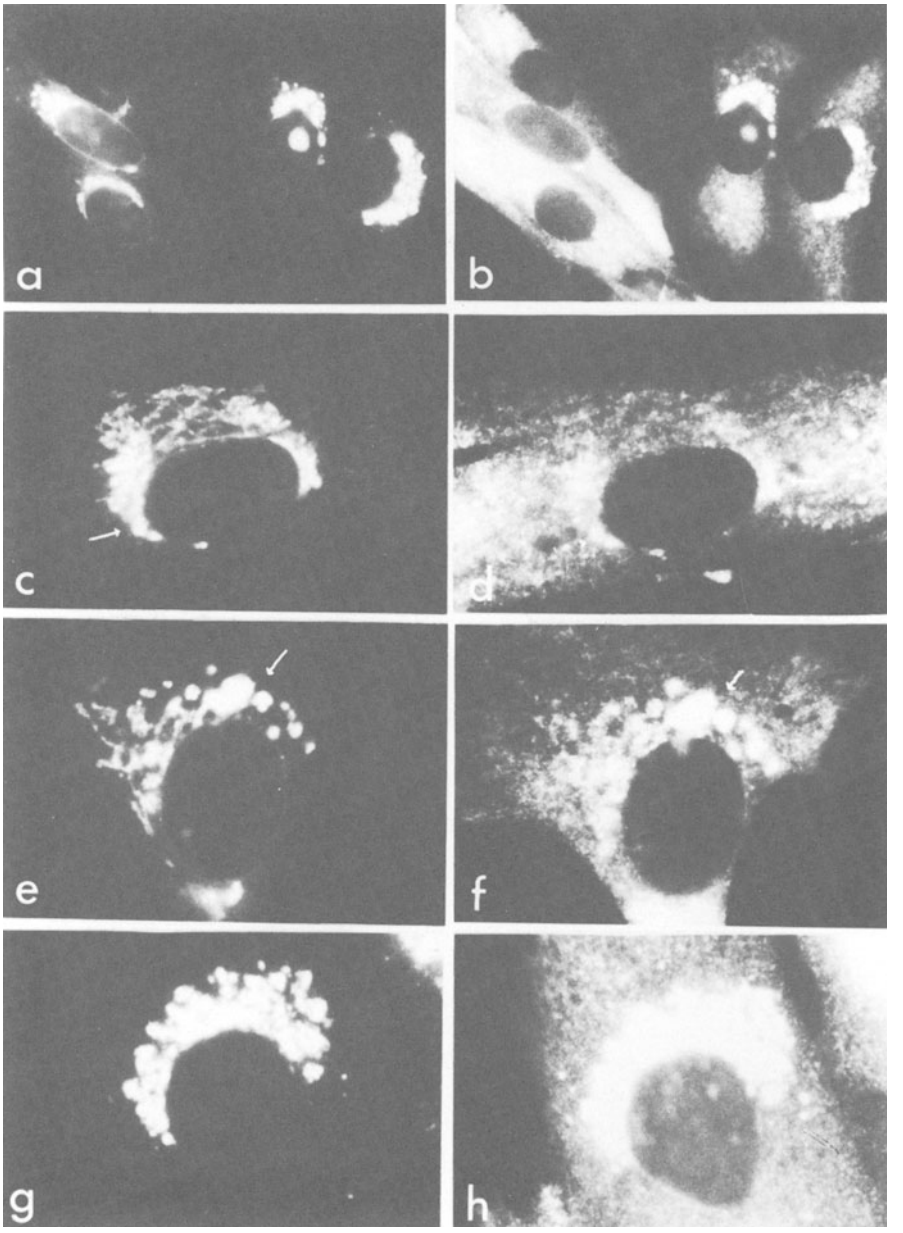


FIGURE 1. Progressive accumulation of Uukuniemi virus glycoproteins G1/G2 (a, c, e, and g) and nucleoprotein N (b, d, f, and h) in the Golgi complex of virus-infected BHK21 cells. The glycoproteins were visualized by indirect immunofluorescence using monoclonal antibodies against G2 (a) or G1 (c, e, and g). The same cells were double-stained with polyclonal antiserum against the nucleoprotein (b, d, f, and h). Note the progressive vacuolization of the Golgi stack concomitant with the accumulation of glycoproteins and nucleocapsids. The monoclonal antibodies used here preferentially recognize the Golgi conformation of G1 and G2. (From Kuismanen *et al.*, 1984).

inhibits the transport of proteins out of the ER and induces the redistribution of resident Golgi proteins to the ER, Punta Toro (PT), a phlebovirus, was found to bud into the ER. Such particles remained in dilated ER vacuoles and were not transported to the cell surface (Chen *et al.*, 1991a).

As is the case for other viruses with a segmented genome, each infectious virion has to package at least one copy of each of the three ribonucleoprotein (RNP) segments. How this is ensured and to what extent it is a regulated process is unknown. Another open intriguing question is whether there is an active transport of nucleoproteins to the site of budding in the Golgi. Because of the helical structure, the RNPs are morphologically identified only as more electron-dense thickenings underneath the Golgi membrane in budding profiles (Kuismanen *et al.*, 1982; Anderson and Smith, 1987). In thin sections of intracellular virus particles, the RNPs appear to lie in close contact with the lipid bilayer, while the interior of the particle appears empty (von Bonsdorff and Pettersson, 1975).

During UUK virus infection, the Golgi complex typically undergoes a morphological change. The stack of flat cisternae is progressively distorted, the Golgi vacuolizes, and large and small vesicles become partly dispersed in the cytoplasm (Fig. 1) (Kuismanen *et al.*, 1984; Gahmberg *et al.*, 1986b). Whether the morphological change that is observed for UUK virus is a hallmark for the *Bunyaviridae* in general has not been systematically studied. The vacuolization is probably caused by the accumulation of the glycoproteins in the Golgi complex, as shown by using a *ts* mutant defective in virus maturation at the restrictive temperature (Gahmberg *et al.*, 1986b). The morphologically altered Golgi is still functional in that it can terminally glycosylate Semliki Forest virus glycoproteins and transport them to the plasma membrane (Gahmberg *et al.*, 1986a). Budding of *Bunyaviridae* members is inhibited by the ionophore monensin (Cash, 1982; Kuismanen *et al.*, 1985; Schmaljohn *et al.*, 1986; Chen *et al.*, 1991a), whereas the association of the nucleocapsids with the Golgi-derived vesicles seems to be unaffected (Kuismanen *et al.*, 1985). Since monensin exchanges protons for sodium ions, this suggests that the pH or ionic conditions prevailing in the Golgi complex are important for virus budding. Budding may thus not only be dependent on a certain critical concentration of glycoproteins, but also on a conformational change of the glycoproteins induced by the milieu present in the Golgi complex. It is still not clear whether budding of *Bunyaviridae* members can occur throughout the Golgi stack or just in a subcompartment.

Vacuoles of varying sizes containing one or several particles are thought to be transported to the plasma membrane (Fig. 2), where virions are released on fusion of the vesicles with the plasma membrane. The nature of the exocytic transport vacuoles/vesicles and their relation to normal exocytic trafficking are unknown. Release of RVF virus (Anderson and Smith, 1987) and PT virus (Chen *et al.*, 1991a) occurs at the basolateral surface of polarized hepatocytes and Vero cells, respectively.

