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Processing and Membrane Topology of the Spike Proteins G1 and G2 of Uukuniemi Virus

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The membrane glycoproteins G1 and G2 of the members of the *Bunyaviridae* family are synthesized as a precursor from a single open reading frame. Here, we have analyzed the processing and membrane insertion of G1 and G2 of a member of the *Phlebovirus* genus, Uukuniemi virus. By expressing C-terminally truncated forms of the p110 precursor containing the whole of G1 and decreasing portions of G2, we found that processing in BHK21 cells occurred with an efficiency of about 50% if G1 was followed by 50 residues of G2, while complete processing occurred if 98, 150, or 200 residues of G2 were present. Surprisingly, processing of all truncated G2 forms was less efficient in HeLa cells. Proteinase K treatment of microsomes isolated from infected cells indicated that the C terminus of G1 is exposed on the cytoplasmic face. Using G1 tail peptide antisera, the tail was likewise found by immunofluorescence to be exposed on the cytoplasmic face in streptolysin O-permeabilized cells. By introducing stop codons at various positions of the G1 tail and at the natural cleavage site between G1 and G2 and expressing these mutants in BHK cells, we found that no further processing of the G1 C terminus occurred following cleavage of G2 by the signal peptidase. This was also supported by the finding that an antiserum raised against a peptide corresponding to the region immediately upstream from the G2 signal sequence reacted in immunoblotting with G1 from virions. Finally, we show that both G1 and G2 are palmitylated. Taken together, these results show that processing of p110 of Uukuniemi virus occurs cotranslationally at only one site, i.e., downstream of the internal G2 signal sequence. G1 and G2 are inserted as type I proteins into the lipid bilayer, leaving the G1 tail exposed on the cytoplasmic face of the membrane. Since the G2 tail is only 5 residues long, the G1 tail is likely to be responsible for the interaction with the nucleoproteins during the budding process, in addition to harboring a Golgi localization signal.

Members of the five genera (*Bunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus*) of the *Bunyaviridae* family have a tripartite, single-stranded RNA genome of negative polarity. For all viruses, the medium-sized segment (M) encodes the membrane glycoproteins G1 and G2 in one single open reading frame (32). Thus, G1 and G2 are synthesized as a precursor that is proteolytically processed to the final products. An isolated report suggesting the possibility of an internal initiation of translation of G2 has recently been published (9). It should be noted that the glycoproteins have originally been named according to their relative mobility on reducing sodium dodecyl sulfate (SDS) gels. Thus, G1 is the slower-migrating protein, and G2 is the faster-migrating protein. To overcome the confusion in nomenclature generated based on protein mobility, a new nomenclature according to which the N-terminally and C-terminally located proteins are called GN and GC, respectively, has recently been proposed (16).

There is a considerable heterogeneity between the genera in regard to the size of G1 and G2 and the presence or absence of nonstructural peptides, called NSm (3, 4, 27). In the case of *Bunyavirus* members, the gene order is NH_2-G2-NSm-G1-COOH. The nonglycosylated NSm is an integral membrane protein, it colocalizes with G2 and G1 to the Golgi complex, but it is not found in virions (16). In contrast, *Hantavirus* members lack an NSm altogether, and the gene order is NH_2-G1-G2-COOH (31, 32). The lack of an NSm may also be true for nairoviruses (gene order, NH_2-G2-G1-COOH), although the processing of the precursor has so far been poorly characterized (21).

Some phleboviruses (e.g., Rift Valley fever and Punta Toro viruses) have an N-terminally located NSm (or pre-G) (4), while Uukuniemi (UUK) virus (formerly the prototype of the *Uukuvirus* genus, but presently a member of the *Phlebovirus* genus lacks an NSm (30).

Finally, the glycoprotein precursor of tospoviruses also lacks an NSm, but an M-encoded protein is synthesized from the complementary strand in an ambisense fashion. This NSm is not a membrane protein and apparently has a function in viral nucleocapsid movement from cell to cell (10).

The function of the membrane-associated NSm has been elucidated for none of the viruses. A minimum function would be to provide an internal signal sequence for translocation of the downstream protein through the endoplasmic reticulum (ER) membrane. In all cases studied, both G1 and G2 are preceded by their own signal peptide. This means that the downstream, C-terminally located glycoprotein (including NSm in the case of *Bunyavirus* members) is preceded by an internal hydrophobic signal sequence. It is likely that signal peptidase is responsible for the cleavage downstream of these sequences to generate the N terminus of the mature protein. The fate of the internal signal sequence has remained unclear, i.e., it has not been elucidated whether the signal peptide is removed by a second proteolytic event on the cytoplasmic face or whether it remains attached to the upstream protein.

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We are using UUK virus as a model to study the biosynthesis of the membrane proteins and the assembly of *Bunyaviridae* members. UUK virus G1 (M_r, 70,000) and G2 (M_r, 65,000) are synthesized as a 110,000-Da precursor (p110) with the gene order NH_2-G1-G2-COOH (11, 30). This p110 is found only in in vitro translates in the absence of microsomal membranes, but it is seen neither in infected cells nor following in vitro translation in the presence of microsomal membranes (35). Both G1 and G2 contain four sites for N-linked glycosylation (30), and they form heterodimers in the ER (24). Whether they are present as heterodimers also in virions is a matter of debate (24, 29). Following folding and assembly, G1 and G2 are transported to the Golgi complex, the site of virus budding, where they accumulate (12, 13, 26). Recently, the Golgi retention signal was mapped to the cytoplasmic tail of G1 of UUK virus (28). This finding has given us a strong impetus to analyze the requirements for cotranslational cleavage of p110 and the structure and topology of the cytoplasmic tail of G1. We found that apart from cleavage by signal peptidase, no further processing of the G1 cytoplasmic tail occurs. Thus, UUK virus does not have an NSm equivalent to the NSm of *Bunyavirus* members. Finally, we show that the tail is palmitylated and exposed on the cytoplasmic face of the lipid bilayer.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 IU of penicillin per ml, and 100 μg of streptomycin (Life Technologies) per ml. BHK21 cells were grown in the same medium, additionally supplemented with 5% tryptose phosphate broth (Gibco). The cells were plated in tissue culture dishes or on coverslips 18 to 24 h before use.

