



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

The membrane glycoprotein G1 of Uukuniemi virus contains a signal for localization to the Golgi complex

Lars Melin, Robert Persson, Agneta Andersson, Anita Bergström,
Ragna Rönnholm, Ralf F. Pettersson *

Ludwig Institute for Cancer Research, Stockholm Branch, Box 240, S-17177 Stockholm, Sweden

Received 24 June 1994; revised 21 December 1994; accepted 27 December 1994

Abstract

Members of the *Bunyaviridae* family acquire their envelopes by budding into the Golgi complex (GC). The accumulation of the membrane glycoproteins G1 and G2 in the GC probably determines the site of maturation. Here we have studied the intracellular transport and targeting to the GC of G1 and G2 of Uukuniemi virus, a member of the *Phlebovirus* genus, and report on their expression from cloned cDNAs either together or separately by using a T7 RNA polymerase-driven vaccinia virus expression system. When G1 and G2 were expressed together from a full-length cDNA as the p110 precursor, both proteins were localized to the Golgi complex, as evidenced by colocalization with the Golgi marker enzyme mannosidase II. Immunofluorescent staining indicated that G1 expressed alone also localized to the GC. However, pulse-chase experiments showed that G1 remained endoglycosidase H sensitive. G2 expressed alone remained associated with the endoplasmic reticulum (ER). G2 could be rescued from the ER and transported to the GC by coexpression with G1 from separate mRNAs. Coexpression also increased the efficiency of G1 transport to the GC. With none of the constructs could the glycoproteins be observed on the cell surface.

These results show that efficient export of G1 and G2 from the ER requires coexpression of both proteins, in conformity with our previous results showing that G1 and G2 form heterodimeric complexes in the ER. Since G1 expressed alone is retained in the GC, we conclude that G1 contains a retention signal for localization to the GC. G2 might thus become associated with the GC indirectly via its interaction with G1.

Keywords: *Bunyaviridae*; *Phlebovirus*; Uukuniemi virus; Golgi complex; Membrane glycoproteins; Protein transport

* Corresponding author. Fax: +46 (8) 33 28 12.

1. Introduction

A number of enveloped viruses mature intracellularly, rather than at the plasma membrane (PM), by budding through endomembranes. Examples of such viruses that have been studied in more detail include rotaviruses (endoplasmic reticulum; ER), coronaviruses (intermediate compartment, Golgi complex; GC), herpesviruses (inner nuclear membrane) and *Bunyaviridae* (GC) (Pettersson, 1991; Griffiths and Rottier, 1992; Hobman, 1993). The molecular basis for utilizing these sites of budding is unknown. One important factor is the targeting to and retention in the budding compartment of one or several of the viral spike proteins. It is thought that the membrane proteins of the above viruses contain a structural motif that defines their compartmentalization. Only in the M (E1) glycoprotein of coronaviruses has such a signal so far been localized, although somewhat conflicting conclusions have been reported (Swift and Machamer, 1991; Armstrong and Patel, 1991). Since many cellular membrane proteins also are transported to and retained in specific intracellular compartments, the viral spike proteins may be used as good models for studying the molecular processes underlying compartmentalization.

Extensive work has indicated that members of the *Bunyaviridae* family bud into the GC (for references see Pettersson et al., 1988; Pettersson, 1991; Matsuoka et al., 1991; Hobman, 1993). Virus particles are thereafter transported in large vesicles, which fuse with the PM, thereby releasing the virus into the extracellular space. As a model, we are using Uukuniemi (UUK) virus, a member of the *Bunyaviridae* family recently reclassified into the *Phlebovirus* genus (Francki et al., 1991). The tripartite single-stranded genome of UUK virus has been completely sequenced from cloned cDNA (Rönholm and Pettersson, 1987; Simons et al., 1992; Elliott et al., 1992). As for all other *Bunyaviridae* (Elliott et al., 1990), the G1 and G2 membrane proteins are encoded by the middle-sized (M) RNA segment as a 110,000-dalton (p110) precursor (Rönholm and Pettersson, 1987) that is cotranslationally cleaved in the ER roughly in the middle (Ulmanen et al., 1981; Kuismänen, 1984). Both proteins have the same size (about 490 amino acids), and each contains four N-linked glycans (Pesonen et al., 1982) and have 26 cysteine residues in their respective ectodomains. Their amino acid sequences are not, however, related.

Pulse-chase experiments have shown that G1 forms its intramolecular disulphide-bonds much faster than G2 (less than 10 min vs. 45–60 min) in the ER, reflecting vastly different kinetics of protein folding. G1 and G2 also heterodimerize in the ER. Our results further indicated that G1 and G2 are dependent on each other for export out of the ER (Persson and Pettersson, 1991). To be able to study the ER to Golgi transport and the retention in the GC of G1 and G2 in the absence of other UUK virus processes, we have expressed the proteins together or separately in BHK21 cells by using the T7 RNA polymerase-driven vaccinia virus system (Fuerst et al., 1986; Elroy-Stein et al., 1989). Our results show that G1 and G2, expressed together are targeted to and retained in the GC. G2 expressed alone is unable to leave the ER on its own, while G1 is able to exit the ER on its

own and become retained in the GC. Thus, a Golgi-retention signal is likely to reside in the G1 glycoprotein.

2. Materials and methods

2.1. Cells and viruses

BHK21/clone-13 cells were cultivated on plastic dishes or bottles, or on coverslips in Eagle's minimum essential medium (EMEM), supplemented with 5% fetal calf serum (FCS) (Gibco Ltd., Middlesex, England), 5% tryptose phosphate broth (TPB), glutamine, penicillin, and streptomycin. HeLa cells were grown in the same medium but supplemented with 10% FCS and without TPB.

The recombinant vaccinia virus (vTF7-3) expressing T7 RNA polymerase (Fuerst et al., 1986; Elroy-Stein et al., 1989) was kindly provided by Dr. Bernard Moss. Virus stocks were prepared from infected HeLa cell homogenates and the titer was determined by plaque assay on HeLa cells according to standard protocols (MacKett et al., 1985).

2.2. Construction of recombinant plasmids

The cDNA inserts encoding p110 (the precursor of G1 and G2), G1 alone, and G2 alone were essentially the same as described by Rönnholm (1992). We first modified the plasmid pTF7-5, generously provided by Dr. B. Moss (Elroy-Stein et al., 1989) by inserting into the unique *Bam*HI site between the T7 promoter and transcription terminator a polylinker containing cleavage sites for 5' *Pst*I, *Kpn*I and *Sal*I 3'. A 3 kb *Pst*I-*Sal*I fragment containing the whole p110 coding sequence and cloned into plasmid pGEM-3 (Promega) was isolated from an agarose gel and ligated into the *Pst*I-*Sal*I-cleaved modified pTF7-5 plasmid. This generated the plasmid pTF-M. The plasmid pTF-G1, containing the whole of G1 coding sequence and the first 98 N-terminal residues of G2, was generated by first cutting pTF-M with *Stu*I (cleaves at nucleotide 1848 within the G2 region) and *Hinc*II (cleaves in the polylinker region downstream of the cDNA insert), followed by blunt-end ligation. To express G2 alone, a construct into which an ATG-codon (also generating an *Nco*I site) was introduced at the N-terminus of the signal sequence of G2, was engineered by using the polymerase chain reaction (Rönnholm, 1992). This fragment was cloned into plasmid pGEM-4 (Promega) downstream from the SP6 promoter to give pGEM-4/G2. An *Xba*I-*Pst*I fragment containing the whole G2 insert was isolated and cloned into pGEM-3Zf(-) (Promega) downstream from the T7 promoter, yielding plasmid pGEM-G2. To increase the translation efficiency of G2, an *Nco*I-*Sal*I fragment containing the G2 cDNA insert was cloned into *Nco*I-*Sal*I-cleaved pTM1 plasmid (Elroy-Stein et al., 1989) to generate pTM1-G2. This plasmid contains the internal ribosome entry site from encephalomyocarditis virus.

