Virology **289,** 224–229 (2001)
doi:10.1006/viro.2001.1171, available online at http://www.idealibrary.com on IDEAL®

The Hantaan Virus Glycoprotein Precursor Is Cleaved at the Conserved Pentapeptide WAASA

Christian Löber,* Bärbel Anheier,* Steffi Lindow,* Hans-Dieter Klenk,* and Heinz Feldmann*,†,[1]

*Institut für Virologie, Robert-Koch-Str. 17, D-35037 Marburg, Germany; and †Canadian Science Centre for Human and Animal Health, 1015 Arlington Street, Winnipeg, Manitoba R3E 3R2, Canada

Received July 16, 2001; returned to author for revision August 16, 2001; accepted August 29, 2001

The medium segment of the tripartite negative-stranded RNA genome of hantaviruses encodes for the predicted glycoprotein precursor GPC. We have demonstrated here the expression of the glycoprotein precursor of Hantaan virus following transfection of mammalian cells. The cleavage of the precursor into the glycoproteins G1 and G2 followed the rules for signal peptides and seemed to occur directly at the pentapeptide motif "WAASA." Our data indicate that the signal peptidase complex is responsible for the proteolytic processing of the precursor GPC of Hantaan virus. The comparison of this region of the glycoprotein precursor, including the absolutely conserved WAASA motif, suggests a similar cleavage event for all hantavirus glycoproteins.

Key Words: Hantaan virus; glycoprotein precursor GPC; cleavage; WAASA motif; signal peptidase complex.

INTRODUCTION

Pathogenic hantaviruses cause two clinical syndromes in man: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). They are rodent-borne viruses and infect their specific reservoir chronically, showing no overt disease. Human infection is thought to be acquired mainly via inhalation of infectious aerosols of rodent excreta (Schmaljohn and Hjelle, 1997; Schmaljohn and Le Duc, 1998; Peters *et al.,* 1999; Feldmann, 2000; Schmaljohn and Nichol, 2001).

Hantaviruses, family Bunyaviridae, genus *Hantavirus*, are spherical to oval enveloped particles with a tripartite, single-stranded, negative-sense RNA genome. In general, together with nairoviruses, they appear to have the simplest genome expression strategy among Bunyaviridae. Neither have convincing evidence for an ambisense strategy (genus *Phlebovirus* and *Tospovirus*) nor for the expression of a nonstructural protein by an overlapping open reading frame (ORF) (genus *Bunyavirus*). The three genome segments encode for four structural proteins: the RNA-dependent RNA polymerase (L protein) is encoded by the large (L) segment, the glycoproteins G1 and G2 by the medium (M) segment, and the nucleoprotein (N) by the small (S) segment. G1 and G2, both type I transmembrane proteins, are cleavage fragments of a predicted precursor GPC (Elliott, 1990; Schmaljohn, 1996; Schmaljohn and Le Duc, 1998; Feldmann, 2000; Johnson and Schmaljohn, 2001).

Hantaan virus (HTNV) (strain 76–118) is the prototype virus of the genus *Hantavirus* and was isolated from *Apodemus agrarius* in Korea (Lee *et al.,* 1978). Most data regarding hantaviral glycoproteins were obtained by studying this particular virus strain. The ORF of the M segment is 3408 nucleotides long and encodes for a putative precursor of 1135 amino acids (Schmaljohn *et al.,* 1987). This precursor possesses seven potential sites for the attachment of *N*-linked oligosaccharides (Fig. 1). The *N*-linked glycans largely remain endoglycosidase H sensitive even after incorporation into the virus particle (Schmaljohn *et al.,* 1987; Antic *et al.,* 1992; Schmaljohn and Le Duc, 1998). G1 and G2 form a heterodimer in the endoplasmic reticulum (ER) before they are targeted to the Golgi compartment (Ruusala *et al.,* 1992). The G1/G2 heterodimers form the spikes on the virus particles which mediate receptor binding and fusion (Arikawa *et al.,* 1985, 1989; Dantas *et al.,* 1986). Sequence determination revealed that the amino terminus of the mature G1 starts at position 18 (threonine) and with the G2 at 649 (serine) (Schmaljohn *et al.,* 1987). The putative GPC shows four hydrophobic domains. Domain I extends from amino acid position 1 to 17 and most likely serves as a signal peptide. Domains II (position 441 to 515) and IV (position 1097 to 1127) are the transmembrane domains of G1 and G2, respectively (Spiropoulou *et al.,* 1994). The hydrophobic domain II is unusually long and the actual membrane spanning portion is unknown. Domain III extends from position 627 to 648 and ends with a highly conserved pentapeptide motif "WAASA" at the amino terminus of G2 (Fig. 1) (see review of Spiropoulou, 2001).