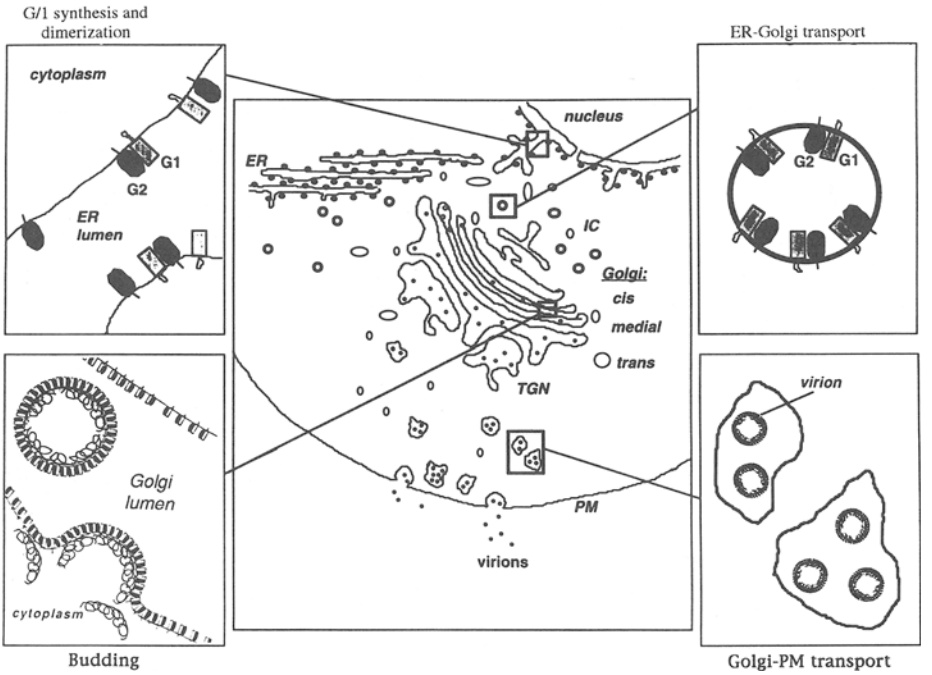


FIGURE 2. Schematic representation of *Bunyaviridae* maturation. The glycoproteins are cleaved cotranslationally in the ER from a precursor encoded by a single ORF in the M RNA segment. Following glycosylation and folding, G1 and G2 heterodimerize (upper left panel). The heterodimers are then transported to the Golgi complex (upper right panel) where further transport is arrested. G1/G2 accumulate in the Golgi and budding of virions is probably triggered by the association of helical nucleocapsids with the cytoplasmic tail of G1 (lower left panel). Virions are transported within vacuoles (lower right panel) to the cell surface where they are released on fusion of the vacuoles with the plasma membrane. IC, intermediate compartment; TGN, trans-Golgi network; PM, plasma membrane.

III. STRUCTURE AND SYNTHESIS OF THE MEMBRANE GLYCOPROTEINS

The spike proteins of all *Bunyaviridae* members are encoded by the medium-sized M RNA segment. Since the details on the structure of the M RNAs and the primary sequences of their protein products will be dealt with in the chapters reviewing the molecular biology of the individual genera, the structure of the glycoproteins will only be summarized here. The coding strategies for the various M RNA segments are depicted in Fig. 3. The spikes of all *Bunyaviridae* members are made of two glycoproteins, G1 and G2. The nomenclature is somewhat confusing in that the equivalent protein in different viruses may have different names. In each case, G1 corresponds to the

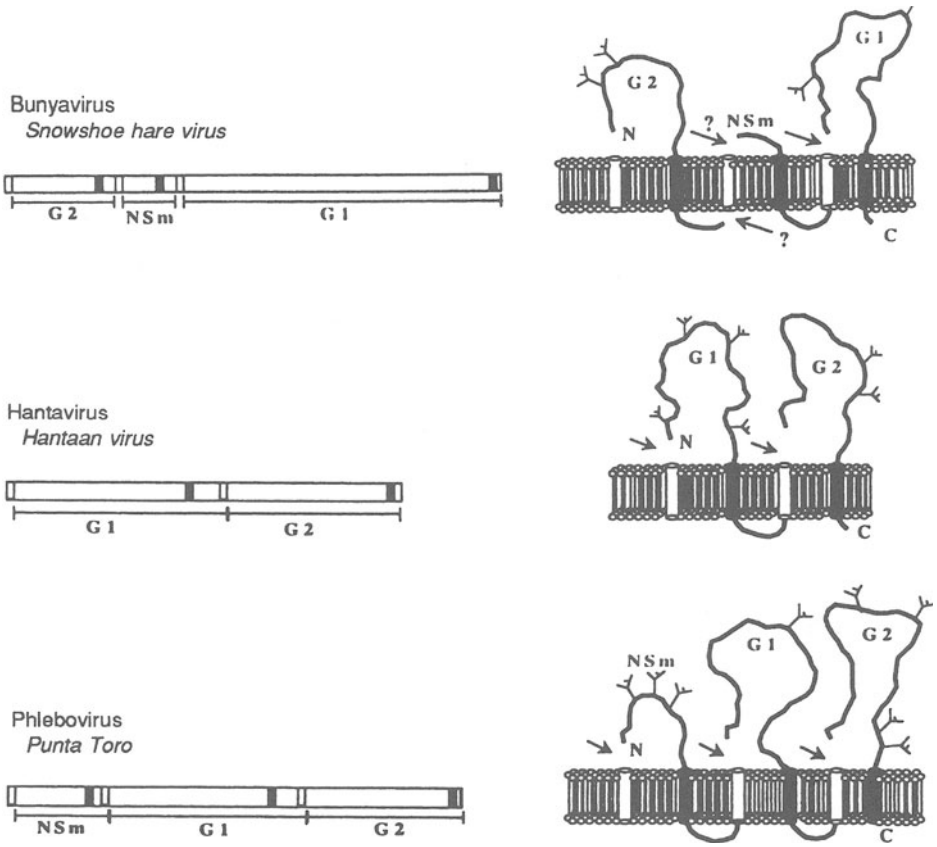


FIGURE 3.

protein with the slower mobility on an SDS–polyacrylamide gel, while G2 is the faster migrating. However, since the size of G1 and G2 in some viruses (e.g., phleboviruses) is quite similar, their mobility may depend on the conditions used during electrophoresis. Thus, under nonreduced conditions, G1 of UUK virus migrates slower than G2 (Kuismanen, 1984), while after reduction and alkylation the order is reversed, G2 now migrating slower than G1 (Persson and Pettersson, 1991). In PT virus, G1 and G2 corresponds to G2 and G1 in RVF virus (Ihara *et al.*, 1985). Recently, it has been proposed that the N-terminally located protein should be named G_N, and the C-terminal one G_C (Lappin *et al.*, 1994). It remains to be seen whether this new nomenclature will be approved by the International Committee on the Taxonomy of Viruses.

As is apparent from Fig. 3, the way the glycoproteins are synthesized, and their sizes, vary considerably between members of the different genera. Following a brief summary of the structure and synthesis of the glycopro-

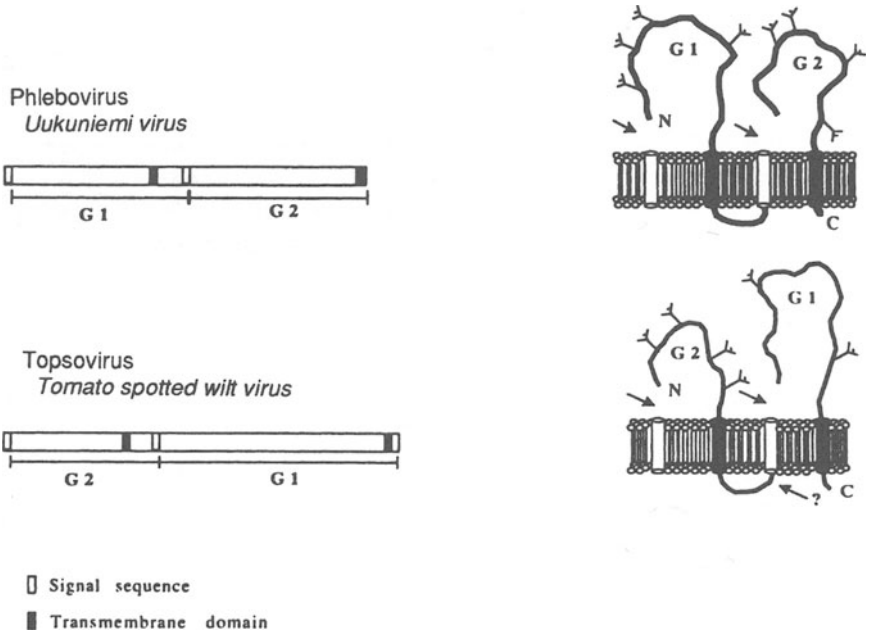


FIGURE 3. Schematic representation of the gene organization of the M RNA-encoded open reading frame of *Bunyaviridae* and the suggested processing and membrane insertion of their products. Representative examples of four genera are shown. The localization of the G1, G2, and NSm genes in the precursor are indicated. Open box depicts the N-terminal signal sequence and closed box the transmembrane anchor. Arrows on the luminal side indicate cleavage by signal peptidase. The exact site for processing the N-terminus of NSm of bunyaviruses is not known. The number of potential glycosylation sites are indicated.