2.3. Infection and transfection

Monolayers of BHK21 cells were usually grown to 60–80% confluency before infection with the recombinant vaccinia virus vTF7-3. Before infection, the cells were washed once with EMEM containing 0.04% bovine serum albumin (BSA) and serum-free EMEM was added. Cells were infected with vTF7-3 at a multiplicity of about 10–20 PFU/cell and incubated at 37°C for 45 min. The virus was then removed and replaced by 2 ml of Optimem medium (Gibco BRL). The cells were transfected with plasmid DNA (1–5 µg) using 5–20 µg LipofectinR according to the protocol recommended by the manufacturer (Gibco BRL).

2.4. Metabolic labelling and pulse-chase

Monolayers of BHK21 cells grown in 6 cm dishes were infected with recombinant vTF7-3 virus and transfected with the plasmid constructs as described above. At 4 or 5 h post transfection, cells were incubated with methionine- and serum-free EMEM containing 0.2% BSA for 45 min, followed by pulse-labelling with 100 µCi/ml of [³⁵S]methionine (Amersham International, Buckinghamshire, England) for 20 min. In chase experiments, an excess of unlabelled methionine (10 mM) was added to the cultures for the indicated time periods. Cytoplasmic extracts were prepared as described previously (Persson and Pettersson, 1991) by lysing the cells in solubilization buffer (1% Triton X-100, 5 mM EDTA, 150 mM NaCl, 100 IU/ml of aprotinin, in 20 mM Tris-HCl, pH 8.0). The solubilized cells were scraped off the dishes and centrifuged in a Biofuge for 5 min at maximum speed. The supernatants were frozen in liquid nitrogen and stored at –80°C until analyzed.

In control experiments, cells were infected with Uukuniemi virus at a m.o.i. of about 10, incubated with methionine-free medium for 45 min at 17 h p.i., and then labelled with [³⁵S]methionine for 10 min and chased with unlabelled methionine as described previously (Persson and Pettersson, 1991).

2.5. Immunoprecipitation and endoglycosidase H treatment

The preparation of polyclonal anti-G1 and -G2 antisera in rabbit, as well as monoclonal antibodies against G1 and G2 have been described previously (Wikström et al., 1989; Persson and Pettersson, 1991). The monoclonal antibodies used in this study are called 6G9 (G1-specific) and 11D4 (G2-specific). Aliquots of solubilized cells, or subcellular fractions, were incubated with preimmune serum together with 10% Pansorbin (Sigma) for 60 min on a rotating device at 6°C (Persson and Pettersson, 1991). When monoclonal antibodies were used, samples were pretreated with a non-relevant ascites fluid. In analyses where only polyclonal antibodies were used, the samples were pretreated with SDS and NaCl at final concentrations of 0.5% and 0.5 M, respectively, and then diluted ten times prior to the addition of preimmune serum. After clearing the samples from bacteria, relevant antibodies were added to each sample followed by incubation at 6°C for 3

h. Protein A-Sepharose 4B-CL (25 μ l of a 50% suspension) was added and the samples were mixed for 60 min at 6°C. The Sepharose-bound immune complexes were pelleted, washed twice with high salt (1% NP-40, 400 mM NaCl, 5 mM EDTA, 100 IU/ml of aprotinin in 50 mM Tris-HCl, pH 8.0) and twice with 10 mM Tris-HCl, pH 8.0, resuspended in 35 μ l sample buffer. After heating to 95°C for 3 min and cooling, the immunoprecipitates were alkylated by adding 5 μ l of 0.5 M iodoacetamide. The samples were analyzed on 10 to 15% polyacrylamide gradient slab gels (Maizel, 1971).

Samples to be treated with endoglycosidase H (endo H) (Boehringer-Mannheim) were first immunoprecipitated and the protein-A Sepharose-bound immune complexes were washed as described above. The Sepharose pellets were resuspended in 25 μ l of 0.3% SDS, in 50 mM Na-acetate, pH 5.6, and heated for 2 min at 95°C. The Sepharose was removed by centrifugation and the supernatants divided into two equal aliquots. Ten μ l of endo H (0.25 U/ml) was added to one of the aliquots, whereas 50 mM Na-acetate was added to the other. After incubation for 17 h at 37°C, additional enzyme and buffer were added, and incubation was continued for another 4 h. Sample buffer was then added followed by analysis by SDS-PAGE.

2.6. Immunofluorescence

BHK21 cells grown on coverslips were infected and transfected as outlined above. At 6 h after transfection, cells were washed twice with ice-cold PBS and fixed with 3% paraformaldehyde in PBS for 15 min at room temperature (RT). After washing, the cells were incubated with 10 mM glycine in PBS for 20 min at RT, washed again and subsequently permeabilized with 0.1% Triton X-100 in PBS for 30 min at RT. For surface immunofluorescence, the permeabilization step was omitted. The cells were incubated for 30 min with appropriate dilutions (in PBS containing 0.1% BSA) of relevant mono- or polyclonal antibodies and bound antibodies were visualized by incubating the cells with FITC-conjugated anti-rabbit IgG or TRITC-conjugated anti-mouse IgG antibodies for an additional 30 min period. The Golgi complex was localized by using a monoclonal antibody against the Golgi marker antigen CTR433 (Jasmin et al., 1989), kindly provided by Dr. Michel Bornens, or a rabbit polyclonal antiserum against mannosidase II, a medial-Golgi marker enzyme (Moremen et al., 1991), kindly provided by Dr. Kelley Moreman and Dr. Marilyn Farquhar.