This study was directed to identify the cleavage of the putative precursor GPC and to address the role of the highly conserved pentapeptide WAASA in this process. This pentapeptide is found with all molecularly characterized hantaviral glycoproteins (Spiropoulou *et al.,* 1994;

[1] To whom correspondence and reprint requests should be addressed. Fax: +1-204-789-2140. E-mail: Heinz_Feldmann@hc-sc.gc.ca.

0042-6822/01 $35.00

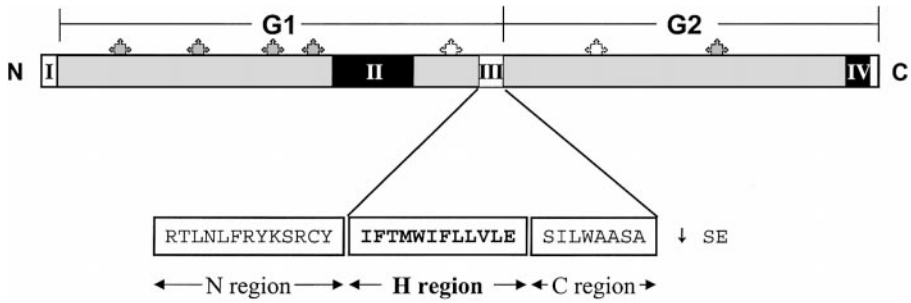

FIG. 1. Schematic illustration of the Hantaan virus glycoprotein precursor and the putative cleavage site. The illustration indicates the hydrophobic domains I to IV of the hantavirus precursor which extend from amino acid position 1–17 (I), 441–515 (II), 627–648 (III), and 1097–1127 (IV) (GenBank Accession No. Y00386). The amino (N) and carboxyl (C)-terminal ends of the glycoprotein precursor are indicated. Seven potential N glycosylation sites (five in G1 and two in G2) are indicated by squares, five of which are most likely to be glycosylated (gray). Two sites are most likely to be nonglycosylated (white): one of the G1 sites because it is facing the cytoplasm and one of the G2 sites because of the proline residue that follows the asparagine residue in the region (Bause, 1983). The arrow indicates the potential cleavage site between G1 and G2. The highly conserved pentapeptide motif WAASA is located at the carboxyl-terminal end of domain III. According to the rules (Perlman and Halvorson, 1983; von Heijne, 1983, 1986), potential signal peptides can be divided into three different regions which are indicated here (N = basic, H = hydrophobic, C = polar).

Schmaljohn and Le Duc, 1998). The precursor GPC has never been identified following hantavirus infection nor using recombinant expression from a plasmid containing the M segment ORF (Schmaljohn *et al.,* 1987; Pensiero and Hay, 1992; Schmaljohn, 1996; Spiropoulou, 2001). This together with the current data on glycoprotein processing led to the hypothesis that cleavage occurs cotranslationally and is most likely mediated by a signal peptidase. However, experimental data supporting this hypothesis have not been published for any of the hantaviruses.

RESULTS AND DISCUSSION

Most secretory and type I transmembrane proteins possess a hydrophobic signal peptide at their amino-terminal end. In eukaryotic organisms, this sequence mediates the translocation of the protein into the lumen of the ER, while in prokaryotic organisms, it mediates the transport across the inner membrane into the periplasm (Rapoport *et al.,* 1996; Schatz and Dobberstein, 1996; Kalies and Hartmann, 1998). Subsequently, a signal peptidase complex usually removes the sequence. Several rules have been established defining a particular sequence as a signal peptide (Perlman and Halvorson, 1983; von Heijne, 1983, 1986). According to these rules, the peptide can be divided into three regions: a basic region (N) at the amino-terminal end, a hydrophobic region (H) in the middle, and a polar region (C) at the carboxyl-terminal end. The amino acids at position −1 (alanine > glycine > serine) and −3 (small uncharged amino acids; alanine, serine > valine > cysteine) are especially critical for recognition by a signal peptidase complex. In addition, the region −3 to +1 should lack any proline residue. By applying these rules, hydrophobic domains I (not shown here) and III (Fig. 1) of the HTNV precursor GPC fulfill the requirements for a signal peptide.