teins, a more detailed description of the individual genera will be presented. In all cases studied, G1 and G2 have been found to be type I membrane-spanning glycoproteins. They are thus thought to span the lipid bilayer only once and to have their N-terminus oriented toward the ER lumen, and C-terminus facing the cytoplasm and the interior of the virus. Although this interpretation, which is deduced from the primary sequence and hydropathy plots, may be correct, limited experimental evidence in support of this topology has been published. G1 and G2 (and NSm where present) are cotranslationally cleaved from the primary translation product encompassing the single open reading frame in the M RNA. Each membrane protein is preceded by a separate signal sequence for targetting of the nascent chain to, and facilitating its translocation through, the ER membrane. The cleavages that follow to create mature G1 and G2 (and NSm) are probably all carried out by the lumenally located signal peptidase (Fig. 3). There is no evidence for additional cleavages occurring on the cytoplasmic side. Following transloca-

tion through the ER membrane, folding, disulfide-bond formation, and core-glycosylation at Asn-X-Ser/Thr sites, G1 and G2 appear to heterodimerize. After a considerable lag period, G1 and G2 are transported to the Golgi complex, where they become arrested and undergo trimming of the glycans. Although G1 and G2 accumulate in the Golgi complex, a fraction escapes this retention and is transported to the cell surface. In the Golgi, G1–G2 dimers could form higher oligomers, but such complexes have not yet been demonstrated for any *Bunyaviridae* member.

A. *Bunyavirus* Genus

The M segment of several members of this genus has been cloned and sequenced, and the deduced primary sequence of the membrane proteins compared (reviewed by Elliott, 1990; Elliott *et al.*, 1990; Bouloy, 1991). The gene order is NH_2–G2–NSm–G1–COOH, where NSm is a short 174-residue-long nonglycosylated membrane protein with unknown function (Fig. 3). The exact cleavage sites between G2 and NSm (position 299) and NSm and G1 (position 473) have been determined only for snowshoe hare (SSH) virus by sequencing the C- and N-terminal ends directly (Fazakerley *et al.*, 1988). Since the proteins within this genus are quite well conserved (Elliott, 1990), the cleavage sites for the other sequenced members [La Crosse (LAC), Bunyamwera (BUN), and Germiston (GER) viruses] can also be deduced. G2 (M_r 29–41 kDa) is preceded by a typical signal sequence that is cleaved off in the ER, while an internal signal sequence for G1 (M_r 108–125 kDa) is apparently located at the C-terminus of NSm (Fig. 3). What proteolytic trypsin-like protease releases NSm from G2, and on which side of the ER membrane this takes place is not clear. The most likely hypothesis is that all cleavages are carried out by the ER luminal signal peptidase. None of the processing events require the concomitant expression of any other viral proteins (Nakitare and Elliott, 1993). The organization and processing of the *Bunyavirus* precursor proteins are quite similar to those of the alphavirus membrane proteins, which also contain an internal (6K) protein located between the p62 and E1 spike proteins (Garoff *et al.*, 1980). The 6K peptide has been found to be dispensible, although its presence enhances virus production (Liljeström *et al.*, 1991). The role of the NSm peptide is not known, but it may, in analogy to the alphavirus 6K peptide (Liljeström and Garoff, 1991), provide the signal sequence for the downstream G1. The fact that it localizes to the Golgi in BUN virus-infected cells suggests that it may have a role in virus maturation (Nakitare and Elliott, 1993; Lappin *et al.*, 1994). That processing of *Bunyavirus* glycoproteins occurs cotranslationally is inferred from the fact that no precursor has been found in pulse–chase experiments (Pennington *et al.*, 1977; Fazakerley *et al.*, 1988). It has not been possible to study the processing *in vitro*, since translation of the M mRNA has for some reason been unsuccessful.

Cysteine residues, N-glycosylation sites, and hydropathy profiles are well conserved between bunyaviruses (Elliott *et al.*, 1990), suggesting a highly conserved three-dimensional structure. Both G1 and G2 are acylated (shown only for LAC virus; Madoff and Lenard, 1982) and mature virions contain primarily N-linked complex-type glycans (Vorndam and Trent, 1979; Madoff and Lenard, 1982; Pesonen *et al.*, 1982b). Partial sequences of the glycans from Inkoo bunyavirus have been determined and found to be of three types: high-mannose, complex, and small endoglycosidase H-resistant intermediate glycans (Pesonen *et al.*, 1982b).

By analogy to the phleboviruses, one would assume that the G1 and G2 proteins of bunyaviruses would also form heterodimeric complexes. However, attempts to demonstrate such dimers have so far failed (Gerbaud *et al.*, 1992).

B. *Hantavirus* Genus

The M segment of several hantavirus strains have been cloned and sequenced (for references see Parrington *et al.*, 1991; Antic *et al.*, 1992a; Spiropoulou *et al.*, 1994). Hantaan (HTN) virus G1 and G2 have similar sizes (M_r about 65 and 55 kDa, respectively) and are synthesized in the order NH_2–G1–G2–COOH (Schmaljohn *et al.*, 1987). There is no evidence for an NSm protein. G1 and G2 are thought to be cotranslationally cleaved in the ER, since no full-length precursor has been detected in virus-infected cells (Schmaljohn *et al.*, 1986, 1990), nor in cells expressing the M segment from a cloned cDNA (Pensiero *et al.*, 1988; Ruusala *et al.*, 1992). Both proteins are preceded by a hydrophobic signal sequence (Fig. 3). That the internal signal sequence preceding G2 is functional is supported by the fact that it can target G2 correctly to the ER in the absence of G1 (Schmaljohn *et al.*, 1990; Pensiero and Hay, 1992; Ruusala *et al.*, 1992). In a recent report, Kamrud and Schmaljohn (1994) found that translation of G2 could be initiated internally, albeit inefficiently, in the full-length M mRNA from an AUG codon preceding G2. They propose that initiation occurs via a leaky scanning, rather than direct internal ribosome entry. The physiological importance, if any, of this internal initiation is unclear.

In the ectodomain of G1 there are four, and in G2 two, potential sites for N-linked glycosylation; however, one of the sites in G2 probably cannot be used (Bause, 1983). Pulse–chase experiments, as well as analyses of extracellular virions indicate that the glycans are primarily of the high-mannose, endo H-sensitive type (Schmaljohn *et al.*, 1986; Ruusala *et al.*, 1992). The fact that HTN virus G1- or G2-specific monoclonal antibodies can precipitate both proteins either without or after prior cross-linking (Arikawa *et al.*, 1989; Antic *et al.*, 1992b), and that G1 and G2 have to be coexpressed to enable exit of both proteins from the ER (Ruusala *et al.*, 1992), suggests that G1 and G2 form heterodimers already in the ER.

C. *Nairovirus* Genus

The structure and synthesis of nairovirus glycoproteins have so far been poorly studied. Only the M RNA segment of Dugbe (DUG) virus has been cloned and sequenced (Marriott *et al.*, 1992). In nairovirus-infected cells large nonstructural glycoproteins have been identified (Clerx and Bishop, 1981; Cash, 1985; Watret and Elliott, 1985). Sequence analysis of the DUG virus M segment (4888 nucleotides) and identification of its products by specific antisera prepared against fusion proteins, indicate that the processing of the primary translation product is more complex than for other *Bunyaviridae* members. The M segment harbors a single ORF corresponding to 1551 amino acids (173,300 Da). The gene order is NH_2–G2–G1–COOH. Following cleavage (probably) by signal peptidase, an 85-kDa glycosylated precursor seems to be further processed to the mature G1 (655 residues, M_r 70 kDa). A 110-kDa G1-related product has also been found in infected cells, but this is probably not a precursor of gp85 or G1. G2 (M_r 35 kDa) is probably also cleaved from a larger (M_r 70 kDa) precursor. The further fate of G1 and G2 in infected cells is not known, except that they are found in virions. A third virion glycoprotein (M_r 45 kDa) has been reported for Hazara virus, a member of the Crimean-Congo hemorrhagic fever serogroup (Foulke *et al.*, 1981).