The origin and cultivation of the prototype strain S23 of UUK virus have been described previously (25). The recombinant vaccinia virus (vTF7-3) expressing T7 RNA polymerase (5) was kindly provided by 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.

Antisera. Two peptides corresponding to amino acids 57 to 69 (PREQN NPARAVAR, peptide A) and 72 to 83 (VRQKMFNLTRLS, peptide B) (see Fig. 1) in the C terminus of G1 were synthesized on an Applied Biosystems Peptide Synthesizer, model 431A (Perkin-Elmer), using 9-fluorenylmethoxycarbonyl chemistry. The peptides contained an additional cysteine residue at the N terminus to allow for coupling of the peptides to the carrier keyhole limpet hemocyanin (Sigma) by using SPDP (*N*-succinimidyl-3-[2-pyridyldithio]propionate) (Pharmacia Biotech) as described previously (15). Antisera were raised in rabbits by immunizing 0.5 mg of coupled peptide in Freund's adjuvant at weekly intervals followed by bleedings 10 days after each immunization. The preparation of monoclonal and polyclonal antisera against G1 and G2 has been described previously (24, 36).

Construction of recombinant plasmids. Full-length cDNA encoding G1 (named $G1_{98}$) and two progressive deletions of the G1 cytoplasmic tail ($G1_{81}$ and $G1_{49}$) (see Fig. 1) were made by PCR using specific sense and antisense primers and the p110 cDNA as a template. The primers used for synthesizing the antisense strands carried two in-frame TGA stop codons (ACT in the antisense orientation) in tandem, a cleavage site for *Sal*I, and four extra clamp residues at the 5′ end. The primers used were as follows: sense, 5′ GCAGTGAGCTCCTT GTGC 3′ (complementary to residues 1222 to 1239 [30]); antisense (complementary sequences are underlined, and stop codons are in italics), 5′ CGGTG TCGAC*TCATCA*ACTTTCAACTGGGCAAGC 3′ (positions 1557 to 1540) ($G1_{98}$), 5′ CGGTGTCGAC*TCATCA*TCTGGTCAAGTTAAACA 3′ (positions 1506 to 1489) ($G1_{81}$), 5′ CGGTGTCGAC*TCATCA*TCCTTCTTCCAGGGA GTG 3′ (positions 1410 to 1393) ($G1_{49}$). The plasmid pTF-M, containing the full-length p110 cDNA cloned into plasmid pTF7-5 (22), was cut with *Nhe*I in the transmembrane domain of G1 and with *Sal*I in the downstream polylinker region. The small *Nhe*I-*Sal*I fragment was replaced by the correspondingly cut PCR fragments, using standard techniques. All inserts generated with PCR were sequenced by the dideoxy chain termination method. The G2 C-terminally truncated constructs of p110 were made in a similar way by PCR, using antisense primers with termination codon sequences inserted directly after amino acid residues 50, 98, 150, and 200, counting from the N terminus of mature G2 (see Fig. 1).

Virus infection, isolation of virions, and transfection. Subconfluent BHK21 cells were washed with adsorption medium (MEM, 0.2% bovine serum albumin, 20 mM HEPES [pH 7.2], 100 IU of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine) before infection with UUK virus at a multiplicity of infection (MOI) of about 10. After 1 h at 37°C, the virus was removed and replaced with adsorption medium. The infection was continued for 15 to 17 h, at which time the medium was collected and the cell debris was removed by centrifugation at 11,300 × g for 30 min at 4°C. The supernatant was diluted with phosphate-buffered saline (PBS) and purified over a cushion of 30% sucrose in TNE buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA) by centrifugation at 45,000 rpm for 45 min at 4°C with a Beckman L7-55 ultracentrifuge equipped with an SW50.1 rotor. The virus pellet was resuspended in PBS.

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

Immunoblotting. UUK virus proteins from purified virions were separated under nonreducing conditions by SDS–10% polyacrylamide gel electrophoresis (PAGE) (20) and electroblotted onto a nitrocellulose membrane (Bio-Rad Laboratories). Polyclonal rabbit anti-G1 serum, at a dilution of 1:500, anti-G2 serum (1:500), and anti-peptide B serum (1:1,000) were used as primary antibodies. Following washing, the membranes were incubated with alkaline phosphatase–anti-immunoglobulin (Ig) conjugate, washed again, and visualized with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (Promega).

Immunoprecipitation. Cytoplasmic extracts were prepared from UUK virus-infected or transfected cells metabolically labeled with [^{35}S]methionine as described previously (22, 24). Cells were lysed in solubilization buffer (1% Triton X-100, 5 mM EDTA, 150 mM NaCl, 100 IU of aprotinin per ml in 20 mM Tris-HCl, pH 8.0). The solubilized cells were scraped off the dish, and the lysate was centrifuged in a Biofuge for 5 min at maximum speed. The supernatants were used directly or were frozen in liquid nitrogen and stored at −80°C until analyzed.

Samples were subjected to immunoprecipitation exactly as described before (22), using anti-G1 or anti-G2 monoclonal or polyclonal antisera and a 10% suspension of Pansorbin (Calbiochem) to collect the immunocomplexes. Following reduction, denaturation, and alkylation with iodoacetamide, the samples were analyzed by SDS-PAGE.