To experimentally test whether domain III contains a signal sequence, the ORF of the HTNV glycoprotein precursor was cloned into the plasmid vector pSP72 for bacteriophage T7-driven expression (pSP72-HTNV/GPC) and into pSC11 for the production of a recombinant vaccinia virus (VV-HTNV/GPC). To verify expression and subcellular localization of G1 and G2, HeLa cells were infected either with a recombinant "*M*odified *V*accinia virus *A*nkara" expressing the bacteriophage T7 RNA polymerase (MVA-T7), followed by transfection with pSP72-HTNV/GPC, or with VV-HTNV/GPC. The use of indirect immunofluorescence demonstrated that recombinant G1 and G2 expressed from a single ORF could be colocalized with mannosidase II, a Golgi marker protein (Fig. 2A). Neither G1 nor G2 could be detected at the plasma membrane when analyzed by surface immunofluorescence (Fig. 2B) or by biotinylation (data not shown). Since antibodies specific to G1 and G2 were limited, we introduced an immunogenic epitope from the influenza hemagglutinin ("HA tag") at the carboxyl terminus of the precursor ORF and subcloned it into the T7-driven expression plasmid pTM1 (pTM1-HTNV/GPC-HA). Following infection of HeLa cells with MVA-T7 and subsequent transfection with plasmid pTM1-HTNV/GPC-HA, G2 expression could be demonstrated by immunoblot analysis using an anti-HA tag monoclonal antibody (Fig. 2C). As shown in previous studies and by unpublished data of our group, G2 can only reach the Golgi compartment following heterodimerization with G1 (Antic *et al.,* 1992; Pensiero and Hay, 1992; Ruusala *et al.,* 1992). In our studies, G2 was localized in the Golgi complex by immunofluorescence (Fig. 2A), confirming proper transport of G1 and G2 into this cellular compartment (Ruusala *et al.,* 1992). Furthermore, G2 detection by immunoblotting confirmed the processing of the precursor GPC into the fragments G1 and G2 (Fig. 2C). Together, all of these data clearly showed that expression, process-