3. Results

3.1. Construction and expression of recombinant plasmids

To express the membrane glycoproteins G1 and G2 of UUK virus in BHK21 cells, we have utilized the T7 RNA polymerase-driven vaccinia virus expression system (Fuerst et al., 1986; Elroy-Stein et al., 1989). The details of constructing the

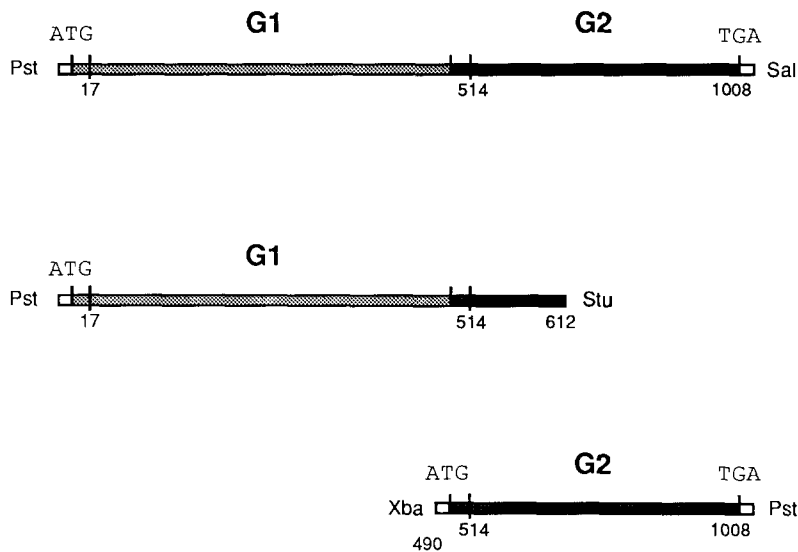


Fig. 1. Schematic representation of the cDNA inserts derived from the Uukuniemi virus M RNA segment that was used to generate the recombinant plasmids pTF-M, pTF-G1, and pGEM-G2 (for details see Section 2). Numbers refer to positions of amino acids starting from the initiating methionine codon of G1 (Rönholm and Pettersson, 1987). Restriction enzyme sites used for cutting the inserts prior to cloning into the plasmids are as indicated. Vertical bars at the N-termini of G1 and G2 indicate the borders of the signal sequences.

three different plasmids used below are described in Section 2. We first transferred the cDNA encoding p110, the precursor of G1 and G2 from the plasmid pGEM-G1/G2 (Rönholm, 1992), to a modified pTF7-5 plasmid. In the plasmid, called pTF-M, the insert is expressed under the control of the T7 promoter and it contains the whole p110 open reading frame (Fig. 1).

A G1-expressing plasmid called pTF-G1 was constructed by deleting the bulk of the G2 region from pTF-M. In this plasmid, the coding sequence of the first 98 N-terminal amino acids of G2 downstream from G1 are retained (Fig. 1). By expressing various tail deletion mutants we have found that the presence or absence of this 98-residue fragment does not affect the transport or localization of G1 (unpublished data).

The plasmids pGEM-G2 and pTM1-G2 were constructed by first introducing an ATG-codon immediately upstream of the internal signal sequence of G2 (Fig. 1) using PCR technology, followed by cloning into the expression plasmids pGEM-3Zf(-) and pTM1. The PCR-amplified fragments were completely sequenced to check for mutations.

The size of the products generated from plasmids pTF-M, pTF-G1 and pTM1-G2 were analyzed in BHK21 cells infected with vTF7-3 and transfected with different plasmids followed by labelling with [³⁵S]methionine for 3 h (5 to 8 h post transfection). The products were immunoprecipitated with polyclonal anti-G1

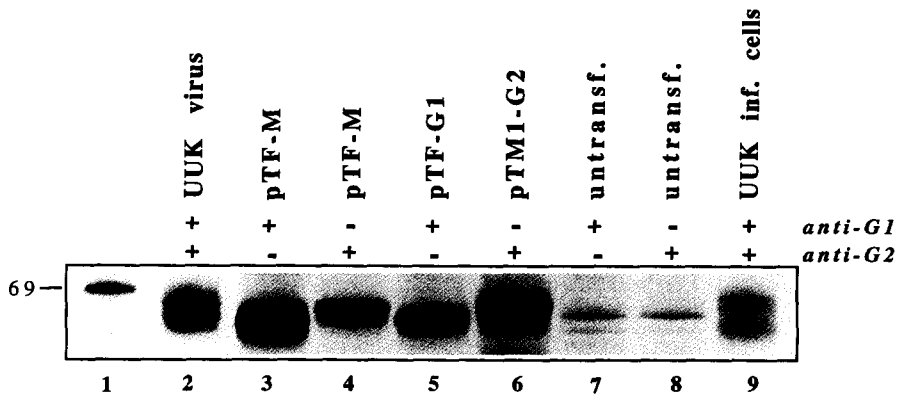


Fig. 2. SDS-PAGE analysis of metabolically labelled G1 and G2 expressed from various recombinant plasmids. Cells were infected with vTF7-3 recombinant vaccinia virus and transfected with plasmids pTF-M (lanes 3 and 4), pTF-G1 (lane 5), and pTM1-G2 (lane 6) or left untransfected (lanes 7 and 8). The cells were then labelled between 5 and 8 h post transfection with [³⁵S]methionine followed by immunoprecipitation with the indicated antibodies and SDS-PAGE. G1 and G2 immunoprecipitated from UUK virus-infected cells pulse-labelled for 10 min (lane 9), or from virions (lane 2), served as controls. The position of the 69 kD molecular weight marker is indicated (lane 1).

and/or -G2 antisera and analyzed by SDS-PAGE. Products with sizes corresponding to those present in virions (Fig. 2, lane 2) and synthesized in UUK virus-infected cells were identified (lane 9). Note that reduced and alkylated G1 migrates ahead of G2, while the order is reversed under non-reduced conditions (Persson and Pettersson, 1991).

The polyclonal anti-G1 and anti-G2 antisera unspecifically coprecipitated a vaccinia virus protein that migrated between G1 and G2 (Fig. 2, lanes 7 and 8). Thus, we conclude that the proteins expressed from all three plasmids had the expected sizes indicating glycosylation and proper cleavage.

3.2. Immunolocalization of G1 and G2 expressed together or separately

Since vaccinia virus causes cytopathic effects and in particular at later stages of the infectious cycle may interfere with the biosynthesis and transport of cellular and heterologous membrane proteins, we have carried out all experiments at early time points, usually between 4 and 8 h p.i. Time-course experiments indicated that G1 and G2 were first detected about 3.5–4 h post transfection. We have previously found that the reactivity of our poly- (Pab) and monoclonal (Mab) antibodies towards immature (ER-localized) and mature (Golgi complex-localized) forms of the glycoproteins vary considerably (Kuismanen et al., 1984; Wikström et al., 1989). These differences most likely reflect conformational maturation during folding in the ER and subsequent intracellular transport. The reactivity of the antibodies has to be taken into account when assessing the intracellular distribution and transport of the expressed proteins. In the present study, we have used two G1-specific and