Treatment of microsomes with proteinase K. BHK21 cells, infected with a MOI of 20, were labeled for 30 min with 100 μCi of [^{35}S]methionine (Amersham) per ml at 16 h postinfection (p.i.). The cells were washed twice with ice-cold PBS and allowed to swell in RSB buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) for 10 min on ice, scraped off the dish, and homogenized with 20 strokes in a tight-fitting Dounce homogenizer. Nuclei were removed by centrifugation at 1,200 rpm for 15 min at 4°C with a Sorvall RT6000B centrifuge equipped with a 1000B rotor. The supernatant was split in five aliquots. Two served as controls, one was treated with 50 μg of proteinase K per ml, one was treated with 1% Triton X-100 and 50 μg of proteinase K per ml, and one was incubated with 50 μg of proteinase K per ml and 1.15 mM phenylmethylsulfonyl fluoride (PMSF) (Boehringer Mannheim). All samples were incubated for 30 min on ice. For immunoprecipitation, the samples were treated with solubilization buffer containing PMSF (24), split in half, and subjected to immunoprecipitation with either anti-G1 or anti-G2 antisera. After precipitation, the samples were treated with endoglycosidase H (endo H) as described previously (22). Following reduction and alkylation, the samples were analyzed by SDS-PAGE.

Pulse-chase labeling. Monolayers of BHK21 or HeLa cells grown on 6-cm-diameter dishes were infected with the recombinant vaccinia virus vTF7-3, followed 45 min later by transfection with plasmid constructs encoding various mutant forms of p110. Pulse-chase labeling was carried out as previously described (22, 24). At 5 h posttransfection, the cells were first incubated in methionine-free MEM for 45 min, followed by labeling for 60 min with [^{35}S]methionine and chases with an excess of unlabeled methionine (10 mM) for 0, 60, or 120 min. Cytoplasmic extracts, prepared as previously described (22), were subjected to immunoprecipitation with a G1-specific polyclonal antiserum (36) followed by analysis on an SDS–10% polyacrylamide gel and fluorography.

Permeabilization of cells with SLO. BHK21 cells were infected with vaccinia virus, transfected with the different cDNA constructs, and then washed three times with cold PBS and once with cold streptolysin O (SLO) buffer (115 mM potassium acetate, 25 mM HEPES-KOH [pH 7.4], 2.5 mM $MgCl_2$) containing 1 mM dithiothreitol. Following the addition of 1 U of SLO (Sigma) per ml, the cells were incubated on ice for 10 min and thereafter washed twice with cold SLO buffer. Prewarmed SLO buffer was added, and the cells were incubated for 30 min at 37°C, put on ice again, and washed once with cold SLO buffer and once with cold PBS. The cells were fixed in 3% paraformaldehyde and prepared for indirect immunofluorescence microscopy.

Immunofluorescence microscopy. Localization of the G1 glycoprotein by indirect immunofluorescence was carried out as described previously (22). BHK21 cells were infected with vTF7-3 and transfected with G1, or G1- and G2-encoding plasmids. At 6 h posttransfection, the cells were fixed with 3% paraformaldehyde, permeabilized either with SLO or Triton X-100 as described above, and stained with either a G1-specific monoclonal antibody (6G9 [36]) diluted 1:100 or a polyclonal antiserum against the G1 tail (peptide B [see Fig. 1]) diluted 1:200. The bound antibodies were visualized by incubation with fluorescein isothiocya-

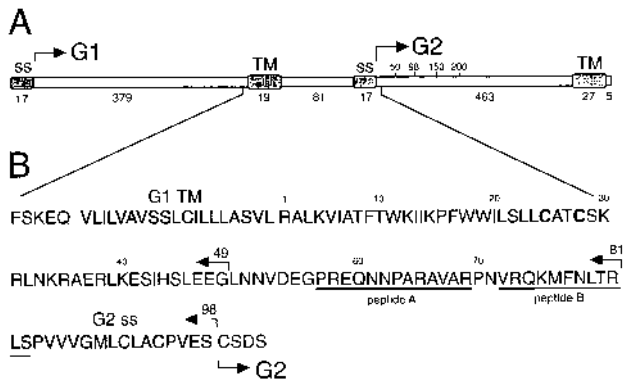


FIG. 1. Schematic representation of the p110 precursor of G1 and G2 and the amino acid sequence of the carboxy-terminal region of G1. (A) The p110 precursor is represented by the bar, with the signal sequences (ss) and transmembrane domains (TM) shown as shaded boxes. The number of amino acid residues of each domain is shown under the bar. The positions of the stop codons introduced in G2 are shown above the bar. The cleavage sites downstream of the ss to generate mature G1 and G2 are indicated. (B) The amino acid sequence spanning the last 122 residues of the G1 C terminus is shown in single-letter code. The G1 TM domain and the G2 ss are shown as shaded boxes. The numbering of the residues in the cytoplasmic tail of G1 is shown above the sequence. The positions of stop codons introduced to generate truncations of the tail are also indicated. The sequence of the peptides (peptides A and B) used to generate antisera are underlined. The two cysteines at positions 25 and 28 are shown in boldface type.

nate-conjugated anti-rabbit IgG or tetramethyl rhodamine isothiocyanate-conjugated anti-mouse IgG antibodies (Sigma). Immunofluorescence micrographs were taken, using an Axiophot microscope (Zeiss).