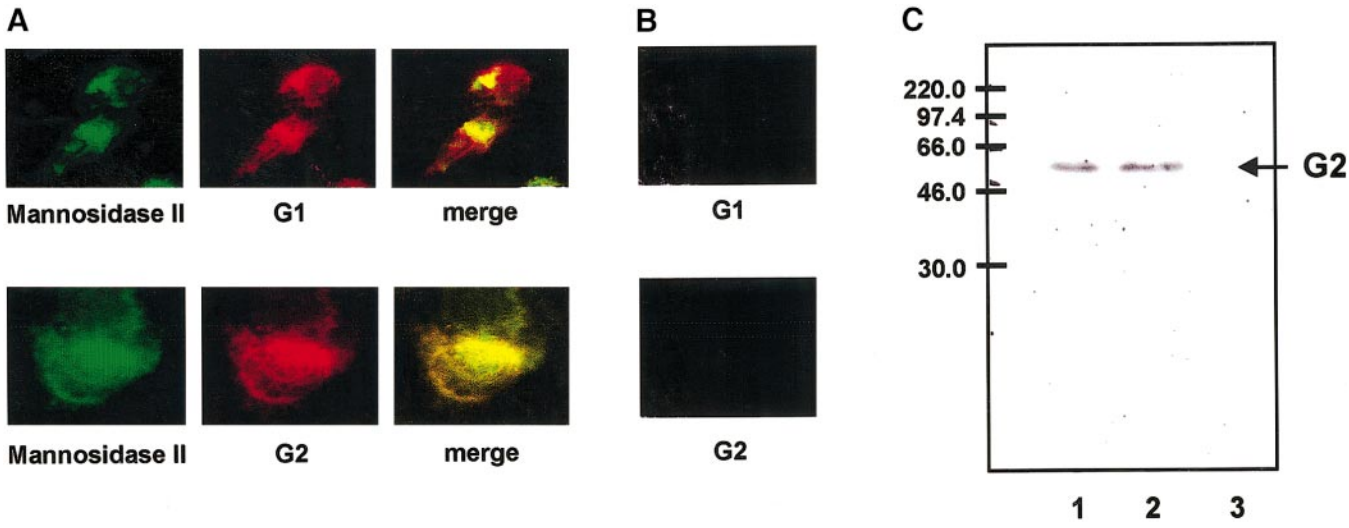

FIG. 2. Expression of the Hantaan virus glycoproteins. (A) Subcellular localization using intracellular immunofluorescence. HeLa cells (70–80% confluence) were infected with a recombinant vaccinia virus expressing the hantaviral glycoproteins G1 and G2 from a single open reading frame (VV-HTNV/GPC) (m.o.i. of 10). Cycloheximide treatment (50 μg/ml) was performed for 5 h prior to fixation. The cells were washed with PBS and fixed with methanol/acetone (1:1, v/v) for 15 min. Immunodetection was done either with a rabbit antiserum directed against mannosidase II (Golgi marker) at a 1:100 dilution or with the appropriate monoclonal antibodies directed against G1 (mAB 6D4) and G2 (mAB 8E10) at a 1:25 dilution. This was followed by an incubation with a species-specific secondary antiserum labeled either with FITC (dilution of 1:50) or with Texas Red (dilution of 1:75). Colocalization ("merge") with the marker protein was performed on a laser scanning microscope. (B) Subcellular localization using surface immunofluorescence assay. The infection of the cells was performed as described above. The incubation with the protein-specific antibodies (G1 or G2; dilution of 1:25) was done directly on ice after washing with PBS. Subsequently the cells were washed, fixed with 3% paraformaldehyde, and analyzed with an FITC-labeled species-specific secondary antibody (dilution of 1:50). (C) Immunoblot detection. HeLa cells were infected with MVA-T7 (m.o.i. of 3) and subsequently transfected with 2.5 and 5.0 μg pTM1-HTNV/GPC-HA (lanes 1 and 2) and 5.0 μg pTM1 control (lane 3). The cells were lysed and the proteins subjected to 10% SDS–PAGE and blotted onto PVDF membrane. The immunoreaction was performed with an anti-HA monoclonal antibody (dilution of 1:2500) followed by an species-specific HRP-conjugated antiserum (dilution of 1:5000). Detection was done using SuperSignal ULTRA chemiluminescent substrate (Pierce, Rockford, IL).

ing, and transport occurred in the same way as in HTNV-infected mammalian cells. Thus, a reliable recombinant mammalian expression system was established to study the proteolytic processing of the HTNV glycoprotein precursor GPC.

To identify the cleavage site of the precursor, we generated single- or double-site mutants at position −1 and −3 of the putative signal peptide (domain III; Fig. 1) in pTM1-HTNV/GPC-HA, which were either in accordance with or in discordance with the rules for signal peptides (see above). Expression of the different mutants in HeLa cells was performed following MVA-T7 infection and subsequent plasmid DNA transfection as described above. Immunoblot analysis of cell lysates using an anti-HA monoclonal antibody identified three major protein bands after separation on SDS–PAGE (Fig. 3). The bottom band represented G2 with a molecular weight of ~55 kDa in its mature glycosylated form. The 120-kDa band represented the noncleaved glycosylated precursor GPC, which based on sequence information was expected to have a molecular weight of ~123 kDa (Schmaljohn *et al.,* 1987; Schmaljohn, 1996). In addition, a higher molecular weight band was detected which may represent aggregates or specific oligomers of the uncleaved precursor. G1 (~68 kDa) was not detected in this assay system because the HA tag was introduced at the carboxyl-terminal end of the precursor. According to our studies, wild-type GPC (WAASA) and the cleavage site mutant WAVSG, which is a mutation in accordance with the rules for signal peptides, were efficiently cleaved without any remaining GPC (Fig. 3, lanes 2 and 3). In contrast, all mutants in discordance with the rules either showed a reduction (WARSA, WAESE, and WAESR) or an almost complete loss (WAASR) in the cleavage of GPC (Fig. 3, lanes 4–7). Immunofluorescence analyses were performed using an anti-HA monoclonal antibody after cycloheximide treatment (5 h) of infected and transfected cells. Cycloheximide inhibits protein synthesis in eukaryotic cells by blocking translation of messenger RNA on the ribosome and therefore allows previously synthesized proteins to be fully processed. Expression of all mutants under these conditions showed a Golgi staining pattern indicating proper subcellular targeting (data not shown).