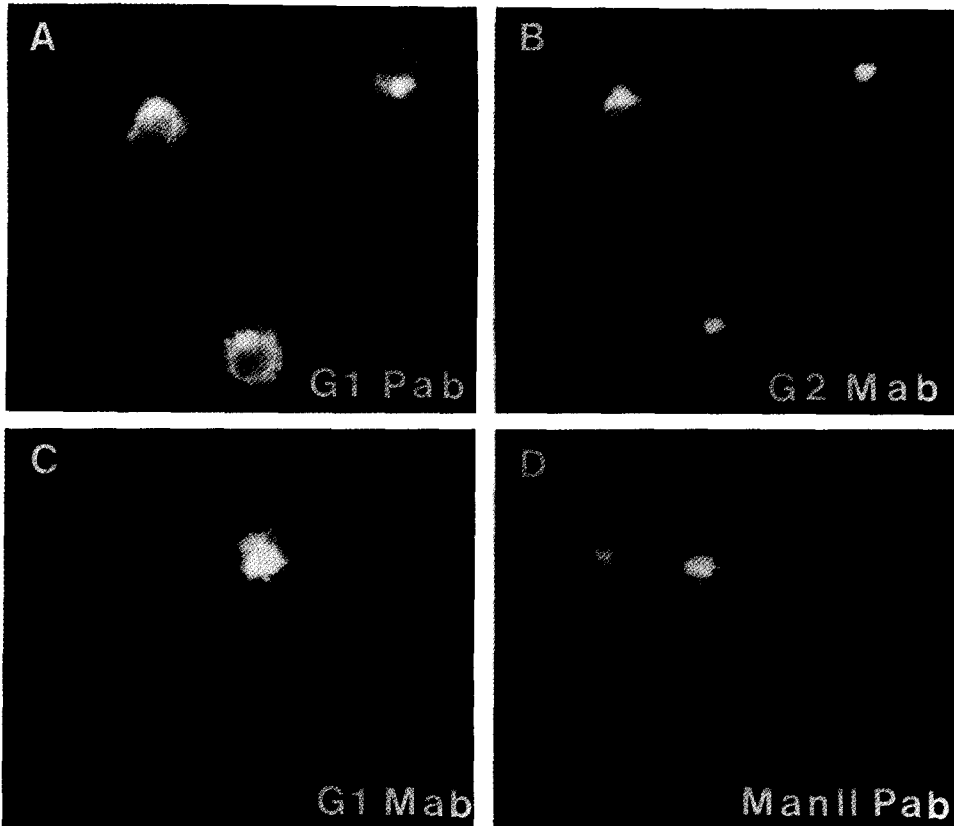


Fig. 3. Immunolocalization of G1 and G2 expressed from pTF-M. BHK21 cells were infected with vTF7-3 followed by transfection with pTF-M. The G1 and G2 proteins were localized by indirect double-immunofluorescence at 6 h after transfection. The cells were stained with anti-G1 Pab (A) and anti-G2 Mab (B), or anti-G1 Mab (C) and anti- α -mannosidase II (D), a marker for the Golgi complex.

two G2-specific antisera: (a) rabbit polyclonal anti-G1 or anti-G2 antisera prepared against SDS-PAGE-purified G1 or G2 (Wikström et al., 1989), which preferentially recognize the ER-forms of the glycoproteins; (b) monoclonal antibodies that preferentially recognize the mature Golgi-forms of the glycoproteins (Persson and Pettersson, 1991). The different reactivities of the G1 antibodies are illustrated, e.g., in Figs. 6A–6C and 6E, where cells expressing G1 alone have been double-stained with the two types of antibodies.

We first analyzed the localization of G1 and G2 expressed from pTF-M, encoding the p110 precursor. As shown by double immunofluorescence (Figs. 3A and 3B), using anti-G1 Pab and anti-G2 Mab, both proteins colocalized to a juxtannuclear region. Double-staining with anti-G1 Mab (Fig. 3C) and a rabbit polyclonal antiserum against mannosidase II (Fig. 3D), a medial-Golgi marker (Moremen et al., 1991), showed that this region corresponds to the Golgi complex.

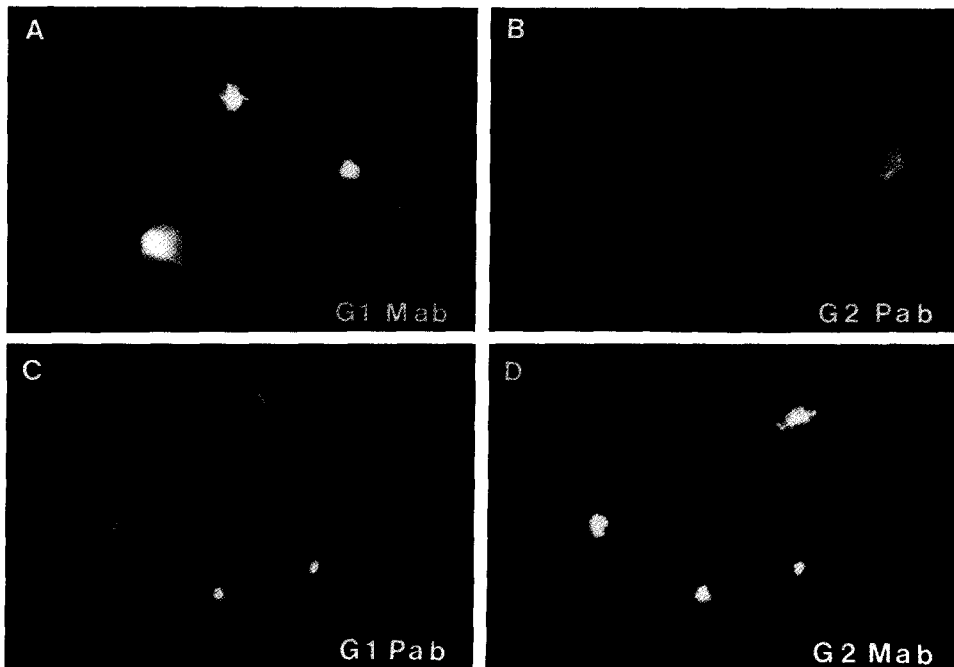


Fig. 4. Immunolocalization of G1 and G2 expressed separately from plasmids pTF-G1 and pGEM-G2. BHK21 cells were infected with vTF7-3 and transfected with the respective plasmids (A and B), or cotransfected with both plasmids (C and D) followed by indirect immunofluorescence at 6 h after transfection. The cells were stained with anti-G1 Mab (A), anti-G2 Pab (B), anti-G1 Pab (C), or anti-G2 Mab (D).

G1 and G2 could not be chased out from the GC as indicated by treating the cells with cycloheximide (50 $\mu\text{g}/\text{ml}$) for 3 h (6–9 h post transfection). The treatment did not alter the intracellular localization of G1 or G2, nor was any immunoreactivity observed on the cell surface (data not shown). Thus, we conclude that G1 and G2 when expressed from the same mRNA, and in the absence of other viral proteins, are targeted to and retained in the GC.