Palmitylation analysis. Subconfluent BHK21 cells grown in 3.5-cm-diameter plastic dishes were infected with UUK virus as described above. Duplicate dishes were labeled in parallel with either 0.5 mCi of [9,10(*n*)-^{3}H]palmitic acid/ml (at 3 h p.i.) or 0.1 mCi of [^{35}S]methionine (Amersham) per ml and 5 μM unlabeled L-methionine (Merck). In the latter case, the cells were first incubated at 6 h p.i. in methionine-free MEM for 45 min. Both sets of infected cells were labeled overnight, followed by purification of extracellular virions as described above.

RESULTS

The length of G2 required for efficient processing of p110 into G1 and G2. Previous work has shown that G1 and G2 are cotranslationally processed in the ER. To analyze how much of G2 has to be made before this processing can occur, we introduced translation termination codons at various points in the N-terminal half of G2. The positions of these stop codons, as well as the general organization of the p110 precursor, are shown in Fig. 1A. These constructs were expressed in either HeLa or BHK21 cells using the T7 vaccinia virus expression system, and the efficiency of processing was analyzed by pulse-chase radiolabeling. As shown in Fig. 2A, processing was quite inefficient (about 20% cleavage) in HeLa cells if G1 was followed by only 50 residues from G2 (lanes 4 to 6). Processing increased substantially (to about 80%) if the G2 portion was extended to 98 residues (Fig. 2A, lanes 7 to 9). Extension to 150 and 200 residues further increased efficiency. A small fraction of the molecules was not cleaved even in cells expressing the complete p110 coding region (Fig. 2A, lanes 16 to 18). The faint band migrating slightly slower than G1 in p110-transfected cells most likely represents G2, which forms a heterodimer with G1 and thus is coprecipitated with the G1 antiserum (24).

Interestingly, processing was much more efficient in BHK21 cells (Fig. 2B). With only 50 G2 residues, cleavage occurred with an efficiency of about 50% (Fig. 2B, lanes 4 to 6), while complete cleavage was observed with all of the longer constructs. The two bands migrating faster than G1 represent vaccinia virus-derived contaminating proteins (22). We were unable to detect the processed, truncated, G2 fragments derived from the various constructs by immunoprecipitation using either G1- or G2-specific antisera. This could be due to either rapid degradation, secretion out of the cell, or inability of the antiserum to react with the truncated fragments. In addition, the shortest fragment (50 residues) lacks a methionine and would thus not be labeled.

As noted before (22), G1 was found to be unstable in the absence of full-length G2 in both cell types and was rapidly degraded during the 2-h chase. Stabilization of G1 was observed if 200 residues of G2 were allowed to be made (Fig. 2 [both panels], lanes 13 to 15) and in p110-transfected cells (Fig. 2A, lanes 16 to 18).

From these results, we conclude that processing of p110 is indeed cotranslational and that more than 50 residues of G2 have to be made to achieve efficient processing.

The C-terminal tail of G1 is accessible to protease digestion in microsomes. From the primary sequence, it has been deduced that G1 and G2 are likely to be type I membrane proteins and that the C termini of G1 and G2 should be exposed on the cytoplasmic face of the membrane (Fig. 1A). To verify this experimentally, microsomal membranes were isolated from metabolically labeled virus-infected cells and subjected to proteinase K digestion. To allow for a more accurate estimation of the shift in molecular weights of the products, the immunoprecipitates were subjected to endo H treatment prior to SDS-PAGE. As shown in Fig. 3, the mobility of proteinase K-treated G1 increased, the shift being equivalent to about 6 kDa (Fig. 3A, lanes 2 and 3). This shift could be prevented if the protease inhibitor PMSF was included during incubation (Fig. 3A, lane 5). G1 was completely degraded if the membranes were solubilized with Triton X-100 (Fig. 3A, lane 4).

In contrast to the case for G1, the mobility of G2 was not affected by proteinase K treatment (Fig. 3B, lanes 2 and 3).

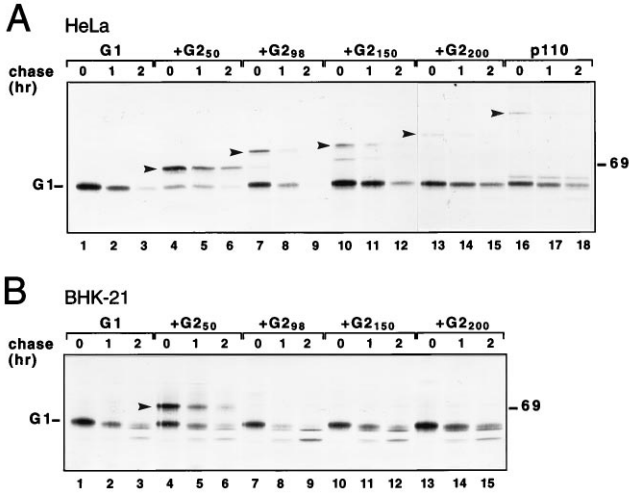


FIG. 2. Cotranslational cleavage of G1 and G2. HeLa (A) or BHK21 (B) cells were infected with recombinant vaccinia virus vTF7-3, followed by transfection with recombinant plasmids as described in Materials and Methods. The plasmids encoded either the entire coding region of G1 (lanes 1 to 3), G1 plus increasing portions of G2 ($G2_{50}$ to $G2_{200}$; lanes 4 to 15), or the complete precursor (p110; lanes 16 to 18). At 5 h after transfection, cells were pulse-labeled for 60 min with [^{35}S]methionine, followed by a 1- or 2-h chase with unlabeled methionine. At the end of the chase, G1 was precipitated from cytoplasmic lysates, using a polyclonal rabbit G1-specific antiserum, followed by analysis on an SDS–10% polyacrylamide gel. The positions of G1 and the 69-kDa molecular mass marker are shown to the left and right, respectively. Arrowheads indicate the positions of uncleaved G1-G2 molecules.

