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Hantavirus Gn and Gc Glycoproteins Self-Assemble into Virus-Like Particles

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How hantaviruses assemble and exit infected cells remains largely unknown. Here, we show that the expression of Andes (ANDV) and Puumala (PUUV) hantavirus Gn and Gc envelope glycoproteins lead to their self-assembly into virus-like particles (VLPs) which were released to cell supernatants. The viral nucleoprotein was not required for particle formation. Further, a Gc endodomain deletion mutant did not abrogate VLP formation. The VLPs were pleomorphic, exposed protrusions and reacted with patient sera.

Hantaviruses are rodent-borne, enveloped, negative-strand, tripartite RNA viruses that form a separate genus in the *Bunyaviridae* family. Their transmission to humans can produce severe diseases, such as hemorrhagic fever with renal syndrome caused by Puumala hantavirus (PUUV) in Northern Europe and hantavirus pulmonary syndrome caused by Andes hantavirus (ANDV) in Argentina and Chile (1–3).

Hantavirus virions are roughly spherical and highly heterogenic, varying from 120 to 160 nm in size. They expose glycoprotein spikes locally ordered into tetramers and also contain naked membrane patches on their surfaces (4, 5). The viral envelope membrane is acquired from infected cells during virus budding and encloses the three single-stranded RNA (ssRNA) segments that encode four structural proteins: the Gn and Gc glycoproteins, nucleocapsid protein (N), and RNA-dependent RNA polymerase. As other bunyaviruses, hantaviruses do not have a matrix protein that mediates assembly and budding; hence, a role for the cytoplasmic tails of glycoproteins has been proposed (6–8). Previous studies of orthobunyavirus mutant glycoproteins showed that the endodomains of both glycoproteins are required for virus-like particle (VLP) and virus assembly (9). Further, studies on the Uukuniemi phlebovirus Gn tail showed that the Gn endodomain plays a crucial role in genome packaging into virus particles (10). Bunyavirus glycoproteins originate from a single glycoprotein precursor (GPC) through cotranslational cleavage in the endoplasmic reticulum (11–14). Hantaviruses are believed to bud at internal membranes, most probably derived from the Golgi apparatus, and exit cells via exocytosis; alternatively, they may bud directly from the plasma membrane (11, 15, 16).

To study viral assembly and budding processes, individual or isolated viral components are expressed in cells to test their release into the culture medium as VLPs corresponding to membrane-containing viral structures (17). Previously, it has been reported that hantavirus VLPs are produced when Gn, Gc, and N proteins are coexpressed (18, 19). However, not all of these proteins may be necessary for VLP production. This notion is supported by the observation that animals elicit high neutralizing antibody re-

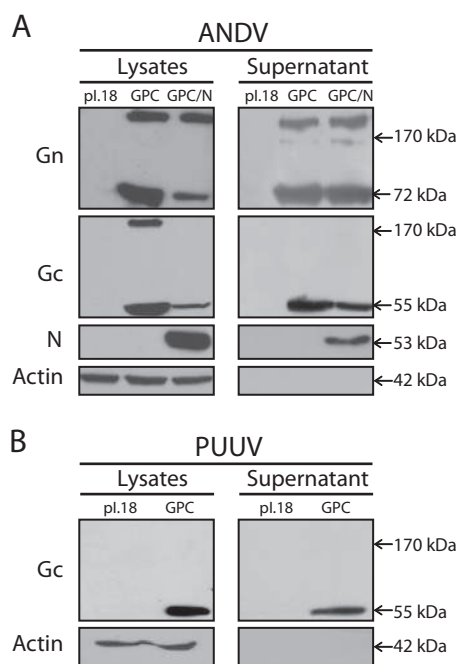


FIG 1 Detection of viral proteins in cell lysates and supernatants. Western blots of lysates and concentrated supernatant of 293FT cells transfected with different plasmids. (A) Transfection of empty plasmid, pL18/ANDV-GPC, or cotransfection of pL18/ANDV-GPC and pCMV-Bios/ANDV-N. (B) Transfection of empty plasmid or pWRG/PUUV-M(s2). ANDV Gn and N proteins were detected with anti-Gn 6B9/F5 and anti-N 7B3/F7, respectively. ANDV and PUUV Gc was detected with MAb anti-Gc 2H4/F6. No MAb against PUUV Gn was available.