D. *Phlebovirus* Genus

Based on sequence relationships, the previously independent *Uukuvirus* genus has been merged with the phleboviruses, forming the present *Phlebovirus* genus (Francki *et al.*, 1991). The strategy of M segment gene expression differs between UUK virus and other phleboviruses in that the mature glycoproteins are preceded by an NSm presequence only in the latter viruses (Fig. 3). The M segment of RVF (Collett *et al.*, 1985; Takehara *et al.*, 1989), PT (Ihara *et al.*, 1985), and UUK (Rönnholm and Pettersson, 1987) viruses have been cloned and sequenced.

The gene order in RVF is NH_2–NSm–G2–G1–COOH and in PTV NH_2–NSm–G1–G2–COOH, the confusing nomenclature being the result of the differences in mobility on SDS gels between G1 and G2 of the two viruses (see above). NSm, which has not been found in virions, is much larger for PT virus (30 kDa) than for RVF virus (14 kDa). Expression of wild-type and mutant forms of PT virus G1 and G2 has shown that both G1 and G2 are inserted in the membrane as type I proteins (Chen *et al.*, 1991b). Both proteins are preceded by a signal sequence that is cotranslationally processed, and they are anchored in the lipid bilayer by a hydrophobic transmembrane C-terminal domain. Whereas the transmembrane domain of G2 can be deduced to span residues 1282 to 1300, the corresponding domain has not as yet been exactly defined in G1, since this putative region encompasses some 50 amino acids (residues 684–733) (Ihara *et al.*, 1985).

The expression of the RVF virus M segment has been studied in greater detail than that of PT virus. *In vitro* translation of the M mRNA of RVF virus yields a 133-kDa uncleaved primary translation product, which in the presence of microsomal membranes is cotranslationally cleaved to yield G1 and G2, as well as 78-, 21-, and 14-kDa products (Suzich and Collett, 1988). In addition to G1 (M_r 65 kDa) and G2 (M_r 56 kDa), a glycosylated 78-kDa and a nonglycosylated 14-kDa protein (but no 21-kDa product) were found in cells infected with RVF virus, or with a recombinant vaccinia virus harboring a complete M cDNA (Kakach *et al.*, 1988; Wasmoen *et al.*, 1988). Mature G2 is preceded by 5 (in PT virus by 13) in-frame AUG initiation codons. *In vitro* mutagenesis followed by expression of the mutants from vaccinia virus recombinants and analyses of the products using peptide antibodies indicate that the first AUG is utilized for the synthesis of the 78-kDa product, whereas the second AUG is utilized for the synthesis of the 14-kDa product (Kakach *et al.*, 1988, 1989; Wasmoen *et al.*, 1988; Suzich *et al.*, 1990). The 78-kDa product, which contains the whole of G2, the 14-kDa peptide, and some upstream residues, was not found to be the precursor for mature G2. Thus, G2 and the 14-kDa presequence may be generated by cotranslational proteolytic cleavage of the protein initiated at the second AUG. The first and fourth (or fifth) AUG codons precede typical signal peptide-like sequences that could direct the 78-kDa protein and G2 through the ER membrane. The second and third AUG codons are not, however, followed by such hydrophobic sequences, making it unclear how the 14-kDa protein could be translocated through the ER membrane. It is therefore not clear whether the 14-kDa protein is indeed translocated into the ER lumen and whether it becomes an integral membrane protein. The finding that the potential N-glycosylation site at Asn_{88} is utilized only in the 78-kDa product, but not in the 14-kDa product (Kakach *et al.*, 1989), suggests that the latter protein is not translocated through the ER membrane. Mature G2 contains only one N-linked glycan, while three out of four sites in G1 seem to be utilized (Kakach *et al.*, 1989). The presequence of the 30-kDa NSm of PT virus shows no apparent sequence homology with the 14-kDa NSm of RVF virus and has not been detected in infected cells.

The role of NSm in the life cycle of phleboviruses is still obscure. Using specific antisera, Wasmoen *et al.* (1988) located G1, G2, and the 78- and 14-kDa proteins to the Golgi complex and to a reticular network, probably representing the ER, both in cells infected with RVF, and with mutant recombinant vaccinia viruses expressing the proteins. In the absence of the 78- and 14-kDa products, G1 and G2 were also localized to, and retained in, the Golgi, indicating that the information for Golgi localization resides in G1/G2 (see below). Thus, the 14-kDa product is not required for proper processing and transport of the mature G1 and G2 proteins. Possible functions for the NSm polypeptide could be that it simply provides a suitable signal sequence for translocation of the downstream protein through the ER membrane. Alternatively, it could play a role in the folding and assembly of the viral spikes in

the ER, or budding in the Golgi complex. The lack of an NSm protein in UUK virus (and hantaviruses) suggests that it is dispensible for the formation of infectious virions. Whether this is true for RVF and PT viruses will, however, have to await the development of a reverse genetics system that would enable the engineering of a virus from which the NSm sequence has been deleted.

Using the T7 polymerase-driven vaccinia virus expression system, Chen and Compans (1991) found that G1 and G2 form a noncovalently linked heterodimeric complex in the ER within minutes after synthesis. Evidence for low levels of G2 disulfide-linked homodimers was also found. The complexes were identified by cross-linking, polyacrylamide gel electrophoresis, and sucrose gradient centrifugation. Both types of complexes were transported to and retained in the Golgi complex. Disulfide-linked heterodimers between a full-length G1 and a membrane anchor minus (soluble) G2 mutant were also observed and these were transported out of the ER, suggesting that the cytoplasmic tail and transmembrane domain of G2 are not required for dimerization or transport.

The primary product made *in vitro* by translating the full-length M mRNA of Uukuniemi virus in the absence of microsomal membranes is a 110-kDa precursor (p110) of G1 and G2. In the presence of dog pancreas microsomes, p110 is cotranslationally cleaved to G1 and G2 (Ulmanen, 1981). As for other *Bunyaviridae* members, the precursor has not been found in infected cells. Pulse–chase analysis (Kuismanen, 1984) and direct sequencing (Rönnholm and Pettersson, 1987) have shown that the gene order is NH_2–G1–G2–COOH. Recent indirect evidence indicates that there is only a single cleavage event between G1 and G2. This cleavage takes place between a serine and a cysteine residue and is probably carried out by the ER signal peptidase. Expression of mutant cDNAs, analyses using peptide antibodies directed against different portions of the C-terminal cytoplasmic tail of G1, and treatment of microsomes isolated from infected cells with proteases, all suggest that the internal signal sequence for G2 remains attached to the tail of G1 (Figs. 3 and 4) (unpublished results). This means that the cytoplasmic tail of G1 consists of an 81-residue largely hydrophilic region followed by a 17-residue hydrophobic region (the G2 signal sequence) (Fig. 4). That the C-terminus of G1 is exposed on the cytoplasmic face of the lipid bilayer has been shown by immunofluoresence of streptolysin O-permeabilized cells by using tail-specific peptide antibodies (unpublished results). The G1 tail is likely to interact with the nucleoproteins to facilitate budding. It also seems to contain a signal for targetting of G1 to the Golgi complex (see below). The tail of G2 is very short (5 residues) and hydrophilic, and may therefore not have any other function than to prevent G2 from slipping into the ER lumen.