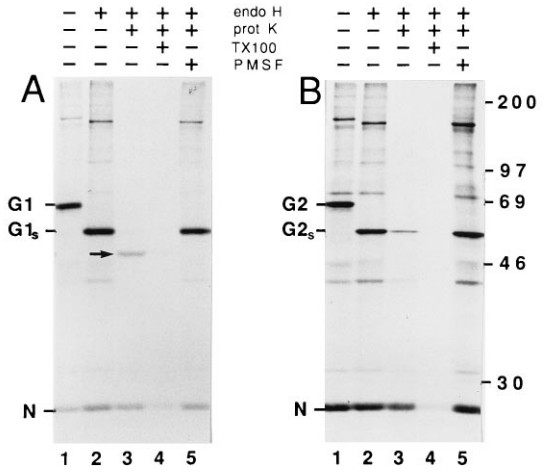


FIG. 3. Protease sensitivity of the cytoplasmic tails of G1 and G2. BHK21 cells infected with UUK virus for 16 h were labeled with [^{35}S]methionine for 30 min. Microsomes from cell homogenates were collected by centrifugation and treated with proteinase K, endo H, Triton X-100, and/or PMSF in the combinations indicated. Following treatment, G1 (A) or G2 (B) was precipitated by using polyclonal G1- or G2-specific antisera, respectively. The immunoprecipitates were analyzed on an SDS–10% polyacrylamide gel, followed by fluorography. The positions of glycosylated (G1 and G2) and endo H-sensitive ($G1_s$ and $G2_s$) glycoproteins, the nucleocapsid protein N, as well as the molecular weight markers, are indicated.

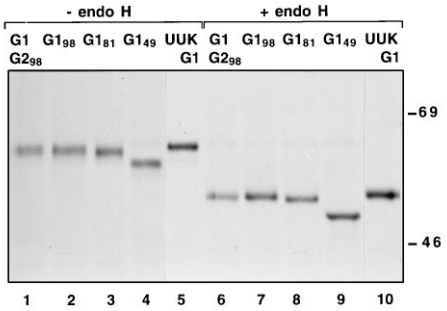


FIG. 4. Analysis of mobility differences of C-terminally truncated G1. BHK21 cells were either infected with UUK virus (lanes 5 and 10) or infected with vTF7-3 followed by transfection with plasmids encoding G1 plus 98 residues of G2 (lanes 1 and 6), mature G1 (lanes 2 and 7), or G1 truncated at position 81 (lanes 3 and 8) or 49 (lanes 4 and 9). Cells were labeled with [^{35}S]methionine for 30 min, lysed, and subjected to immunoprecipitation using anti-G1 antiserum. Samples were either left untreated (lanes 1 to 5) or treated with endo H (lanes 6 to 10), followed by analysis on an SDS–10% polyacrylamide gel. The positions of molecular weight markers are indicated on the right.

This was expected, since the G2 cytoplasmic tail is predicted to be only 5 residues long (-KVKKS-COOH).

Thus, we conclude that the cytoplasmic tail of G1 is exposed on the cytoplasmic face of the lipid bilayer.

The p110 precursor is processed at only one site to generate G1 and G2. We have previously proposed that p110 is cleaved by the ER signal peptidase immediately following the internal signal sequence of G2. We also speculated that a secondary cleavage to remove the G2 signal sequence from the tail of G1 might take place (30). This would generate a small peptide analogous to the 6-kDa protein of alphaviruses (33). Since direct sequencing of the C terminus of G1 has repeatedly been unsuccessful, we decided to take indirect approaches to analyze the possibility of a secondary cleavage. To this end, translational stop codons were introduced at the known cleavage site at the N terminus of mature G2 (called $G1_{98}$), at the N-terminal end of the G2 signal sequence ($G1_{81}$) and, as a migration control, also in the middle of the G1 tail ($G1_{49}$). The amino acid residues are numbered starting from the predicted cytoplasmic border of the transmembrane domain of G1 (Fig. 1B). The constructs described above were expressed in BHK21 cells, again using the T7 vaccinia virus system, and the metabolically labeled products were analyzed by SDS-PAGE. One portion of the samples was left untreated, while the other portion was treated with endo H to allow for better detection of mobility differences on the SDS gel. As a control, we used either the construct containing the complete G1 followed by 98 residues of G2 ($G1 + G2_{98}$ [Fig. 4, lanes 1 and 6]) or G1 precipitated from virus-infected cells (Fig. 4, lanes 5 and 10). In both cases, proper cleavage to authentic G1 occurs, as shown in Fig. 2B.

The mobilities of $G1 + G2_{98}$ (Fig. 4, lanes 1 and 6), $G1_{98}$ (Fig. 4, lanes 2 and 7), and G1 from virus-infected cells (Fig. 4, lanes 5 and 10) were indistinguishable from each other, while $G1_{81}$ migrated slightly faster (Fig. 4, lanes 3 and 8). The control $G1_{49}$ migrated clearly faster than $G1_{81}$. The surprisingly small shift in mobility between $G1_{98}$ and $G1_{81}$ is discussed below (see Discussion).

To confirm the results discussed above, we prepared an antiserum against a synthetic peptide corresponding to residues 72 to 83 (using the numbering described above), i.e., a sequence just preceding the G2 signal sequence (peptide B [Fig. 1B]). This antiserum was used in immunoblotting to probe purified virus proteins. As shown in Fig. 5 (lane 4), the peptide antiserum reacted with G1, but not with G2, showing that this peptide sequence is part of the cytoplasmic tail of G1. The same result was obtained with an antiserum against peptide A (Fig. 1B, residues 57 to 69) located N terminal to peptide B (data not shown).