Our experiments demonstrated clearly the expression of a hantavirus glycoprotein precursor in cells (Fig. 3). Previous evidence for the expression of such a precursor in the family Bunyaviridae had only been obtained through *in vitro* translation experiments for the genus *Phlebovirus* (Ulmanen *et al.,* 1981; Suzich and Collett, 1988). Detection of the precursor GPC could only be achieved through complete or partial cleavage inhibition

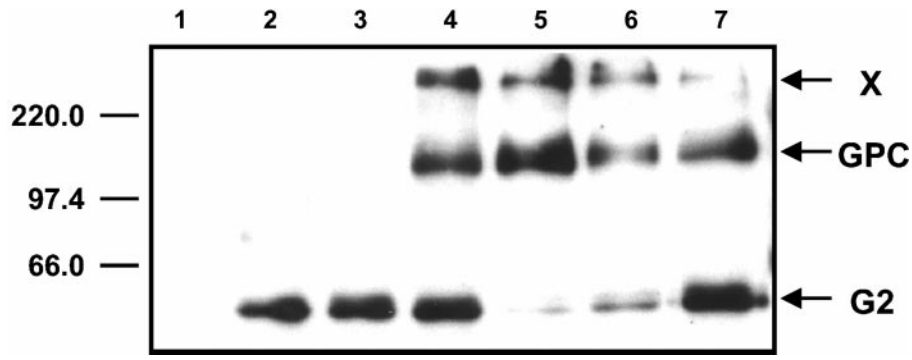

FIG. 3. Expression of cleavage site mutants of the Hantaan virus glycoprotein precursor. Mutagenesis was performed on plasmid pTM1-HTNV/GPC-HA at the putative cleavage site as described under Materials and Methods. HeLa cells were infected with MVA-T7 (m.o.i. of 3) and subsequently transfected with 5 μg of the different plasmids encoding for the mutated glycoprotein precursors. The cells were lysed and the proteins subjected to 10% SDS–PAGE and blotted onto PVDF membrane. GPC and G2 were detected using an anti-HA monoclonal antibody (dilution of 1:2500) as described in the legend of Fig. 2. *Key:* X indicates the high molecular weight aggregates or oligomeric forms of the glycoprotein precursor GPC. Lane 1, control (no DNA transfected); lane 2, WAASA (wild-type); lane 3, WAVSG mutant; lane 4, WARSA mutant; lane 5, WAASR mutant; lane 6, WAESE mutant; lane 7, WAESR mutant.

which resulted in the formation of higher molecular weight complexes. These complexes may consist of aggregates or specific oligomers of the precursor glycoprotein. Hantaviral glycoproteins and glycoproteins of other bunyaviruses show a high content of cysteine residues (5.7% for HTNV; 4–7% for other Bunyaviridae) (Schmaljohn *et al.,* 1987; Schmaljohn and Le Duc, 1998) and for hantaviruses these residues are highly conserved (Antic *et al.,* 1992; Spiropoulou *et al.,* 1994). This indicates that disulfide bridge formation in the ER may be important for the conformation of these glycoproteins. Since signal peptide cleavage occurs prior to disulfide bridge formation, GPC may have a different conformational structure than the G1/G2 heterodimer. Thus, GPC could be misfolded and associate with ER resident proteins leading to aggregation (Fig. 3) and degradation. Since degradation was not observed in our studies, the presence of specific oligomeric forms has also to be considered and has to be further investigated.

The mutational analyses clearly demonstrated that cleavage follows the rules for signal peptides and may occur at the highly conserved pentapeptide WAASA (Fig. 3). This supports the concept of a signal peptidase complex mediating the proteolytic processing of the glycoprotein precursor GPC of hantaviruses. Inappropriate changes at position −1 had a larger inhibitory impact on cleavage than those at position −3. Interestingly, double mutations at position −1 and −3 did not result in a greater inhibitory effect but showed an increased cleavage over the single-site mutants (Fig. 3, lanes 6 and 7). This could be explained by the fact that a potential neighboring cleavage site for a signal peptidase complex was created (. . .$SIL_{-3}WA_{-1}$ ↓ ESE. . .). Additional mutagenesis of the leucine into an argine residue (. . .$SIR_{-3}WA_{-1}$ ↓ ESE. . .) reduced cleavage of the GPC in comparison to WAESE, supporting this hypothesis (data not shown).