Following translocation and cleavage, both G1 and G2 undergo core glycosylation at asparagine sites. There are four potential sites in both G1 and G2, and all of them are probably used (Kuismanen, 1984; Rönnholm and Pettersson, 1987). During intracellular transport, the glycans are trimmed to

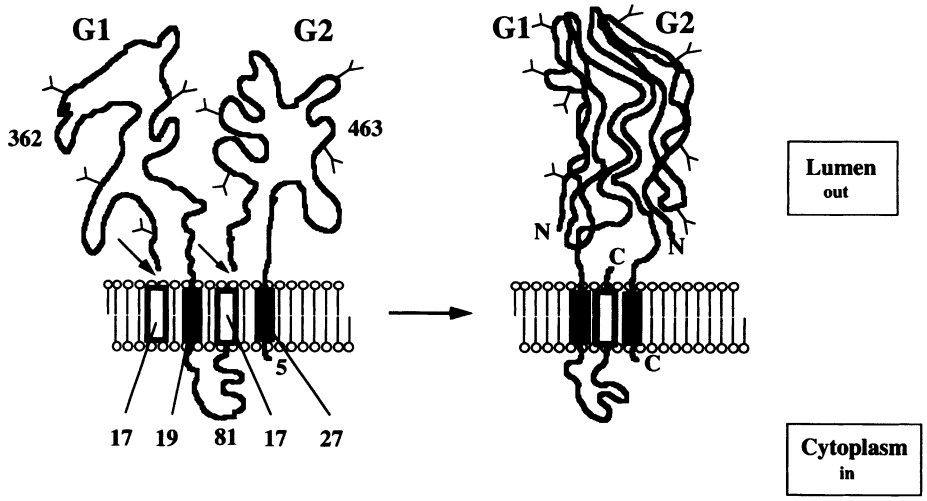


FIGURE 4. Processing and dimerization of Uukuniemi virus G1 and G2. G1 and G2 are cleaved cotranslationally in the ER from the precursor p110 and probably inserted in the ER membrane as depicted. Following glycosylation and folding, G1 and G2 heterodimerize already in the ER. Mature G1 and G2 are thought to be processed at two sites by the signal peptidase (arrows). The internal signal sequence of G2 remains covalently attached to the C-terminus of G1. However, it is not known whether this hydrophobic region remains membrane-associated as shown. The number of residues comprising the different domains as well as the glycosylation sites are indicated.

varying degrees. In purified virions, G1 contains primarily complex, sialylated, endo H-resistant glycans, while G2 contains a mixture of complex, intermediate (both endo H-resistant), and high-mannose (endo H-sensitive) chains (Pesonen *et al.*, 1982a; Kuismanen, 1984). The same pattern of glycans is also found in Inkoo virus (*Bunyavirus* genus) (Pesonen *et al.*, 1982b). Pulse–chase experiments have shown that G1 acquires endo H-resistant glycans slowly with a $t_{1/2}$ of about 45 min, while the acquisition of partial endo H resistance in G2 occurs even more slowly (Kuismanen, 1984). The kinetics of folding, as monitored by disulfide-bond formation, also differs between the two proteins. G1 was found to fold rapidly within minutes ($t_{1/2}$ about 10 min), while folding of G2 was much slower ($t_{1/2}$ about 45–60 min) (Persson and Pettersson, 1991; Pettersson *et al.*, 1993). Since both proteins have 26 cysteine residues in their respective ectodomain, and therefore the potential to form 13 disulfide bonds, this indicates that the number of disulfide bonds *per se* does not determine the folding kinetics of membrane proteins. During folding, both G1 and G2 are transiently associated with the IgG heavy chain-binding protein (BiP), an ER luminal chaperone. In conformity with the different folding kinetics, G1 is bound to BiP for a shorter time than G2. Protein disulfide isomerase (PDI) can also be coprecipitated with the glycoproteins, in agreement with the role for PDI in catalyzing disulfide-bond

formation (Persson and Pettersson, 1991; Bardwell and Beckwith, 1993). Having folded correctly, G1 and G2 form heterodimers in the ER (Fig. 4). Pulse–chase experiments showed that newly made G1 rapidly dimerizes with G2. Since G2 folds much slower than G1, this means that newly synthesized G1 is unable to dimerize with its companion G2 made from the same p110 molecule. Thus, G1 and G2 cleaved from a p110 enter a glycoprotein pool in the ER from which partners are drawn into heterodimeric complexes. This conclusion is also supported by experiments in which G1 and G2 were expressed from separate cDNAs (Melin *et al.*, 1995). Following dimerization, there is a lag of some 30 to 45 min ($t_{1/2}$), before the G1–G2 heterodimers appear in the Golgi complex. It is at present unclear where the proteins reside during this long delay, as is also the mechanism for the transport delay. Both G1 and G2 become palmitylated (Pettersson, unpublished), and no evidence for O-linked glycosylation has been found (Pesonen *et al.*, 1982a). As discussed below, G1 and G2 accumulate in the Golgi complex, where G1 acquires endo H-resistant glycans, while G2 remains largely endo H-sensitive (Kuismanen, 1984). A minor fraction of the spike proteins will end up at the plasma membrane, in particular late in infection (Kuismanen *et al.*, 1985). This may represent glycoproteins left over from the budding. The fate of this surface expressed fraction is not known. It may recycle back to the Golgi complex, or be transported to the lysosomes for degradation.

Recently, it has been suggested that G1 and G2 in UUK virions are present as homodimers (E. Kuismanen, personal communication). On treatment of virions with low pH, the G2 homodimers dissociate into monomers. This suggests that the spike proteins of UUK virus undergo conformational changes and rearrangements analogous to those demonstrated for Semliki Forest virus (an alphavirus) E1 and E2 (Wahlberg and Garoff, 1992).

Whether G1/G2 forms higher-order complexes is not known. Cross-linking experiments and sucrose gradient centrifugations have failed to demonstrate such complexes (Persson and Pettersson, unpublished data). In virions G1/G2 are seen by EM as hollow cylinders organized in a T = 12 icosahedral surface lattice. This architecture is most apparent at low pH. The symmetry indicates the presence of 720 structural units (60 × T), each of which probably is made up of a G1–G2 heterodimer. This means that each particle should contain 720 molecules each of G1 and G2 (von Bonsdorff and Pettersson, 1975).

E. *Tospovirus* Genus

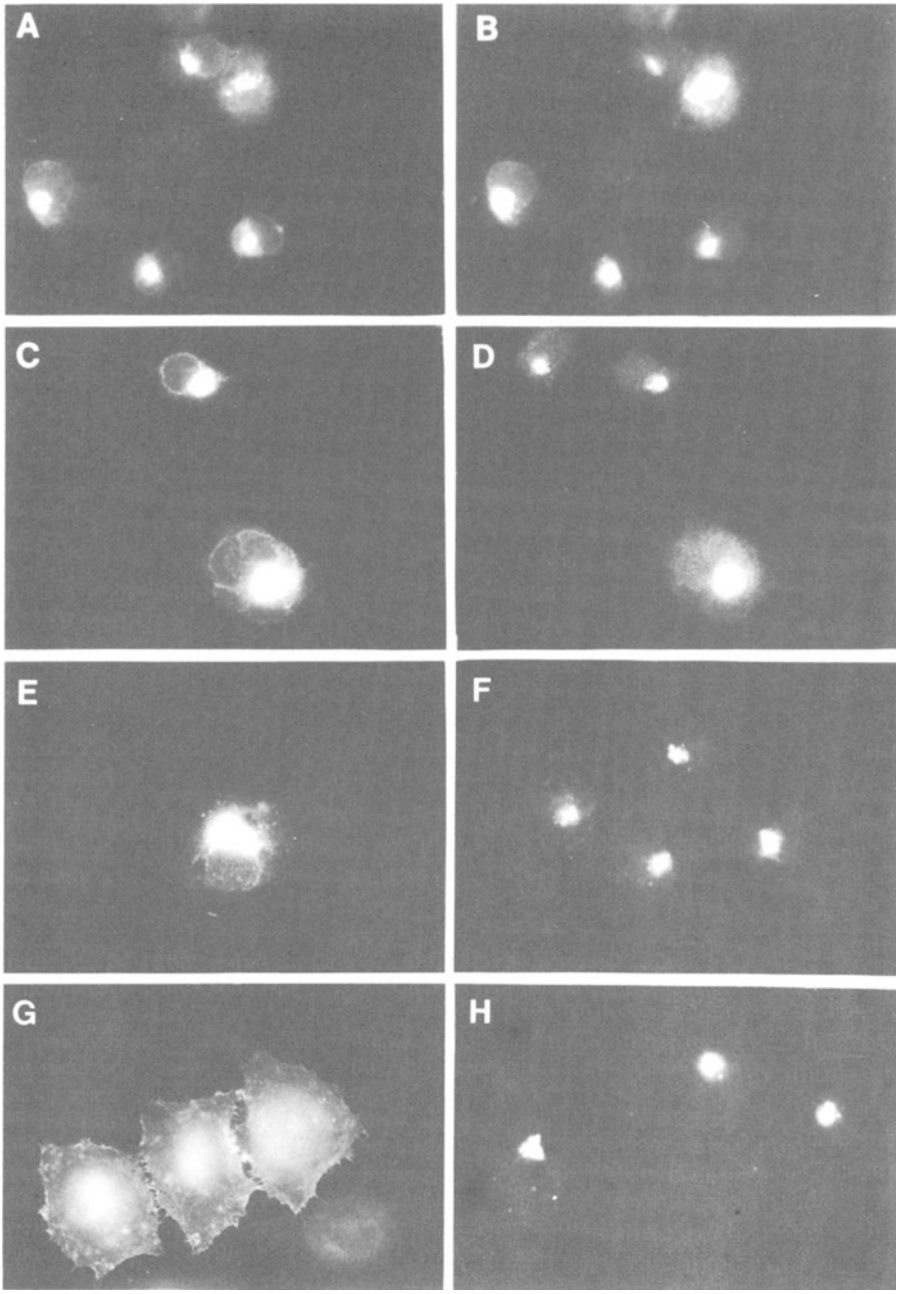
Although the M RNA segments of two distinct tospoviruses have been cloned and sequenced, little is known about the synthesis, transport, or intracellular localization of their products. The M segments of tomato spotted wilt (TSW) virus and impatiens necrotic spot (INS) virus are 4821 (Kormelink *et al.*, 1992) and 4972 (Law *et al.*, 1992) residues in size. In contrast to all