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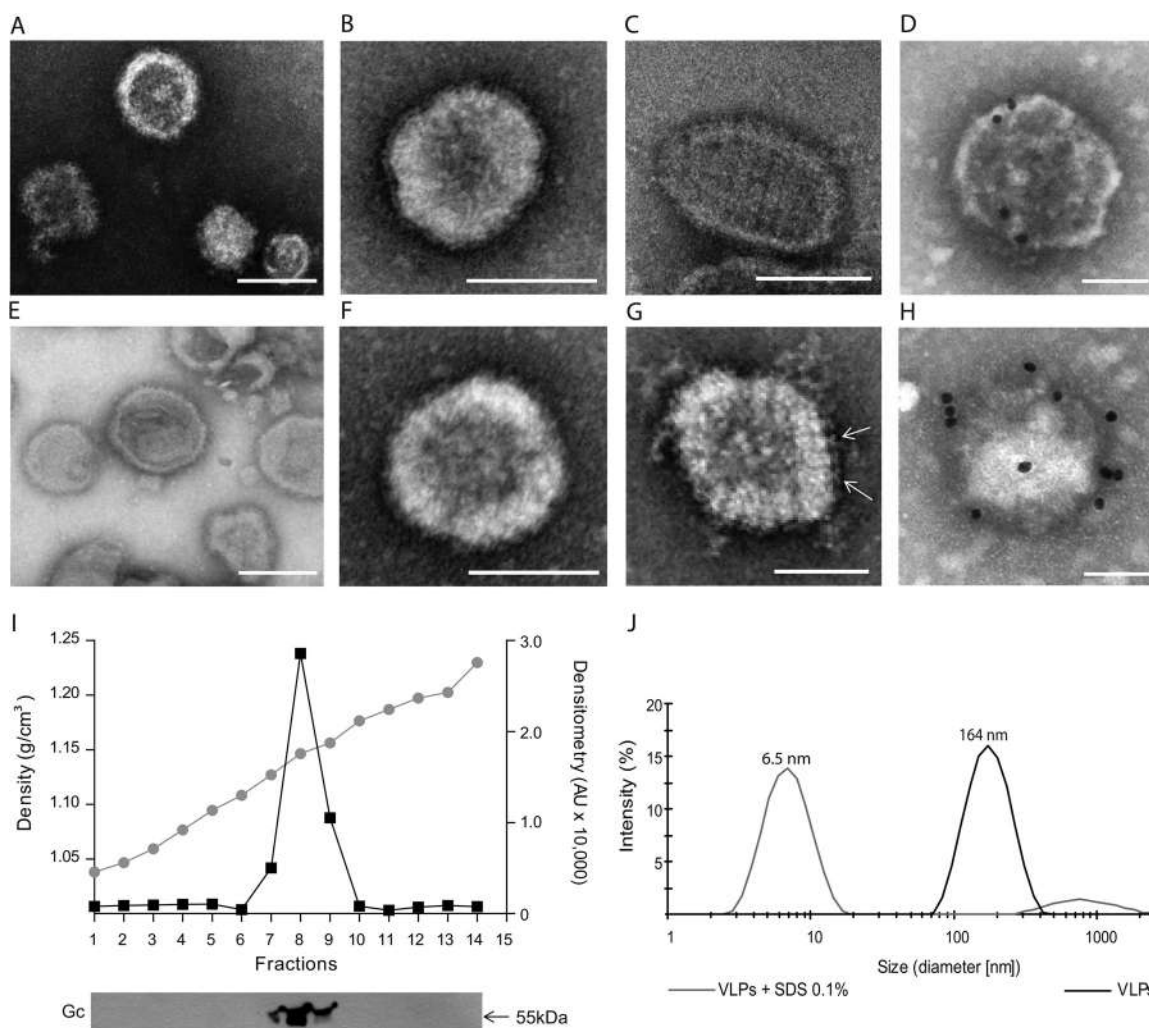