Amino acid sequence comparison of bunyavirus glycoprotein precursors in this particular cleavage region indicated that the same concept for cleavage is not only strongly favored for all hantaviruses but also for many other members of the family Bunyaviridae (Fig. 4). Our mutational analyses, however, did not provide an explanation for the conservation of the pentapeptide WAASA among all known hantaviral glycoproteins (Spiropoulou *et al.,* 1994). As demonstrated here (mutant WAVSG; Fig. 3A, lane 3), changes in accordance to the rules for signal peptides would not interfere with proper cleavage of the precursor GPC. Therefore, unknown evolutionary pressure may be applied, which conserves this region. Hantaviruses are rodent-borne agents and the conservation of this particular pentapeptide may be driven by measures that are important for maintaining the virus in the natural host. In addition, previous studies using antipeptide antisera determined the carboxyl terminus of the mature HTNV G1 to amino acid position 588 to 614 (Schmaljohn *et al.,* 1987). This would leave a peptide of at least 38 amino acids, including the conserved WAASA motif. Generation of such a peptide would require a secondary cleavage event but a 4-kDa peptide has yet to be described. Nevertheless, the conservation of the pentapeptide WAASA may be explained by the existence of such a protein.

In conclusion, we have shown the expression of a hantaviral precursor glycoprotein GPC in mammalian cells. The cleavage of the precursor GPC followed the rules for signal peptides, indicating that a signal peptidase complex is responsible for the processing into the glycoproteins G1 and G2. Future studies may show if cleavage is required for the infectivity of hantavirus particles. This seems likely since uncleaved precursor molecules form aggregates which may not be able to promote the functions of the G1/G2 heterodimer in receptor binding and fusion.

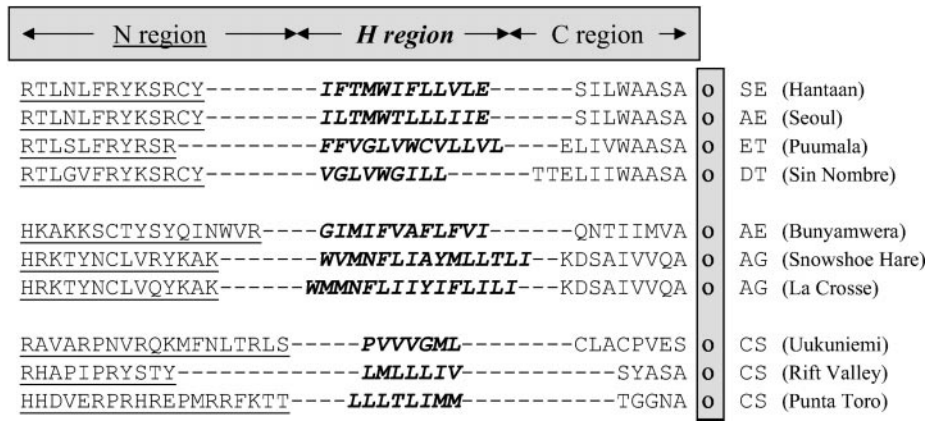

FIG. 4. Sequence comparison of potential signal peptidase cleavage sites of different bunyavirus glycoprotein precursors. The arrows indicate the potential cleavage sites between either the two glycoproteins G1 and G2 (genera *Hantavirus* and *Phlebovirus*) or NS_M and G1 (genus *Bunyavirus*). According to the rules (Perlman and Halvorson, 1983; von Heijne, 1983, 1986), potential signal peptides can be divided into three different regions which are indicated here (N = basic, H = hydrophobic, C = polar). Protein sequence accession numbers: Genus *Hantavirus*—Hantaan, P08668; Seoul, BAA04514; Puumala, P41266; Sin Nombre, AAA75530; genus *Bunyavirus*—Bunyamvera, NP_047212; Snowshoe Hare, P04875; La Crosse, AAB62804; genus *Phlebovirus*—Uukuniemi, GNVUUK; Rift Valley, NP_049342; Punta Toro, P03517.

MATERIALS AND METHODS

Viruses and cell cultures

HeLa and Vero E6 cells were cultured in Dulbecco's modified essential medium (DMEM) (Gibco BRL, Eggenstein, Germany) containing 10% fetal calf serum (FCS) (Gibco BRL). Hantaan virus, strain 76–118, and Modified Vaccinia virus Ankara expressing the bacteriophage T7 polymerase (MVA-T7) (Wyatt *et al.,* 1995) were used in this study.

RNA isolation

Vero E6 cells were infected with HTNV at an m.o.i. of 10^{-2}. Cells were harvested at a 3+ cytopathogenic effect and RNA was isolated from the cells using the RNeasy kit (QIAGEN, Düsseldorf, Germany) according to the protocol of the manufacturer.