other *Bunyaviridae*, the M segment of tospoviruses displays an ambisense coding strategy. An mRNA transcribed from the 3′ part of the virion RNA encodes a precursor (TSW virus: 1136 residues, 127.4 kDa; INS virus: 1110 residues, 124.9 kDa) to the two glycoproteins G1 (M_r 78 kDa) and G2 (M_r 58 kDa) in the order NH_2–G2–G1–COOH, while a subsegmental mRNA derived from the viral-complementary strand encodes a nonstructural protein [NSm; 302 residues (33.4 kDa) and 303 (34.1 kDa) residues, respectively]. The NSm is not a membrane protein and has recently been found to associate with nucleocapsids and plant cell plasmodesmata, and to be responsible for viral nucleocapsid movement from cell to cell (Kormelink *et al.*, 1994; R. Goldbach, personal communication). Because of these properties, NSm will therefore not be discussed here further.

Based on hydropathy plots, it seems likely that tospovirus G1 and G2, similar to those of other *Bunyaviridae* members, are both type I proteins spanning the membrane once (Fig. 3). G1 of TSW virus shows clear sequence homology with G1 of BUN virus (45% similarity, 22% identity), while G2 is much less homologous (Kormelink *et al.*, 1992). G2–G1 of INS virus displays 36 and 39% similarities to PT and RVF virus glycoproteins, respectively (Law *et al.*, 1992). This indicates an evolutionary relationship between plant and animal bunyaviruses. The synthesis, processing, and transport of tospovirus glycoproteins have not yet been analyzed.

IV. G1 AND G2 ACCUMULATE IN THE GOLGI COMPLEX

As described above, members of all *Bunyaviridae* genera with the possible exception of the tospoviruses, mature by budding through membranes of the Golgi complex (Pettersson *et al.*, 1988; Matsuoka *et al.*, 1991; Pettersson, 1991; Hobman, 1993). Evidence from several bunyaviruses indicates that the site of maturation is determined by the accumulation of viral glycoproteins in the Golgi. Following synthesis, folding, glycosylation, and dimerization in the ER, G1 and G2 move to the Golgi, where further transport is arrested (Fig. 1). Accumulation of G1/G2 in the Golgi complex has been analyzed in infected cells or in cells expressing G1 and G2 from cloned cDNA by immunofluorescence, and in a few cases by immuno-EM (Kuismanen *et al.*, 1982, 1985; Anderson and Smith, 1987), and subcellular fractionation (Persson and Pettersson, 1991; Ruusala *et al.*, 1992). Colocalization of viral glycoproteins with markers for the Golgi apparatus, such as thiamine pyrophosphatase (nucleoside diphosphatase) (Kuismanen *et al.*, 1982; Pensiero *et al.*, 1988), wheat germ agglutinin (WGA) (Kuismanen *et al.*, 1982; Wasmoen *et al.*, 1988; Chen and Compans, 1991; Nakitare and Elliott, 1993; Lappin *et al.*, 1994), CTR433 (Rönnholm, 1992), Golgizone (Pensiero and Hay, 1992), or mannosidase II (Kuismanen *et al.*, 1984; Gahmberg *et al.*, 1986b; Melin *et al.*, 1995), have unambiguously shown that G1/G2 indeed accumulate in this organelle (Fig. 5). However, it has to be stressed that the exact sublocalization


A
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in the Golgi complex has not been determined on the EM level using appropriate markers. Because of the altered morphology induced by the viral glycoproteins (Fig. 1) (Kuismanen *et al.*, 1984; Gahmberg *et al.*, 1986b), it is very difficult to identify the *cis*, *medial*, and *trans* cisternae of the Golgi complex. It is therefore not known whether the glycoproteins are localized to particular cisternae of the Golgi, and whether localization is exactly the same for all members of the family. There is thus a great need for localization of G1/G2 on the EM level.

Proteins en route to the plasma membrane become transiently concentrated in the Golgi complex. This is seen by immunofluorescence as an apparent accumulation of the proteins in the Golgi. To distinguish between this type of Golgi localization and true retention and accumulation, chase in the presence of cycloheximide to stop further protein synthesis is necessary. Proteins passing through the Golgi will be chased out of the Golgi, while truely retained proteins will remain. In the case of *Bunyaviridae* glycoproteins, many investigators have shown that G1/G2 cannot be chased out of the Golgi even after 4- to 6-hr chases with cycloheximide (Gahmberg *et al.*, 1986b; Matsuoka *et al.*, 1994). In most cases no or very little glycoprotein has been localized to the plasma membrane. However, the glycoproteins of RVF virus grown in primary rat hepatocytes were expressed on the cell surface (Anderson and Smith, 1987), and UUK virus glycoproteins are present on the cell surface at later stages of infection (Kuismanen *et al.*, 1982). In the latter case, cell-associated virions may account for most of the observed patchy immunofluorescence.

V. MAPPING THE GOLGI-RETENTION SIGNAL IN G1/G2

G1 and G2 accumulate in the Golgi complex when expressed in the absence of any other viral proteins, showing that Golgi retention is an inherent property of G1/G2 (Pensiero et al., 1988; Matsuoka *et al.*, 1988, 1994;

FIGURE 5. Intracellular localization of Uukuniemi virus G1, G2, and chimeric G1 proteins in transfected cells. The cDNA constructs were expressed in HeLa (panels A–E) or BHK21 (panels F–H) cells using the T7 polymerase-driven vaccinia virus system. Proteins were localized by indirect immunofluorescence using polyclonal antiserum against G1 (A), CD4 (F–H), or mannosidase II (D), and monoclonal antibodies against G2 (B) or G1 (C, E). In panels A and B, G1 and G2 were coexpressed from two different plasmids and the cells were double-stained to detect the proteins in the same cells. Cells expressing G1 alone were double-stained with antisera against G1 (C) and mannosidase II (D), a Golgi marker. Panel E shows the localization of G1 in which its transmembrane domain and 10 flanking residues on both sides were exchanged for the corresponding domains of VSV G protein. Panels F–H show localization of chimeras between G1 and CD4: Golgi localization is apparent for chimeras in which the CD4 ectodomain and transmembrane domain were fused to the whole cytoplasmic tail of G1 (98 residues) (F), or the 81 proximal residues, i.e., with the G2 signal sequence deleted (H) (see Fig. 3). In contrast, a chimera between the CD4 ectodomain and cytoplasmic tail, and the G1 transmembrane domain resulted in surface expression (G), indicating that the G1 transmembrane domain is not necessary for Golgi localization.