FIG 2 Characterization of hantavirus VLPs. Negative-stain EM of concentrated supernatants from cells transfected with pI.18/ANDV-GPC (A to D) or pWRG/PUU-M(s2) (E to H). Negative-stain EM using phosphotungstic acid of unfixed ANDV VLPs (B), PUUV VLPs (E, F), glutaraldehyde-fixed ANDV VLPs (A, C), and PUUV VLPs (G). Immunogold EM of ANDV VLPs (D) and PUUV VLPs (H) stained with uranyl acetate using patient sera (1:100) and protein A conjugated to gold beads (20 nm). (I) Sucrose gradient sedimentation of ANDV VLPs and detection of VLPs through Western blotting using anti-ANDV Gc MAb 2H4/F6. Buoyant density was determined by refractometry, and relative intensity of Gc bands was quantified as arbitrary units using ImageJ (40). (J) Dynamic light scattering of ANDV VLPs at pH 7.4 and ANDV VLPs treated for 30 min with SDS 0.1%. Bars, 100 nm.

sponses after DNA vaccination solely using a hantavirus Gn and Gc coding plasmid (20–23), which may be indicative for VLP formation *in vivo*, in the absence of the N protein. In addition, for other members of the *Bunyaviridae*, such as phleboviruses, it has been reported that the glycoproteins are the only viral components required for the formation of VLPs (24, 25). To test whether the hantavirus N protein is required for the assembly and budding of hantavirus-like particles, in the present work, hantavirus VLP formation was assessed by plasmid-driven expression of hantavirus Gn and Gc glycoproteins. To this end, 293FT cells (Invitrogen) grown in 10-cm dishes were transfected by the calcium phosphate protocol (26) using 8 µg of pI.18/ANDV-GPC plasmid coding for ANDV-GPC under the control of the cytomegalovirus promoter (27). As a positive control for hantavirus VLP formation, 293FT cells were cotransfected with pI.18/ANDV-GPC and pCMV-Bios/N (28) coding for ANDV-N under the cytomegalovirus promoter. The expression of ANDV Gn, Gc, and N was analyzed by

Western blotting of cell lysates at 48 h posttransfection (Fig. 1A). To this end, the monoclonal antibodies (MAbs) anti-Gn 6B9/F5 (29), anti-Gc 2H4/F6 (30), and anti-N 7B3/F7 (31) and secondary antibody anti-mouse IgG peroxidase conjugate (Sigma) were used. In the lysates of cells transfected with one or both plasmids, ANDV Gn monomers were detected at ~70 kDa and ANDV Gc monomers at ~55 kDa (Fig. 1A, left). Higher-molecular-mass bands of Gn and Gc were also observed as reported previously (27, 29). N could be detected in cells cotransfected with pCMV-Bios/N with a size of ~50 kDa. To analyze whether the viral proteins were also present in cell supernatants, they were concentrated by ultracentrifugation at 100,000 × g, as described previously (27). As seen in Fig. 1A (right), Gn and Gc were detected in concentrated supernatants of cells expressing both envelope proteins alone or in combination with the viral N protein, confirming our hypothesis that N was not required for the release of glycoproteins into the cell supernatant. To test whether the release of ANDV glycopro-

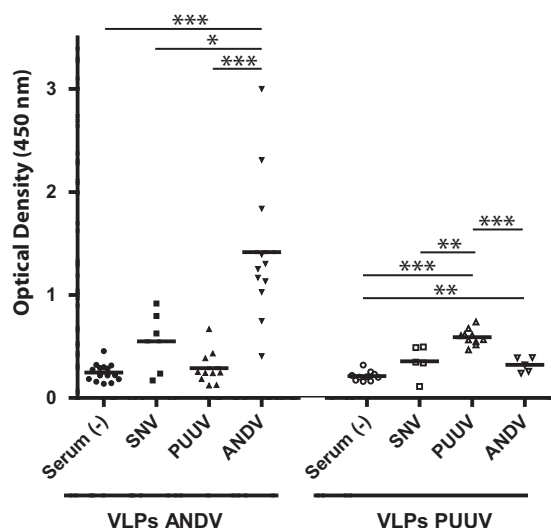


FIG 3 Antigenicity of hantavirus VLPs. ELISA plates were activated with concentrated ANDV or PUUV VLPs, and their reactivity was tested with sera derived from patients infected with different hantavirus species (ANDV, PUUV, Sin Nombre virus [SNV]). A Student *t* test was used for statistical evaluation: ***, $P < 0.00025$; **, $P < 0.0025$; *, $P < 0.025$.