Generation of wild-type glycoprotein precursor

The ORF of the HTNV M segment was generated in two pieces by RT-PCR. Subsequently, both amplification products were ligated into the plasmid vector pSP72 (Promega, Mannheim, Germany) using a single *Hin*dIII site (nucleotide position 1411) in the G1-ORF to generate pSP72-HTNV/GPC. An epitope for antibody detection was incorporated using PCR. This was done with pSP72-HTNV/GPC plasmid DNA using a primer upstream of the internal *Hin*dIII site and a downstream primer which included the sequences for the 3′ end of the HTNV/GPC ORF, the HA epitope, a stop codon, and recognition sites for *Xho*I and *Mlu*I. The appropriate part of the M segment ORF in pSP72-HTNV/GPC was exchanged with the newly synthesized PCR product to generate pSP72-HTNV/GPC-HA. The entire ORF HTNV/GPC-HA was then subcloned into the plasmid vector pTM1 (pTM1-HTNV/GPC-HA). This decision was made based on the observation that the MVA-T7-mediated transient expression was not well established using constructs based on plasmid pSP72. In addition, the M segment ORF was subcloned from pSP72-HTNV/GPC into pSC11 (pSC11-HTNV/GPC). The generation of the recombinant vaccinia virus (VV-HTNV/GPC) was done as described previously (Becker *et al.,* 1994). All of the generated intermediate clones, as well as the final construct, were verified by sequence determination prior to use in expression experiments.

Mutagenesis

The Quick Change Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany) was used to introduce the mutations into plasmid pTM1-HTNV/GPC-HA at positions −1 and −3 of the potential cleavage site (WAASA motif). Appropriate primers were synthesized and mutagenesis was performed according to the protocol of the manufacturer. All clones used in further expression studies were verified by sequence analysis.

Expression studies

For the expression using the recombinant vaccinia virus VV-HTNV/GPC, HeLa cells (70–80% confluence) were infected with an m.o.i. of 10. Indirect immunofluorescence assays were performed 24 h postinfection as described below. For T7-driven expression in mammalian cells, HeLa cells (70–80% confluence) were infected with MVA-T7 at an m.o.i. of 3. One hour later the cells were transfected with a mixture of 5 μg plasmid DNA and 5 μl DOTMA (1 μl/μg DNA) (GIBCO BRL) according to the protocol of the manufacturer. For immunoblot analysis, the cells were lysed 24 h posttransfection. The proteins were subjected to 10% SDS–PAGE, blotted onto PVDF membrane (Millipore Immobilon P; Millipore Corp.,

Bedford, MA), and analyzed using an anti-HA monoclonal antibody (Berkeley Antibody Co., BABCO, Richmond, USA) at a 1:2500 dilution followed by species-specific HRP-conjugated antiserum (DAKO, Hamburg, Germany) at a 1:5000 dilution. For indirect immunofluorescence assays, infected and transfected cells on coverslips were treated with cycloheximide (50 μg/ml) 5 h prior to acetone/methanol (1:1 v/v) fixation (24 h posttransfection). For subcellular localization, the glycoproteins were detected with either a single primary protein-specific antibody [anti-HA monoclonal; 1:500 dilution; Berkeley Antibody Co., BABCO, anti-G1 (mAB 6D4), 1:25 dilution or anti-G2 (mAB 8E10), 1:25 dilution] or together with a rabbit antiserum directed against mannosidase II (Golgi marker; 1:100 dilution). This step was followed by an incubation with the species-specific secondary antiserum [swine anti-rabbit FITC-labeled, 1:50 dilution (DAKO) and goat anti-mouse Texas Red labeled, 1:75 dilution (Dianova, Hamburg, Germany)]. Colocalization with the marker protein was analyzed using a laser scanning microscope (Zeiss, Germany).

ACKNOWLEDGMENTS

We thank Connie S. Schmaljohn for critical review of the manuscript and Daryl Dick for editorial comments. The G1 (6D4) and G2 (8E10)-specific monoclonal antibodies were kindly provided by C. S. Schmaljohn, U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID), Fort Detrick, Frederick, MD. The rabbit anti-mannosidase II antiserum was kindly provided by K. Moreman, Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA. This work was supported by grants of the Deutsche Forschungsgemeinschaft (SFB 286 and Fe 286/5-1) and the Volkswagen-Stiftung (I/72087).

REFERENCES

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

Arikawa, J., Takashima, I., and Hashimoto, N. (1985). Cell fusion by haemorrhagic fever with renal syndrome (HFRS) viruses and its application for titration of virus infectivity and neutralizing antibody. *Arch. Virol.* **86,** 303–313.