Taken together, these results indicate that there is only one processing site between G1 and G2, namely, the signal peptidase cleavage site following the G2 signal sequence.

Peptide antisera react with the cytoplasmic tail of G1 in SLO-permeabilized cells. To analyze if the G1 cytoplasmic tail is exposed on the cytoplasmic face of the lipid bilayer also in intact cells, we permeabilized BHK21 cells expressing either G1 and G2 or G1 alone with SLO. Under proper conditions, SLO is known to permeabilize the plasma membrane, but not the internal membranes (2). We used the two polyclonal peptide antisera against peptides A and B as reagents for the cytoplasmic tail (Fig. 1B). The latter antiserum is the same as

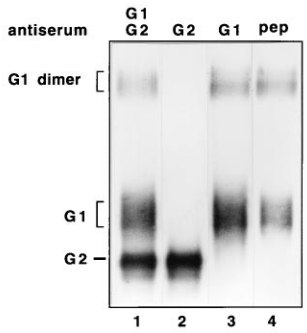


FIG. 5. Immunoblotting with a peptide antiserum against the C terminus of G1. [^{35}S]methionine-labeled UUK virus proteins were separated on an SDS–10% polyacrylamide gel and blotted onto a nitrocellulose filter. Filter strips were incubated with antisera against G1 and G2 (lane 1), G2 (lane 2), G1 (lane 3), or peptide B, corresponding to the region upstream of the G2 signal sequence (lane 4) (Fig. 1). Bound antibodies were detected with alkaline phosphatase-conjugated secondary antibody.

that used for Fig. 5. To check that the ER and Golgi membranes were not permeabilized, we used a monoclonal antibody directed against the ectodomain of G1. This allowed for double staining of cells with both the tail and the luminal antibodies.

Irrespective of whether G1 and G2 were coexpressed (Fig. 6A) or G1 was expressed alone (Fig. 6B), the results were the same. In SLO-permeabilized cells, the peptide B tail antiserum gave a strong positive immunoreactivity mainly localized to a reticular network and a juxtanuclear region, reminiscent of the ER and the Golgi complex, respectively (Fig. 6A-3 and B-3). Peptide A immunoreactivity was somewhat weaker but otherwise indistinguishable from that of the peptide B antiserum (data not shown). The G1 monoclonal antibody showed no immunoreactivity, indicating that the internal membranes were not permeabilized by SLO. If, however, the cells were permeabilized with Triton X-100, both the monoclonal and polyclonal antibodies displayed strong colocalizing immunoreactivity.

Thus, at least the distal half of the G1 tail (residues 57 to 83) is exposed on the cytoplasmic face of the ER and Golgi complex membranes in intact cells.

G1 and G2 are palmitylated. Viral membrane proteins are frequently acylated, a modification which is likely to affect the structure and topology of cytoplasmic tails. To analyze whether G1 or G2 might also be acylated, we metabolically labeled infected cells with [^{3}H]palmitic acid and analyzed virions purified from the culture medium by SDS-PAGE. Virions labeled with methionine served as a control. As shown in Fig. 7, both G1 and G2 were readily labeled, while the nucleocapsid protein was not. The latter protein served as an internal control for the specificity of labeling. Thus, both G1 and G2 are palmitylated.

DISCUSSION

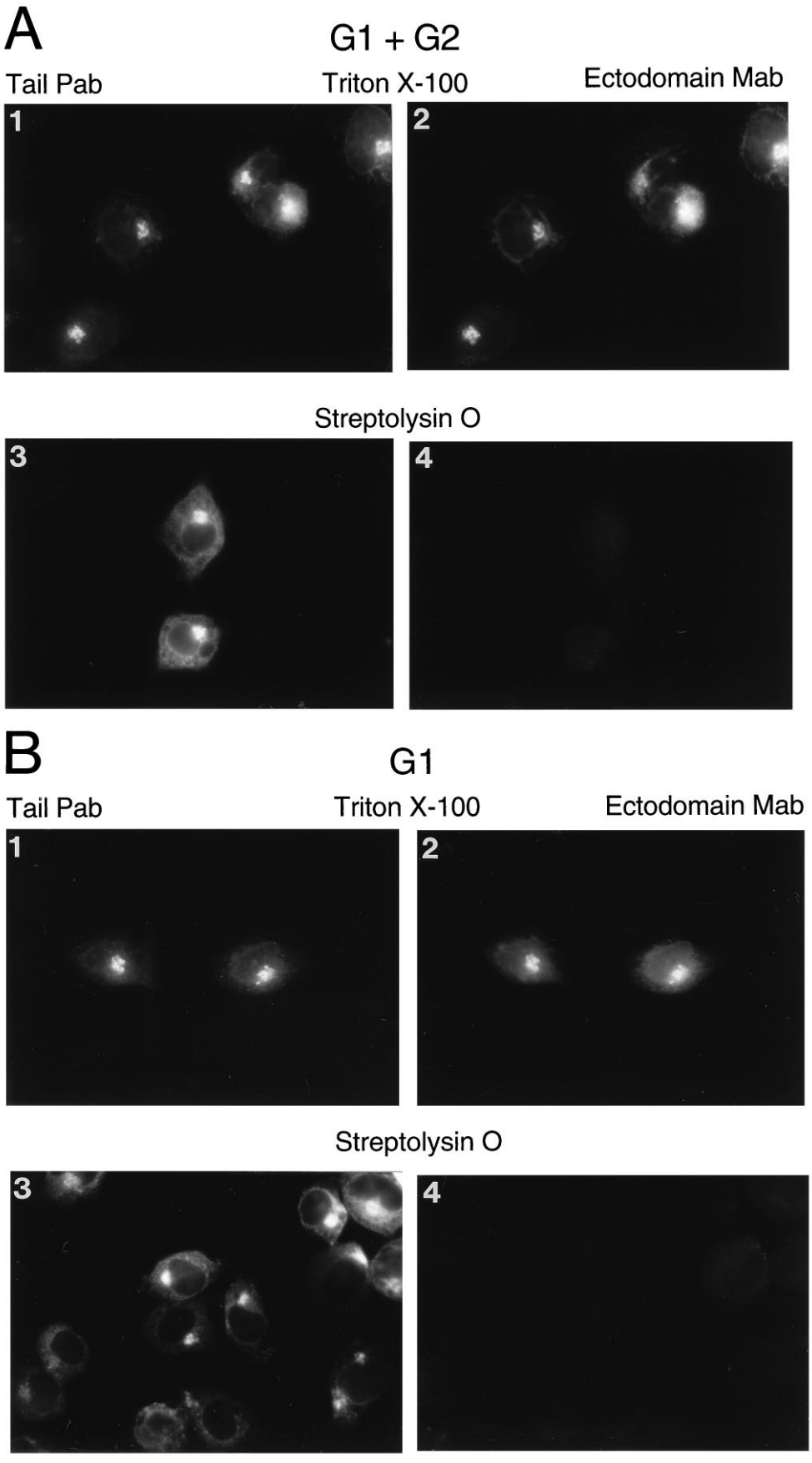
Based on the results obtained in this paper, we present the following model for the processing and membrane insertion of the Uukuniemi virus glycoproteins G1 and G2 (Fig. 8). Cleavage of p110, the precursor of G1 and G2, takes place cotranslationally in the ER, and only a rather short stretch of the downstream G2 has to be made before cleavage, presumably by the signal peptidase, can occur. The G1 cytoplasmic tail is not further processed; instead, the internal signal sequence of G2 remains covalently attached to G1. The G1 tail, which becomes palmitylated, remains at least partially exposed on the cytosolic side of the membrane. The G2 protein, which also becomes palmitylated, has a very short five-residue-long positively charged cytoplasmic tail, which is unlikely to serve any function other than to prevent translocation of G2 into the lumen. As we have shown previously (24), G1 and G2 then fold independently, followed by heterodimerization, exit from the ER, and accumulation in the Golgi complex (12, 13, 23).