teins was a particular property of ANDV or if it was a more general feature of hantaviruses, plasmid-driven expression of PUUV glycoproteins was next tested. To this end, 293FT cells were transfected with 8 μ g of the plasmid pWRG/PUU-M(s2) coding for PUUV-GPC under the cytomegalovirus promoter (32). When cell lysates and the concentrated supernatant of pWRG/PUU-M(s2)-transfected cells were analyzed by Western blotting, PUUV Gc was detected in both fractions with its expected molecular mass (Fig. 1B), indicating that, as for ANDV, the glycoproteins of PUUV were also released into the cell supernatant in the absence of additional viral molecules. To test whether the presence of viral glycoproteins in cell supernatants was related to virus-like structures, concentrated supernatants of cells transfected with plasmids coding for ANDV-GPC or PUUV-GPC were next analyzed by electron microscopy (EM) using phosphotungstic acid at a pH of ~ 7.4 for negative staining, as described previously (33). Electron micrographs of concentrated supernatants of ANDV or PUUV glycoprotein-expressing cells showed virus-like structures that were variable in size and shape (Fig. 2A to D and E to H, respectively). No apparent morphological differences could be detected between ANDV VLPs produced with or without the N protein (data not shown). It has been well described that EM of hantaviruses reveals a characteristic grid-like pattern (34–36). Negative-staining EM of ANDV and PUUV VLPs fixed with 0.5% glutaraldehyde allowed to discern a grid-like pattern (Fig. 2C and G, respectively). Further, isolated surface projections could be distinguished (Fig. 2G, arrows), resembling a Y-shape similar to the described molecular structure of Hantaan virus and Tula virus spikes that are composed of a tetrameric, globular head domain connected to the membrane by a thinner, central stalk region (4, 5).

The observed virus structures were further characterized by immunogold EM. For this purpose, VLPs were adsorbed to Formvar/carbon-coated copper grids and blocked with 5% bovine serum albumin. Subsequently, the immobilized VLPs were incu-

bated with hantavirus patient sera (1:100) derived from Chilean patients infected with ANDV or ANDV-related species or with sera from European patients infected with PUUV or PUUV-related species. After 3 washes with Tris-buffered saline (TBS), primary antibody binding was detected with protein A conjugated to gold beads (Sigma). As can be seen in Fig. 2D and H, ANDV and PUUV VLPs were recognized by the respective specific patient sera. Normal patient sera were used as negative controls ($n = 3$; data not shown). To further characterize the VLPs in terms of density, sucrose gradient sedimentation was performed by ultracentrifugation for 16 h at 38,000 rpm using an SW55 rotor. The refractive index of each fraction was analyzed at 20°C, and the presence of VLPs was examined by Western blotting using MAb anti-Gc 2H4/F6. VLPs derived from ANDV glycoprotein expression peaked in fraction 8, corresponding to a buoyant density of 1.15 g/ml (Fig. 2I) that coincides with the density range of 1.15 to 1.18 g/ml, which has been reported for infectious hantaviruses and other bunyaviruses (34, 37). The size range of particles generated by glycoprotein expression was next determined by dynamic light scattering (Zetasizer Nano ZS; Malvern Instruments). The size of over 90% of VLPs varied within the range of 90 to 255 nm (Fig. 2J). When VLPs were incubated with 0.1% SDS, their size diminished below 20 nm, confirming their membranous composition (Fig. 2J). Taken together, these data indicate that the release of glycoproteins into cell supernatants in the form of virus-like structures does not require the participation of the viral N protein. Further, the hantavirus glycoproteins are the only viral components required for the assembly and release of VLPs.