When cells were transfected with pTF-G1, G1 could also be visualized in the GC with the anti-G1 Mab (Fig. 4A). In contrast, G2 expressed alone from pGEM-G2 (not shown) or pTM1-G2 (Fig. 4B) was localized by the anti-G2 Pab to a reticular perinuclear region, including the nuclear membrane. This pattern is reminiscent of the ER. The anti-G2 Mab stained cells only very weakly, with no Golgi-profiles evident (data not shown). Double-staining with anti-G2 (Fig. 5A) and CTR433 (Fig. 5B), a Golgi marker (Jasmin et al., 1989), also showed that G2 is not concentrated in the GC. These results suggested that G2 was retained in the ER. With none of the constructs was any immunofluorescence detected on the cell surface (data not shown). To confirm that G1 expressed alone was indeed localized to the region of the GC, transfected cells were double-stained either with anti-G1

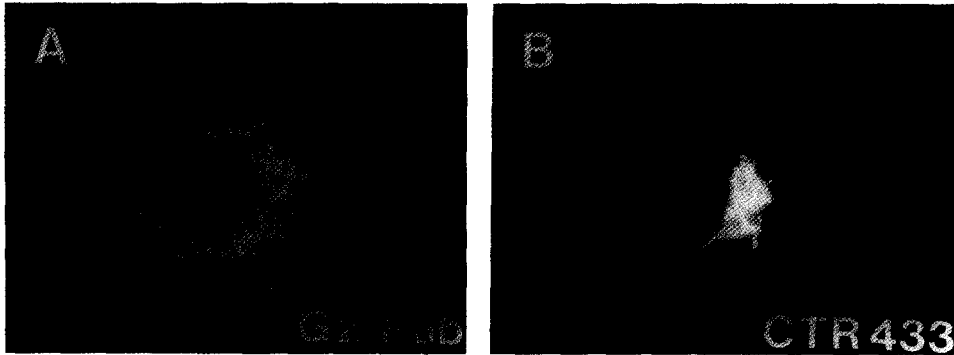


Fig. 5. Colocalization of G2 and CTR433, a marker for the Golgi complex. BHK21 cells were infected with vTF7-3 and transfected with pTM1-G2. The cells were stained with anti-G2 Pab (A) and CTR433 Mab (B).

Mab and mannosidase II (Figs. 6C and 6D), or anti-G1 Pab and anti-CTR433 Mab (Figs. 6E and 6F). In both cases, G1 colocalized with the Golgi-markers.

To analyze whether G2 expressed from pTM1-G2 could be rescued out from the ER by coexpression with G1, cells were cotransfected with pTF-G1 and pTM1-G2. It should be noted that G1 and G2 are now expressed from separate mRNAs. As shown in Fig. 4, both G1 and G2 were found in the GC, as evidenced by double-staining with the anti-G1 Pab (Fig. 4C) and the anti-G2 Mab (Fig. 4D). Thus, G1 was apparently able to interact with G2 and thereby to facilitate the exit of G2 from the ER.

3.3. Pulse-chase analyses of the intracellular transport of G1 and G2

Previous studies have indicated that in UUK virus-infected cells G1 acquires endoglycosidase H-resistant glycans during its transport through the GC, while the glycans of G2 remain largely endo H-sensitive (Kuismanen, 1984). Thus, the kinetics and efficiency of transport can be studied by pulse-chase only for G1. Cells infected with vTF7-3 and subsequently transfected with pTF-M, pTF-G1, pGEM-G2 separately, or pTF-G1 and pGEM-G2 together were pulse-labelled for 20 min with [³⁵S]methionine followed by chases with an excess of unlabelled methionine for different time periods as indicated in Fig. 7. Samples from cytoplasmic extracts were subjected to immunoprecipitation with the anti-G1 Pab, and half of the immunoprecipitates were treated with endo H. Finally, the samples were analyzed by SDS-PAGE and autoradiography. G1 expressed from pTF-M started to acquire endo H-resistance from 30 min on (Fig. 7A), reaching a level of about 70% resistance by the end of the chase. During the chase, no degradation of G1 was observed. The appearance of one fully and three partially resistant bands is most likely explained by the fact that G1 has four sites for N-glycosylation. The observed pattern suggests that only some of the glycans on G1 became terminally modified. The pattern and kinetics of acquisition of endo H-resistance was similar for G1 in

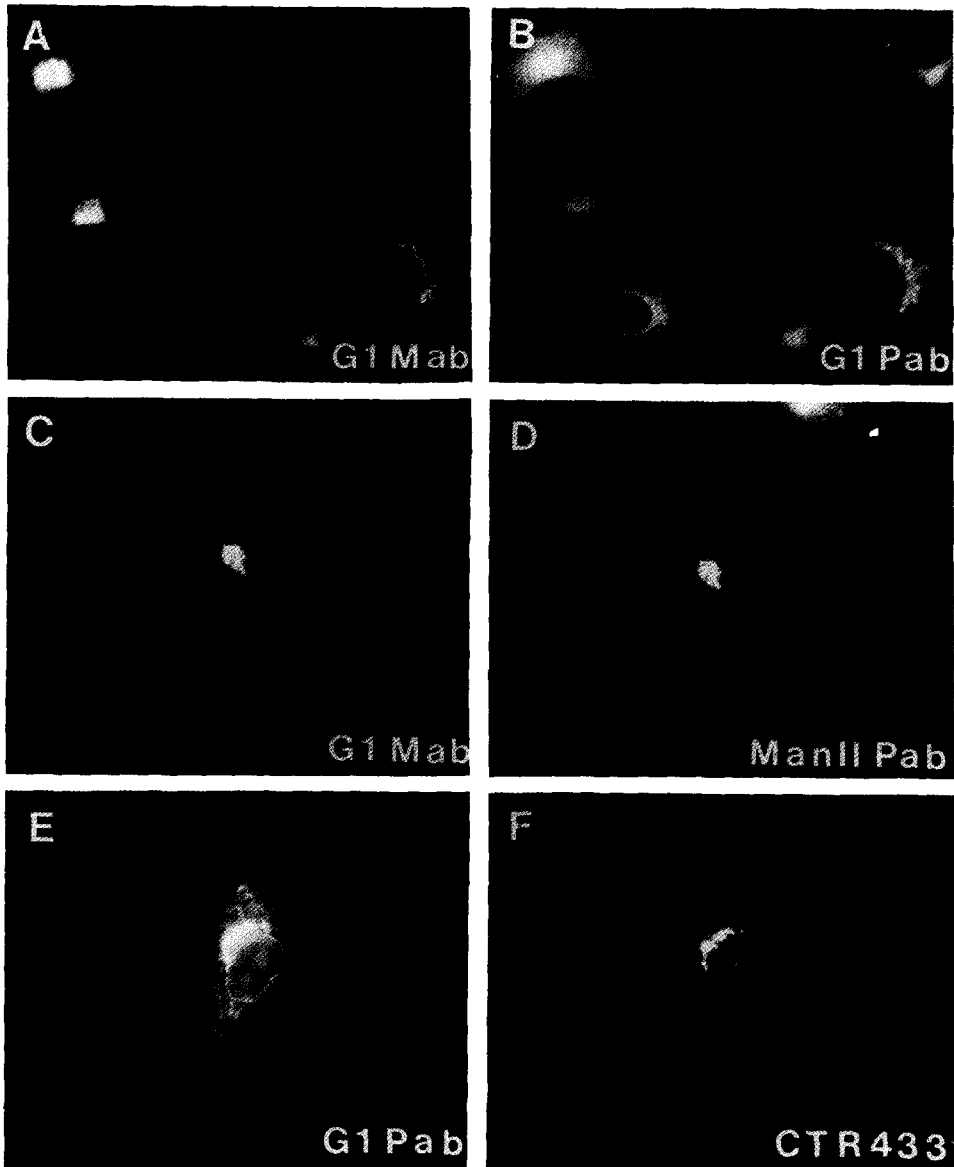


Fig. 6. Colocalization of G1 with markers for the Golgi complex. BHK21 cells were infected with vTF7-3 and transfected with pTF-G1. The cells were double-stained with anti-G1 Mab (A) and anti G1-Pab (B) to show the differential reactivity of the two antisera. Anti-G1 Pab preferentially recognizes the ER-form, while anti-G1 Mab preferentially stains the mature Golgi-form of G1. Double-staining with anti-G1 Mab (C) and anti- α -mannosidase II (D), or anti-G1 Pab (E) and anti-CTR433 (F), show that G1 expressed alone colocalizes with the Golgi markers.