Wasmoen *et al.*, 1988; Chen *et al.*, 1991a,b; Ruusala *et al.*, 1992; Rönnholm, 1992; Pensiero and Hay, 1992; Nakitare and Elliott, 1993; Lappin *et al.*, 1994; Melin *et al.*, 1995). The question thus arises as to which of the two glycoproteins determines Golgi localization, or whether both proteins are necessary.

Cellular proteins destined to different locations in the cells have been found to contain address tags (sorting, targetting, retention signals). Such signals are found on both membrane and secreted proteins, as well as on soluble cytosolic proteins. These include the classical N-terminal signal sequence for ER targetting, signals for import into mitochondria, peroxisomes, and the nucleus (von Heijne, 1990). In the exocytic pathway, signals for sorting proteins from the *trans*-Golgi network (TGN) to the lysosomes, endosomes, secretory granules, apical and basolateral surfaces of polarized cells, as well as retention and recycling signals for keeping proteins in defined compartments, such as the ER and the Golgi, have been identified and in many cases narrowed down to short amino acid stretches. The generally held view is that proteins targetted onto the exocytic pathway are transported out to the cell surface by default (bulk flow) unless they contain retention or sorting signals (Pfeffer and Rothman, 1987). There are no reasons to believe that viral membrane proteins differ from host cell proteins in this respect (Pettersson, 1991; Griffiths and Rottier, 1992).

Against this background, it has been assumed that *Bunyaviridae* glycoproteins also contain a signal for targetting to, and retention in, the Golgi complex. The identification of such a signal has been hampered by the fact that G1 and G2 form heterodimeric complexes. In many cases both proteins have to be coexpressed to become competent to exit the ER (Hurtley and Helenius, 1989). Expression of mutant proteins in these cases requires the coexpression of the other subunit for proper transport. G1 and G2 are complex glycoproteins with 5–6% cysteines in their ectodomains. This makes them vulnerable to misfolding as a consequence of mutagenesis, particularly in their ectodomains. Two approaches can be taken to map the retention signal. One involves removing the signal by mutagenesis resulting in the relief of the Golgi block and transport to the cell surface. The other possibility is to make chimeric proteins by using domains from reporter proteins normally transported to the cell surface. The identification of a Golgi-retention signal in this case relies on the ability of defined domains from either G1 or G2 to retain such reporters in the Golgi. The search for a retention signal for *Bunyaviridae* glycoproteins has just begun and no defined signal has as yet been identified. Only a few *Bunyaviridae* members, notably BUN, PT, HTN, and UUK viruses, have been used as models and the results from these examples are summarized individually below.

A. Bunyamwera Virus

BUN virus glycoproteins localize to the Golgi either in infected cells, or in HeLa cells expressing G1 and G2 from a full-length M cDNA using the T7

polymerase-driven vaccinia virus system (Nakitare and Elliott, 1993; Lappin *et al.*, 1994). In the presence of brefeldin A, the glycoproteins were redistributed to the ER. Recent results by Lappin *et al.* (1994) indicate that G1, G2, and NSm are all localized to the Golgi complex when expressed from the full-length cDNA. G2 and NSm expressed separately also localize to the Golgi, while G1 expressed alone remains in the ER. G1 can be rescued out from the ER by coexpression with G2 (but not with NSm) from a different plasmid. Using recombinant vaccinia viruses, similar results have been reported for LAC virus glycoproteins (Bupp *et al.*, 1994). These results show that G1 requires coexpression of G2 to become transport competent, suggesting heterodimeric interactions between the two proteins. They also indicate that at least G2 contains a Golgi-retention signal.

B. Hantaan Virus

HTN virus G1 and G2 accumulate in the Golgi either in infected cells (Pensiero *et al.*, 1988), or if expressed together from a full-length cDNA clone (Pensiero *et al.*, 1988; Pensiero and Hay, 1992; Ruusala *et al.*, 1992). Regarding the fate of G1 and G2 expressed separately, some inconsistencies have been reported. Pensiero and Hay (1992) found that G1 expressed in CV-1 cells on its own from a recombinant vaccinia virus was targetted to and accumulated in the Golgi complex, while G2 expressed alone remained in the ER. When the two proteins were coexpressed from different plasmids, G2 could be rescued from the ER and was transported to the Golgi. A mutant G1, truncated at its C-terminus by removing 82 residues upstream of the cleavage site of mature G2, was also localized to the Golgi. These results suggest that the first 567 residues of G1 contain a signal for Golgi retention. It should be noted that these localizations were made only on the basis of low-resolution immunofluorescence. Whether G2 accumulates in the Golgi indirectly by binding to G1, or whether it contains its own targetting signal is not clear.

Using similar recombinant viruses (Schmaljohn *et al.*, 1990), Ruusala *et al.* (1992), in contrast, found that neither protein was able to exit the ER on its own in HeLa cells. Only when coexpressed from the same full-length insert, or from separate plasmids, were both proteins localized to the Golgi. These results were confirmed by subcellular fractionation analyses on sucrose gradients. It is not apparent how to reconcile these different results, but differences in host cells, cDNA constructs, or experimental design (in particular the duration of recombinant vaccinia virus infection) are possible explanations.

C. Punta Toro Virus

G1 and G2 expressed together from a recombinant vaccinia virus containing a cDNA lacking most of NSm were found to be targetted to the Golgi

complex. G1 acquired endo H resistance and very little protein was found on the cell surface (Matsuoka *et al.*, 1988). The proteins were retained in the Golgi even after a 6-hr chase in the presence of cycloheximide. In a subsequent report, Chen *et al.* (1991b) found that G2 expressed alone was transported to the cell surface, and an anchor minus, soluble, G2 was secreted into the medium. However, if this truncated G2 was coexpressed with full-length G1, then it was targetted to and retained in the Golgi. These results suggested that G2 lacks a Golgi-retention signal and that it is retained in the Golgi indirectly via its binding to G1. In this report, G1 was not expressed alone and therefore a direct proof that G1 de facto contains the Golgi-retention signal was not obtained. However, Matsuoka *et al.* (1994) have recently reported on the expression of full-length and C-terminally truncated mutants of G1. A mutant with a stop codon at the G2 cleavage site was transported to and retained in the Golgi complex. Progressive deletions of the cytoplasmic tail of G1 up to 10 residues from the G1 transmembrane domain resulted in mutants retained intracellularly, i.e., both in the ER and in the Golgi complex. The ER-retained fraction could not be chased out to the Golgi even after 4 hr of cycloheximide treatment. The inefficient exit of G1 tail deletion mutants from the ER makes it difficult to assess the role of the cytoplasmic tail in keeping G1 in the Golgi. Mutants lacking the complete cytoplasmic tail were transported to the cell surface and a soluble anchor minus mutant was secreted out of the cell. A chimeric molecule containing the ectodomain and cytoplasmic tail of the murine leukemia virus (MCF) envelope protein and the transmembrane domain of G1 was partially retained intracellularly (ER and Golgi), while a chimera in which both the transmembrane domain and cytoplasmic tail were attached to the ectodomain of MCF_{env} was more efficiently localized to the ER and Golgi. A construct containing only the cytoplasmic tail of G1 and the rest from the MCF_{env} was transported to the cell surface. These results, which are partly similar to those obtained for UUK virus G1 (see below), suggest that the Golgi retention signal of PT G1 is complex with contributions both from the transmembrane domain and from the cytoplasmic tail proximal to the membrane. Interpretation of the results is complicated by the difficulty in defining the borders of the transmembrane domain of the PT virus G1. As noted above, the hydrophobic region harboring the putative transmembrane domain is some 50 residues long (Ihara *et al.*, 1985).