The hantavirus VLPs were further characterized in terms of their antigenicity using the sera of hantavirus patients. For this purpose, ANDV or PUUV VLPs contained in concentrated supernatants were immobilized on enzyme-linked immunosorbent assay (ELISA) plates by incubation for 1 h at room temperature (RT). Subsequently, wells were blocked with 4% casein-sucrose for 2 h at RT, and human sera were then added at a dilution of 1:250. After 1.5 h of incubation at RT, the wells were washed 5 times with 0.05% Tween 20–phosphate-buffered saline (PBS). Next, wells were incubated for 1 h with anti-human immunoglobulins G, A, and M conjugated to peroxidase and finally revealed with a tetramethylbenzidine peroxidase substrate (KPL). The reaction was stopped within 10 min by the addition of 1 M phosphoric acid, and the absorbance was read at 450 nm. As seen in Fig. 3, ANDV VLPs reacted with sera derived from Chilean patients. Weak cross-reactivity with ANDV VLPs was detected with the sera of patients from North America infected with Sin Nombre virus or Sin Nombre virus-related species. No reactivity against ANDV VLPs was observed with sera from PUUV-infected patients and with negative-control sera. When PUUV VLPs were incubated with patient sera, reactivity was detected with PUUV-infected patient sera, and a weak cross-reactivity was observed with the sera of patients from America. No reactivity of PUUV VLPs was observed with the negative-control sera. In summary, these data confirm that the ANDV and PUUV VLPs contain glycoproteins on their surfaces that expose epitopes that are recognized by patient sera reactive against the respective native hantavirus.

To test the requirement of the Gc endodomain for VLP assembly, a Gc endodomain deletion mutant (GPC Δ GcCT) was generated, based on the prediction of the Gc transmembrane region (38, 39). DNA mutagenesis was performed by Pfx polymerase (Invitrogen)-driven PCR amplification of the ANDV-GPC coding re-

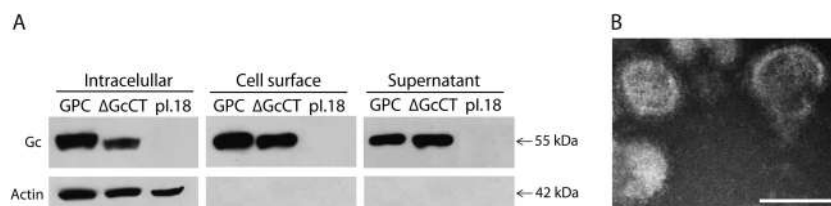


FIG 4 Deletion analysis of ANDV Gc endodomain mutant. (A) Western blot analysis using anti-Gc and anti-actin MABs of different fractions corresponding to the nonbiotinylated fraction (intracellular proteins), the biotinylated fraction (surface proteins), or the concentrated supernatant of 293FT cells that were transfected with pL18/ANDV-GPC, pL18/ANDV-GPCΔGcCT, or empty vector and biotinylated 48 h posttransfection. (B) Negative-stain EM analysis of concentrated supernatants derived from 293FT cells transfected with pL18/ANDV-GPCΔGcCT. Bar, 100 nm.

gion using the forward primer 5'-TAGATCTATTATGGAAGGG TGGTATCTGGTTGC-3' and the reverse primer 5'-CTCGAGCTAGCACAGAACTGAACATTATGATAGAG AG-3'. Subsequently, the PCR product was subcloned into the pL18 expression vector. To test expression and folding of the GPCΔGcCT mutant, 293FT cells were surface biotinylated 48 h posttransfection, using a cell surface protein isolation kit (Pierce) as described previously (29). Previous to biotinylation, the cell supernatant was collected for particle concentration, and the cell lysates were separated into two fractions: the unbiotinylated fraction containing intracellular proteins and the biotinylated fraction containing surface proteins. When these fractions were analyzed by Western blotting, no apparent difference among the wild-type (WT) and mutant proteins was detected in terms of Gc surface localization or the presence of glycoproteins in the concentrated supernatant (Fig. 4A). Further, negative-stain EM of the concentrated supernatant revealed virus structures similar to those of VLPs formed by the WT glycoproteins (Fig. 4B). These data indicate that in contrast to the orthobunyavirus Gc endodomain, the ANDV Gc endodomain is not required for the assembly of virus structures. This difference may be explained in part by the different length of these regions, conserving nine residues among hantaviruses (39) compared to 26 residues among orthobunyaviruses (9).

In the absence of a reverse-genetics system for hantaviruses, the VLP formation system is an important tool for the characterization of the maturation and exit functions as well as cell entry of hantavirus glycoproteins. Further mutagenesis studies are currently assessing glycoprotein properties required for their self-assembly and cell exit. It remains to be determined whether self-assembly is a general feature of glycoproteins of the *Hantavirus* genus and the *Bunyaviridae*.

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