Arikawa, J., Schmaljohn, A. L., Schmaljohn, C. S., and Dalrymple, J. M. (1989). Characterization of Hantaan virus envelope glycoprotein antigenic determinants by monoclonal antibodies. *J. Gen. Virol.* **70,** 615–624.

Bause, E. (1983). Structural requirements of N-glycosylation of proteins. *Biochem. J.* **209,** 331–336.

Becker, S., Huppertz, S., Klenk, H. D., and Feldmann, H. (1994). The nucleoprotein of Marburg virus is phosphorylated. *J. Gen. Virol.* **75,** 809–818.

Dantas, J. R., Okuno, Y., Asada, H., Tamura, M., Takahashi, M., Tanishita, O., Takahashi, Y., Kurata, T., and Yamanishi, K. (1986). Characterization of glycoproteins of a virus causing hemorrhagic fever with renal syndrome (HFRS) using monoclonal antibodies. *Virology* **151,** 379–384.

Elliott, R. M. (1990). Molecular biology of Bunyaviridae. *J. Gen. Virol.* **71,** 501–522.

Feldmann, H. (2000). Hantaviruses. *In* "Encyclopedia of Life Sciences." http://www.els.net, Nature Publishing Group, London.

Johnson, C. B., and Schmaljohn, C. S. (2001). Replication of hantaviruses. *Curr. Top. Microbiol. Immunol.* **256,** 15–32.

Kalies, K. U., and Hartmann, E. (1998). Protein translocation into the endoplasmic reticulum (ER)–Two similar routes with different modes. *Eur. J. Biochem.* **254,** 1–5.

Lee, H. W., Lee, P. W., and Johnson, K. M. (1978). Isolation of the etiologic agent of Korean hemorrhagic fever. *J. Infect. Dis.* **137,** 298–308.

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.

Perlman, D., and Halvorson, H. O. (1983). A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *J. Mol. Biol.* **167,** 391–409.

Peters, C. J., Simpson, G. L., and Levy, H. (1999). Spectrum of hantavirus infection: Hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome. *Annu. Rev. Med.* **50,** 531–545.

Rapoport, T. A., Jungnickel, B., and Kutay, U. (1996). Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu. Rev. Biochem.* **65,** 271–303.

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.

Schatz, G., and Dobberstein, B. (1996). Common principles of protein translocation across membranes. *Science* **271,** 1519–1526.

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

Schmaljohn, C. S. (1996). Bunyaviridae: The viruses and their replication. *In* "Virology," 3rd ed. (B. N. Fields, D. M. Knipe, P. M. Howley, *et al.*, Eds.), pp. 1447–1471. Raven Press, Philadelphia, PA.

Schmaljohn, C. S., and Hjelle, B. (1997). Hantaviruses: A global disease problem. *Emerg. Infect. Dis.* **3,** 95–104.

Schmaljohn, C. S, and Le Duc, J. W. (1998). Bunyaviridae. *In* "Topley and Wilosn's Microbiology and Microbial Infections," 9th ed. (L. H. Collier, Ed.), pp. 601–628. Edward Arnold, London.

Schmaljohn, C. S., and Nichol, S. T. (2001). Hantaviruses. *Curr. Top. Microbiol. Immunol.* **256,** 1–196.

Spiropoulou, C. F., Morzunov, S., Feldmann, H., Sanchez, A., Peters, C. J., and Nichol, S. T. (1994). Genome structure and variability of a virus causing hantavirus pulmonary syndrome. *Virology* **200,** 715–723.

Spriopoulou, C. F. (2001). Hantavirus maturation. *Curr. Top. Microbiol. Immunol.* **256,** 33–46.

Suzich, J. A., and Collett, M.S (1988). Rift Valley fever virus M segment: Cell-free transcription and translation of virus-complementary RNA. *Virology* **164,** 478–486.

Ulmanen, I., Seppala, P., and Petterson, R. F. (1981). In vitro translation of Uukuniemi virus-specific RNAs: Identification of a non-structural protein and a precursor to the membrane glycoproteins. *J. Virol.* **37,** 72–79.

von Heijne, G. (1983). Patterns of amino acids near signal sequence cleavage sites. *Eur. J. Biochem.* **133,** 17–21.

von Heinje, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14,** 4683–4690.

Wyatt, L. S, Moss, B., and Rozenblatt, S. (1995). Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. *Virology* **210,** 202–205.