UUK virus-infected cells, although sialylation known to occur (Kuismanen, 1984) caused smearing of the partially resistant bands (Fig. 7D) to a much larger extent than in G1 expressed from pTF-M.

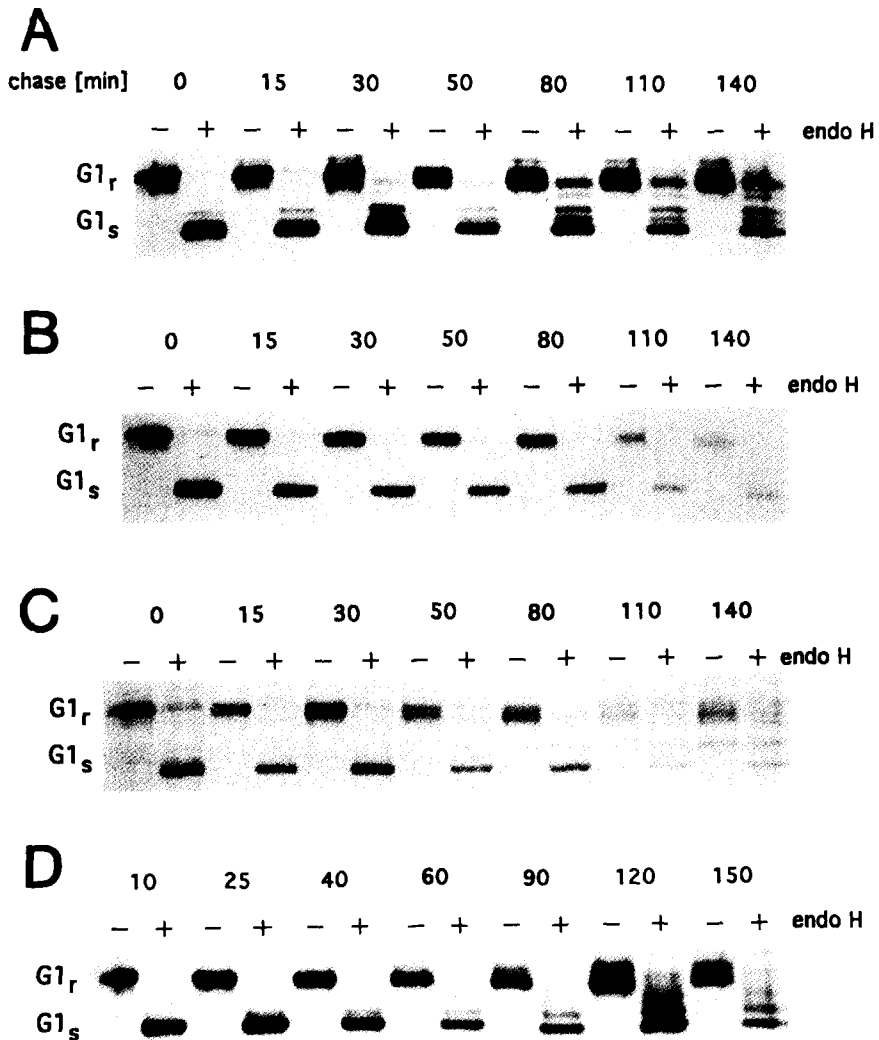


Fig. 7. Acquisition of endoglycosidase H-resistant glycans of G1 expressed from different plasmids. Cells were infected with vTF7-3 and transfected 45 min later with the various plasmids. At 5 h post transfection, cells were labelled with [³⁵S]methionine for 20 min followed by chase periods up to 150 min in the presence of unlabelled methionine. UUK virus-infected cells, pulse-labelled for 10 min, served as a control. Immunoprecipitated G1, either treated with endo H or left untreated, was analyzed by SDS-PAGE and autoradiography. The panels show the results from cells transfected with pTF-M (A), pTF-G1 (B), pTF-G1+pGEM-G2 (C), and from virus-infected cells (D). G1_r = endo H-resistant G1, G1_s = endo H-sensitive G1.

G1 expressed alone from pTF-G1 did not seem to acquire endo H-resistant glycans (Fig. 7B). The decreased recovery of G1 during the chase indicated that G1 is rapidly degraded. When cells were cotransfected with pTF-G1 and pGEM-G2, some G1 again acquired endo H-resistant glycans (Fig. 7C), suggesting that interaction with G2 is required for terminal glycosylation to occur. The fact that G1 also in this coexpression experiment appeared to be degraded faster than when expressed together with G2 from the same mRNA (Figs. 7A and 7D) is likely to be due to the fact that the balanced (equimolar) expression of G1 and G2 in every cell is very difficult to achieve. We have found that G1 is much more efficiently expressed than G2 from their respective plasmid constructs. Therefore, G1 without a G2 partner would be degraded (compare with Fig. 7B).

From these experiments we conclude that G1 becomes partially glycosylated when coexpressed with G2, while terminal glycosylation apparently does not occur when G1 is expressed alone. The interpretation of this finding is discussed below.

4. Discussion

The purpose of the experiments described above was to analyze the intracellular transport and localization of the membrane proteins G1 and G2 of Uukuniemi virus, with the specific aim of finding out whether either of the glycoproteins when expressed separately could be targeted to and retained in the Golgi complex. The results can be summarized as follows. When expressed together from the same or separate mRNA, G1 and G2 both localized to the GC. Similarly, G1 expressed alone was also targeted to and retained in the GC. Exit from the ER was, however, inefficient. G2 expressed alone was apparently unable to exit the ER, but could be rescued onto the exocytic pathway by coexpression with G1. Two further conclusions can be drawn from the results. Firstly, that the hydrophobic sequence preceding G2, as has been postulated previously (Kuismanen, 1984; Rönnholm and Pettersson, 1987), functions as an internal signal peptide for the translocation of G2 through the ER membrane. Secondly, that G1 and G2 translated from separate mRNAs can interact with each other, presumably by forming heterodimers (Persson and Pettersson, 1991), i.e. there is no need to generate G1 and G2 from the p110 precursor by cleavage in order to form a transport competent complex (see below). Similar results have been obtained regarding the formation of mixed influenza virus haemagglutinin trimers (Boulay et al., 1988).

The finding that G1 and G2 expressed together accumulate in the GC is in conformity with our previous results obtained with a temperature-sensitive mutant (ts12). In this mutant, the defect most likely resides in the NSs protein, derived from the S RNA segment (J.F. Simons and R.F. Pettersson, unpublished results), and G1 and G2 are thus wild-type. In cells infected with ts12 at the restrictive temperature (39°C), G1 and G2 were targeted to and retained in the GC in the absence of ribonucleoprotein accumulation in the GC, and virus budding (Gahmberg et al., 1986a, b). This implied that the Golgi-targeting information resides in either G1 or G2.