D. Uukuniemi Virus

G1 and G2 of UUK virus expressed from a full-length cDNA by using an SV40 vector (Rönnholm, 1992), the T7 polymerase-driven VV expression system (Melin *et al.*, 1995), or the Semliki Forest virus (SFV) replicon (Andersson *et al.*, unpublished) are transported to and retained in the Golgi complex. Both proteins colocalize with markers for the Golgi complex. G1 acquires partial endo H resistance with kinetics resembling that of G1 expressed

during virus infection (Kuismanen, 1984; Melin *et al.*, 1995). G2 expressed alone is retained in the ER and may be transported to the cell surface very slowly (Rönnholm, 1992; Melin *et al.*, 1995). Thus, there seems to be a difference between PT and UUK virus G2 in this respect (see above). G1 expressed alone is transported to and retained in the Golgi (Fig. 5, panels C and D) (Rönnholm, 1992), albeit rather inefficiently (Persson and Pettersson, 1991; Melin *et al.*, 1995). This indicates the presence of a Golgi-targetting signal in G1. When G1 and G2 are coexpressed from separate plasmids (mRNAs), the proteins colocalize to the Golgi (Fig. 5, panels A and B). Thus, G2 requires coexpression of G1 to become transport competent. In addition, the efficiency of G1 transport is increased by coexpressing G2. These findings support the notion that G1 and G2 interact with each other (Persson and Pettersson, 1991).

To map the Golgi retention signal in G1, attention has been focused on the transmembrane domain and cytoplasmic tail of the molecule. Chimeras between these domains of G1 and various domains of proteins efficiently transported to the plasma membrane have been expressed using the T7-VV and Semliki Forest virus systems. In addition, mutants with progressive deletions of the cytoplasmic tail have been analyzed. Chicken lysozyme fused to the transmembrane domain and cytoplasmic tail (including the signal sequence for G2) was targetted to and retained in the Golgi. A G1 construct in which the transmembrane domain and 10 flanking residues on each side were exchanged with those from VSV G was retained in the Golgi (Fig. 5E), while G1 with the transmembrane domain and tail from VSV G was efficiently transported to the cell surface. These results clearly suggested that the retention signal resides in the cytoplasmic tail rather than in the ectodomain or the transmembrane domain. The results were confirmed by analyzing chimeras between CD4 (a plasma membrane protein) and G1. CD4 containing the G1 transmembrane domain and the CD4 tail was efficiently transported to the cell surface (Fig. 5G), while CD4 with its own transmembrane domain and the G1 cytoplasmic tail was retained in the Golgi complex (Fig. 5F). Deleting the G2 signal sequence in this latter hybrid had no effect on the Golgi localization (Fig. 5H). In summary, these results show that the Golgi retention signal is localized to the cytoplasmic tail between residues 10 and 50 (counting from the G1 transmembrane domain) (Pettersson *et al.*, 1996). In contrast to the PT virus G1 (see above), we have found very little effect of the transmembrane domain of UUK virus G1 on Golgi retention. Thus, for UUK virus G1 the cytoplasmic tail is both necessary and sufficient for Golgi localization.

VI. CONCLUSIONS AND PERSPECTIVES

There is one interesting property that unites the diverse members of the *Bunyaviridae* family, namely, their site of maturation in the Golgi complex. At present, we do not understand what specifies these viruses to bud into this

organelle. However, it seems clear that the accumulation of the G1/G2 spike protein complex in this organelle is a major determinant. Further, it seems that only one of the two proteins contains a Golgi-targetting and retention signal, while the other becomes Golgi-localized indirectly by interacting (dimerizing) with the other. The sparse information so far obtained indicates that the Golgi retention signal resides in the tail of one of the proteins, with possible contribution from the transmembrane domain. Since there is no apparent sequence homology between the tails or transmembrane domains of different *Bunyaviridae* glycoproteins, retention may depend more on the conformation than on a primary sequence motif.

The mechanism by which G1/G2 are retained in the Golgi complex remains to be elucidated. There are at least three main possibilities. First, the cytoplasmic tail (and part of the transmembrane domain) could be interacting with cytoskeletal components in the Golgi matrix (Slusarewicz *et al.*, 1994), thereby preventing further transport. Second, the tails could interact with each other to form a lattice underneath the membrane. This could result in the formation of large G1/G2 complexes in the Golgi, which because of their size would be excluded from the transport vesicles. Such complexes have as yet not been demonstrated. *Bunyaviridae* members lack a submembranous matrix (M) protein found in, for example, rhabdo-, orthomyxo-, and paramyxoviruses. The relatively long tail (e.g., 100 residues in UUK virus G1) could serve the function provided by M proteins. The tail is likely to interact with the ribonucleoproteins, since the tail of the other protein is usually short (5 residues in the case of UUK virus G2). The fact that G1–G2 of UUK virus form an icosahedral surface lattice in virions (von Bonsdorff and Pettersson, 1975) suggests that such complexes might be formed in the Golgi complex prior to budding. Membrane proteins are also transported from the ER to the Golgi complex in small vesicles (Pryer *et al.*, 1992). This would mean that the formation of large oligomers in the ER should be prevented in order to facilitate inclusion in such transport vesicles. Third, G1/G2 could be transported to the plasma membrane and then efficiently recycled back to the Golgi complex as has been shown for the *trans*-Golgi network-specific protein TGN38/41 (Stanley and Howell, 1993).

What bearing does the retention of *Bunyaviridae* glycoproteins in the Golgi complex have on the problem of compartmentalization of cellular proteins in general, and vice versa? Few cellular compartment-specific retention signals have so far been identified. These include the KDEL sequence at the C-terminal end of resident ER luminal (soluble) proteins (e.g., BiP/grp78, protein disulfide isomerase) (Pelham, 1990), the double-lysine motif at the C-terminus of ER-retained membrane proteins (Jackson *et al.*, 1990), and the transmembrane domain (and flanking luminal stalk region) of Golgi-localized glycosyl transferases (Munro, 1991; Nilsson *et al.*, 1991; Burke *et al.*, 1992). The KDEL proteins that have escaped ER retention are recycled back to the ER with the help of a membrane receptor (Pelham, 1990). The mechanism for the retention of membrane proteins is not yet known, although

binding to the microtubules in the ER (Dahllöf *et al.*, 1991) or a Golgi matrix (Slusarewicz *et al.*, 1994) have been suggested. Glycosyl transferases co-localizing to the same cisternae in the *medial*-Golgi may in addition form large aggregates by binding to each other via the transmembrane domain ("kin recognition"; Nilsson *et al.*, 1994).

In the case of other viral compartment-specific membrane proteins, equally little is known regarding the retention mechanism. The adenovirus E3/19K is retained in the ER by the double-lysine motif (Jackson *et al.*, 1990). The NS28 of rotavirus is retained in the ER by an unknown mechanism, and no retention motif has been identified (Bergmann *et al.*, 1989). The rotavirus ER luminal VP7 has been shown to be retained by an interaction of its own cleaved signal sequence with the first 31 N-terminal residues of the mature protein. Three residues were found to be critical for ER retention within this latter region (Maass and Atkinson, 1994). Finally, the first transmembrane domain of the coronavirus infectious bronchitis virus M (E1) protein specifies its localization to the *cis*-Golgi; retention may be the result of protein oligomerization (Machamer *et al.*, 1990; Weisz *et al.*, 1993). In the murine hepatitis virus (MHV A59), another coronavirus, the cytoplasmic tail also seems to play an important role (Armstrong and Patel, 1991). It is likely that different proteins are targetted to, and retained in, various membranes by different mechanisms. The elucidation of these mechanisms is a challenge both in cell biology and in virology. It is possible that viruses, including the *Bunyaviridae*, budding at various cellular membranes have during evolution adopted host membrane proteins to serve as spike proteins. In the past, enveloped viruses have been excellent models in cell biology and they will continue to play an important role in the future, e.g., in the elucidation of targetting and retention signals in membrane proteins.

Finally, a commonly asked question is whether budding at intracellular membranes offers any advantage to the virus, in particular in regard to the possibility of evading the immune response. With the detailed knowledge available today on how the humoral and cell-mediated immune responses operate on a molecular and cell biological level, we find this unlikely. A simpler explanation is that different viruses have utilized a diversity of alternative strategies offered by the complex cellular organization and metabolism. A similar broad diversity has evolved in regard to the strategies by which viruses replicate and express their genomes.

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