For all members of the *Bunyaviridae* family, the two membrane glycoproteins are encoded in one single open reading frame present in the medium-sized RNA segment (M). Since a precursor has not been found in infected cells, it has been inferred that cleavage between G1 and G2 is likely to be cotranslational. This is also supported by the finding that the precursor can be demonstrated by in vitro translation of the M mRNA in the absence of microsomal membranes, while cleavage occurs in the presence of membranes (35). However, it has not been known at which point during synthesis cleavage of the precursor occurs. In the present study, we found that processing occurs with an efficiency of about 50% in BHK21 cells already, when 50 residues of G2 have been synthesized. This means that processing occurs before the synthesis of G2 has been completed. During translocation, about 30 to 40 residues of the nascent chain are presumed to be buried within the channel of the large ribosomal subunit (37), and some 20 additional residues would span the ER membrane. Thus, about 50 to 60 residues have to be made before cleavage can occur on a growing chain. However, if stop codons are inserted as was done in the present study, then the C terminus of the nascent chain will be released from the ribosome upon translation termination and will most likely flip into the lumen of the ER. The cleavage site may therefore under these circumstances become exposed earlier than it would on a growing chain such as the p110 precursor. The results obtained with HeLa cells, showing less efficient cleavage and the requirement for a longer stretch of synthesized G2 before cleavage occurs, indicate that there are cell-specific differences, the reasons of which are unknown.

Based on several indirect analyses, including sensitivity to protease digestion, mobility shifts of truncated mutant proteins, and immunoblotting with tail peptide antisera, we conclude that the signal sequence of G2 remains covalently attached to the G1 cytoplasmic tail. Thus, no additional cleavage to remove the G2 signal sequence takes place as suggested previously (30). Therefore, there is no internal peptide comparable to the NSm of *Bunyavirus* members (4) or the 6,000-molecular-weight peptide (6K peptide) of alphaviruses (33). The fact that proteinase K treatment resulted in an apparent decrease in the size of G1 corresponding to only 6 kDa, rather than the expected 9 to 10 kDa, could mean that the whole G1 cytoplasmic tail is not accessible to protease digestion. Alternatively, the removal of the hydrophobic G2 signal sequence by the treatment might affect the mobility on an SDS gel aberrantly. This is supported by the finding that only a very small mobility difference between the mutants $G1_{81}$ and $G1_{98}$ was observed, although they differ by as much as 17 (hydrophobic) residues. That the removal of hydrophobic sequences can have only a minor effect on the mobility of proteins has been observed previously for other proteins (1, 7, 15). The signal sequence for a downstream membrane protein has been shown to remain attached to the upstream protein also in the case of some other viruses. Thus, the signal sequence for E2 of rubella virus remains attached to the C terminus of the virus capsid protein (34), and the signal sequence for the 6K protein remains linked to the tail of the upstream E2 membrane protein of alphaviruses (17).