The use of the T7 RNA polymerase-driven transcription of the mRNAs ensured

an efficient expression of G1 and G2. Since vaccinia virus is cytopathic to cells, we carried out the experiments as early as possible in the infectious cycle. However, similar results were obtained also at later time points (about 12 h post transfection; data not shown). In a recent study, results similar to those reported here were obtained for G1 and G2 expressed by using a SV40 expression vector (Rönholm, 1992). However, the transfection efficiency and the level of expression using this system were too low to allow characterization of the efficiency and kinetics of the intracellular transport of G1 and G2. Thus, the proteins could only be localized by immunofluorescence.

Our previous results indicated that G1 and G2 form heterodimeric complexes in the ER. Apparently only properly folded (disulphide-bonded) glycoproteins are able to dimerize. Since newly synthesized G1 folds much faster than newly made G2, it seems unlikely that G1 and G2 synthesized from the same p110 precursor will end up in the same heterodimer. Instead, it seems that a pool of G1 and G2 molecules exists in the ER from which G1/G2 heterodimers can be recruited. It was therefore not surprising to find that G1 and G2 synthesized from separate mRNAs were able to interact and to become transported out of the ER.

As has been observed previously in virus-infected cells (Persson and Pettersson, 1991), the transport to the GC of G1 and G2 expressed together was rather slow and inefficient as documented by the partial acquisition of endo H-resistant glycans in G1. The basis for this is unknown, but may be due to an inherent property of the glycoproteins. We found previously that G2 required the continuous synthesis of G1 in order to become transported to the GC. Whether G1 also required concomitant synthesis of G2 remained less clear (Persson and Pettersson, 1991). The results obtained here confirm the finding that G2 is unable to exit the ER on its own. Presumably transport competence is only acquired after dimerization with G1. It is well known that oligomerization in the ER of protein complexes is usually required to achieve competence to exit the ER (Rose and Doms, 1988; Hurtley and Helenius, 1989). It was therefore somewhat surprising to find that G1 was able to exit the ER and to reach the GC on its own. The same observation was made for G1 expressed by using the SV40-based system (Rönholm, 1992). As shown in Figs. 3A, 5C and 5E, G1 was clearly localized to a juxtannuclear region identified as the GC by double-staining with antibodies against Golgi markers. Based on immunofluorescence, only a fraction of G1 was transported to the GC. G1 expressed alone was rapidly degraded and no evidence for acquisition of endo H-resistant glycans was obtained. This latter finding was unexpected and could mean (1) that the amount of endo H-resistant G1 transported to the GC was below detection level as studied by this method, (2) that G1 did not reach the medial-Golgi where endo H-resistance is acquired, or (3) that the G1 transported to the GC could not be used as a substrate for N-acetylglucosamine transferase. At present we cannot distinguish between these alternatives. Whether the fraction of G1 that is able to exit the ER is transported as monomers or oligomers (e.g. homodimers) remains another open question.

Since G1 expressed alone was retained in the GC in the absence of G2, we conclude that a signal for Golgi-localization resides in this protein. The question

whether G2 also contains a Golgi-retention signal could not be addressed in this study, since G2 expressed alone remained in the ER. The glycoproteins of Punta Toro virus (PT, another phlebovirus) also dimerize and are retained in the GC (Matsuoka et al., 1988; Chen et al., 1991; Chen and Compans, 1991). Recent results suggest that the Golgi-localization signal of PT virus is present in G1, and that G2 becomes targeted to the GC indirectly by virtue of its interaction with G1. The latter conclusion was drawn from the fact that G2 expressed alone remained in the ER, while an anchor-minus soluble G2 was secreted out of the cell, but became targeted to the GC when coexpressed with a membrane-anchored G1 (Chen et al., 1991). The Golgi-retention signal in G1 of PT was recently mapped to the transmembrane domain and cytoplasmic tail (Matsuoka et al., 1994).

Regarding the behaviour of Hantaan virus (the prototype member of the *Hantavirus* genus) glycoproteins, conflicting results have been obtained. Our own studies indicated that neither G1 nor G2 could exit the ER on their own, while both proteins localized to the GC when coexpressed (Ruusala et al., 1992). In contrast, Pensiero and Hay (1992) found that G1 alone could be targeted to the GC, while G2 remained in the ER. This conclusion was based solely on immunofluorescence localization of Hantaan virus G1 and G2 expressed from recombinant vaccinia viruses late in the infection (17 h p.i.). The reason for the different results remains at present unclear, especially since the same cDNA clones were used in both studies. It is possible that the differences are not real, but are simply due to different interpretations by Pensiero and Hay of results that in fact are the same as those reported by Ruusala et al. (1992).

Using the T7-vaccinia virus expression system, Nakitare and Elliott (1993) found that G1 and G2 of Bunyamwera virus (the prototype member of the *Bunyavirus* genus) expressed from the full-length M segment cDNA localized to the Golgi complex. In a recent paper, they further show that G2 and NSm was localized to the GC when expressed alone, while G1 was retained in the ER. G1 could be rescued out of the ER by coexpressing G2 (Lappin et al., 1994). These results are very similar to those presented here. Thus, the results with several bunyaviruses now clearly indicate that the two spike proteins are targeted and retained in the GC in the absence of other viral proteins, and that the retention signal specifying Golgi localization resides in only one of the glycoproteins.

The nature of the Golgi-retention signal remains to be determined by expressing genetically modified forms of UUK virus G1. The regions of interest include the cytoplasmic tail (which could be as long as about 100 amino acids) (Rönholm and Pettersson, 1987) and the transmembrane domain with its flanking borders (Matsuoka et al., 1994). Mapping of the Golgi-specific coronavirus glycoprotein M (E1) (Swift and Machamer, 1991) and e.g. the cellular Golgi-enzymes sialyl- (Munro, 1991; Wong et al., 1992) and galactosyl- (Nilsson et al., 1991; Teasdale et al., 1992) transferases have shown that the transmembrane domain and the sequences flanking that domain are necessary and sufficient to retain these proteins in the GC. It will be interesting to see whether anchoring of the Bunyaviridae glycoprotein(s) to Golgi-membranes is based on similar domains and mechanisms.

Acknowledgements

We wish to dedicate this article to the memory of Ms. Maj-Greth Nilsson, who regretfully passed away during the course of this work. Maj-Greth was instrumental in carrying out some of the cloning and sequencing work. We thank Yihai Cao, Ulf Eriksson, Jan Fredrik Simons and Sune Kvist for valuable discussions, and Dr. Bernard Moss for the recombinant vaccinia virus vTF7-3, and the plasmids pTF7-5 and pTM1.