At present, it is unclear whether the hydrophobic C terminus of G1 (the G2 signal sequence) remains embedded in the lipid bilayer throughout the intracellular transport of G1 and in the virion (Fig. 8). It is possible that it may at some stage slip out of the membrane to the cytosolic side, as has been suggested

FIG. 6. The cytoplasmic tail of G1 is exposed on the cytosolic side of membranes. BHK21 cells were infected with vaccinia virus vTF7-3 and then transfected with either a combination of G1- and G2-encoding plasmids (A) or the G1-encoding plasmid alone (B). At 6 h after transfection, cells were fixed with paraformaldehyde and permeabilized with either Triton X-100 (panels A-1, A-2, B-1, and B-2) or SLO (panels A-3, A-4, B-3, and B-4). The cells were then double stained for the detection of G1 by using a monoclonal antibody directed against the luminal part of G1 (panels A-2, A-4, B-2, and B-4) and a polyclonal rabbit peptide antiserum directed against the cytoplasmic tail (peptide B [Fig. 1B]) (panels A-1, A-3, B-1, and B-3). Absence of immunoreactivity in panels A-4 and B-4 indicates that SLO did not permeabilize internal membranes. Pab, polyclonal antibody; Mab, monoclonal antibody.


A
G1 + G2
Tail Pab
Triton X-100
Ectodomain Mab
1
2
Streptolysin O
3
4
B
G1
Tail Pab
Triton X-100
Ectodomain Mab
1
2
Streptolysin O
3
4

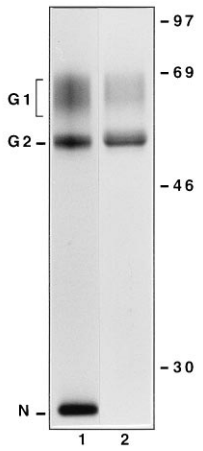


FIG. 7. G1 and G2 are palmitylated. BHK21 cells infected with UUK virus were metabolically labeled with [^{35}S]methionine (lane 1) or [^{3}H]palmitate (lane 2). The purified virions were analyzed on an SDS–10% polyacrylamide gel, followed by fluorography. The positions of molecular weight markers are shown on the right.

for the C terminus of Sindbis virus E2 membrane protein. In the latter case, this topological change has been suggested to be triggered by phosphorylation and to be coupled to virus budding (18, 19).

The finding that G1 is palmitylated most likely has implications for the organization of the tail as has been suggested, e.g., for the cytoplasmic tail of influenza virus hemagglutinin (23) and Sindbis virus E2 protein (8). Posttranslational palmitylation of proteins most frequently occurs by the covalent attachment of fatty acids to cysteines via a thioester bond. There are two cysteines at positions 25 and 28 (Fig. 1B) in the tail of G1. The short G2 tail does not contain any cysteines, whereas there is one cysteine residue in the very C-terminal part of the transmembrane domain (30). Whether any or all of these residues are palmitylated remains to be studied by in vitro mutagenesis.

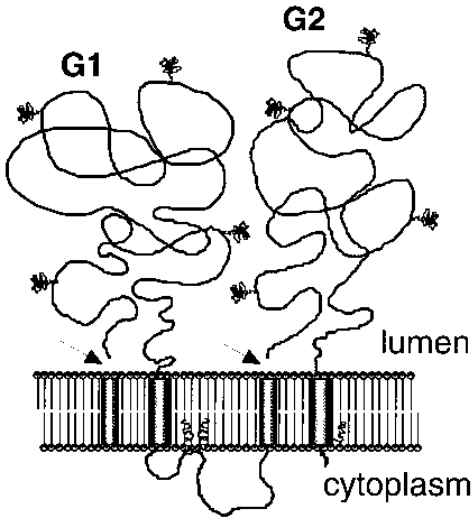


FIG. 8. Model for the processing and topology of UUK virus G1 and G2. Processing of the p110 precursor occurs cotranslationally by signal peptidase cleavage at the N termini of mature G1 and G2 (arrows). Each protein acquires four N-linked glycans and becomes palmitylated, presumably at the cysteine residues located in the cytoplasmic tails.

The cytoplasmic tail of G1 has at least two functions. First, it harbors the signal for retaining the G1-G2 heterodimeric complex in the Golgi complex (22, 28). Second, it probably contains a sequence with which the three helical ribonucleoproteins (RNP) interact to facilitate budding at the Golgi complex membranes (14). These two sequences are likely to be separated. Our recent results have indicated that the Golgi complex retention signal is located within the first 50 membrane-proximal residues (28). Thus, one can speculate that the RNP-interacting domain could be located in the C-terminal half of the tail. Immunofluorescence staining with the G1 tail-specific antibody of SLO-permeabilized cells transfected with G1 or G1 plus G2 showed that at least the C-terminal portion of the tail is exposed on the cytosolic side of the Golgi complex membrane. Unless in vitro assays are developed to analyze RNP-G1 interactions, reverse genetics appears to be the most straightforward approach to study the function of different domains of the tail.

Can one draw conclusions from the results presented here as to the topology of G1 and G2 of some of the other *Bunyaviridae* members? Other phleboviruses, such as Rift Valley fever and Punta Toro viruses (4, 30), as well as hantaviruses (4, 31), have hydropathy profiles around the C-terminal region of the N-terminally located spike protein (also referred to as GN) that are very similar to that of UUK virus G1, and as yet, no second processing of the GN tail has been reported. Therefore, cleavage between G1 and G2 and membrane insertion seem likely to be very similar to those of Uukuniemi virus. The situation for the *Bunyavirus* members is more complicated because of the presence of the NSm peptide between G2 and G1 (3). The C terminus of G2 (GN) and the N terminus of G1 (GC) of snowshoe hare virus have been determined by direct sequencing (6). The C-terminal end of G2 (GN) is hydrophobic and may thus serve as the signal peptide for NSm. Thus, the N-terminally located membrane glycoprotein (GN) of *Bunyaviridae* seems to have a hydrophobic C-terminal sequence similar to the C terminus of UUK virus G1. It is noteworthy that, in the cases studied, the GN protein harbors the Golgi complex retention signal (27) and has a much longer cytoplasmic tail than GC (3, 4). As mentioned above, this tail may serve a dual function: to confer Golgi complex retention and to serve as a receptor for RNP binding.

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