References

- Armstrong, J. and Patel, S. (1991) The Golgi sorting domain of coronavirus E1 protein. *J. Cell Sci.* 98, 567–575.
- Boulay, F., Doms, R.W., Webster, R.G. and Helenius, A. (1988) Posttranslational oligomerization and cooperative acid activation of mixed influenza hemagglutinin trimers. *J. Cell Biol.* 106, 629–639.
- 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.
- Elliott, R.M., Schmaljohn, C.S. and Collet, M.S. (1990) Bunyaviridae genome structure and gene expression. *Curr. Topics Microbiol. Immunol.* 169, 91–141.
- Elliott, R.W., Dunn, E., Simons, J.F. and Pettersson, R.F. (1992) Nucleotide sequence and coding strategy of the Uukuniemi virus L RNA segment. *J. Gen. Virol.* 73, 1745–1752.
- Elroy-Stein, O., Fuerst, T. and Moss, B. (1989) Cap-independent translation of mRNA conferred by encephalomyocarditis virus 5' sequence improves the performance of the vaccinia virus/bacteriophage T7 hybrid expression system. *Proc. Natl. Acad. Sci., U.S.A.* 86, 6126–6130.
- Francki, R.I.B., Fauquet, C.M., Knudson, D.L. and Brown, F. (1991) In: Classification and nomenclature of viruses. Bunyaviridae. *Archives of Virology, Suppl. 2.* Springer-Verlag, Berlin, pp. 273–283.
- Fuerst, T.R., Niles, E.G., Studier, F.W. and Moss, B. (1986). Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci., U.S.A.* 83, 8122–8126.
- Gahmberg, N., Pettersson, R.F. and Kääriäinen, L. (1986a) Efficient transport of Semliki Forest virus glycoproteins through a Golgi complex morphologically altered by Uukuniemi virus glycoproteins. *EMBO J.* 5, 3111–3118.
- Gahmberg, N., Kuismanen, E., Keränen, S. and Pettersson, R.F. (1986b) Uukuniemi virus glycoproteins accumulate in and cause morphological changes of the Golgi complex in the absence of virus maturation. *J. Virol.* 57, 899–906.
- Griffiths, G. and Rottier, P. (1992) Cell biology of viruses that assemble along the biosynthetic pathway. *Sem. Cell Biol.* 3, 367–381.
- Hobman, T.C. (1993) Transport of viral glycoproteins into the Golgi complex. *Trends Microbiol.* 1, 124–130.
- Hurtley, S.M. and Helenius (1989) Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell. Biol.* 5, 277–307.
- Jasmin, B.J., Cartaud, J., Bornens, M. and Changeux, J.P. (1989) Golgi apparatus in chick skeletal muscle: changes in its distribution during end plate development and after conversion. *Proc. Natl. Acad. Sci., U.S.A.* 86, 7218–7222.
- Kuismanen, E. (1984) Posttranslational processing of Uukuniemi virus glycoproteins G1 and G2. *J. Virol.* 51, 806–812.

- Kuismanen, E., Bång, B., Hurme, M. and Pettersson, R.F. (1984) Uukuniemi virus maturation: immunofluorescence microscopy with monoclonal glycoprotein-specific antibodies. *J. Virol.* 51, 137–146.
- 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, 1–11.
- Mackett, M., Smith, G.L. and Moss, B. (1985) The construction and characterization of vaccinia virus recombinants expressing foreign genes. In: D.M. Glover (Ed.), *Dna Cloning*, Vol. 2. Oxford IRL Press, Oxford, pp. 191–212.
- Maizel, J.V., Jr. (1971) Polyacrylamide gel electrophoresis of viral proteins. *Methods Virol.* 5, 179–246.
- Matsuoka, Y., Ihara, T., Bishop, D.H.L. and Compans, R.W. (1988) Intracellular accumulation of the Punta Toro virus glycoproteins expressed from cloned cDNA. *Virology* 167, 251–260.
- Matsuoka, Y., Chen, S.Y. and Compans, R.W. (1991) Bunyavirus protein transport and assembly. *Curr. Topics Microbiol. Immunol.* 169, 161–179.
- Matsuoka, Y., Chen, S.-Y. and Compans, R.W. (1994). A signal for Golgi retention in bunyavirus G1 glycoproteins. *J. Biol. Chem.* 269, 22565–22573.
- Moremen, K.W., Touster, O. and Robbins, P.W. (1991) Novel purification of the catalytic domain of Golgi α -mannosidase, II. Characterization and comparison with the intact enzyme. *J. Biol. Chem.* 266, 16876–16885.
- Munro, S. (1991). Sequences within and adjacent to the transmembrane segment of α -2,6-sialyltransferase specify Golgi retention. *EMBO J.* 10, 3577–3588.
- Nakitare, G.W. and Elliott, R.M. (1993) Expression of the Bunyamwera virus M genome segment and intracellular localization of NSm. *Virology* 195, 511–520.
- Nilsson, T., Lucocq, J.M., Mackay, D. and Warren, G. (1991) The membrane spanning domain of β -1,4 galactosyltransferase specifies *trans* Golgi localization. *EMBO J.* 10, 3567–3575.
- Pensiero, M.N. and Hay, J. (1992) The Hantaan virus M-segment glycoproteins G1 and G2 can be expressed independently. *J. Virol.* 66, 1907–1914.
- Persson, R. and Pettersson, R.F. (1991) Formation and intracellular transport of a heterodimeric viral spike protein complex. *J. Cell Biol.* 112, 257–266.
- Pesonen, M., Kuismanen, E. and Pettersson, R.F. (1982) Monosaccharide sequence of protein-bound glycans of Uukuniemi virus. *J. Virol.* 41, 390–400.
- Pettersson, R.F. (1991) Protein localization and virus assembly at intracellular membranes. *Curr. Topics Microbiol. Immunol.* 170, 67–106.
- Pettersson, R.F., Gahmberg, N., Kuismanen, E., Kääriäinen, L., Rönnholm, R. and Saraste, J. (1988) Bunyavirus membrane proteins as models for Golgi-specific proteins. *Mod. Cell Biol.* 6, 65–96.
- 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 Doms, R.W. (1988) Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 4, 257–288.
- 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.
- Simons, J., Hellman, U. and Pettersson, R.F. (1992) Uukuniemi virus S RNA segment: ambisense coding strategy, packaging of complementary strands into virions, and homology to members of the genus *Phlebovirus*. *J. Virol.* 64, 247–255.
- Swift, A. and Machamer, C.E. (1991) A Golgi retention signal in a membrane-spanning domain of coronavirus E1 protein. *J. Cell. Biol.* 115, 19–30.
- Teasdale, R.D., Dágostaro, G. and Gleeson, P.A. (1992) The signal for Golgi retention of bovine β 1,4-galactosyltransferase is in the transmembrane domain. *J. Biol. Chem.* 267, 4084–4096.
- Ulmanen, I., Seppälä, P. and Pettersson, R.F. (1981) In vitro translation of Uukuniemi virus-specific RNAs: identification of a nonstructural protein and a precursor to the membrane glycoproteins. *J. Virol.* 37, 72–79.

- Wikström, L., Persson, R. and Pettersson, R.F. (1989) Intracellular transport of the G1 and G2 membrane glycoproteins of Uukuniemi virus. In: D. Kolakofsky and B.W.J. Mahy (Eds.), *Genetics and Pathogenicity of Negative Strand Viruses*. Elsevier, New York, pp. 33–41.
- Wong, S.H., Low, S.H. and Hong, W. (1992) The 17-residue transmembrane domain of β -galactoside α 2,6-sialyltransferase is sufficient for Golgi retention. *J. Cell Biol.* 117, 245–258.