Aromatic and polar residues spanning the candidate fusion peptide of the Andes virus Gc protein are essential for membrane fusion and infection

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Hantaviruses infect human cells through cell attachment and subsequent fusion of viral and cellular membranes at low pH. This largely unknown entry process is mediated by the Gn and Gc glycoproteins, anchored at the viral envelope membrane. Performing bioinformatic analysis and peptide-liposome-binding assays we suggested in a former report that Gc of Andes virus (ANDV) and other hantaviruses corresponds to the viral fusion protein sharing characteristics with class II fusion proteins. To gain insights into the fusion protein of hantaviruses, residues within the previously predicted fusion peptide of ANDV Gc were substituted and mutant proteins tested in fusion and infection assays. To ensure proper folding of mutant proteins, they were first characterized for trafficking to the plasma membrane and incorporation on to ANDV Gn/Gc-pseudotyped lentiviral particles. Cell attachment of these particles was assessed using a newly developed binding assay and their subsequent entry properties determined by FACS analysis of transduced cells expressing the GFP reporter gene. Furthermore, a three-colour-based cell–cell fusion assay of ANDV Gn/Gc expressing cells was performed. The results indicate an essential role of conserved Gc residues W115 and N118 in membrane fusion. Conversely, substitutions of the non-conserved Gc residue G116 did not considerably affect fusion and infection. Altogether, the findings are fully consistent with our earlier prediction suggesting Gc residues 115–121 as an internal fusion peptide and further emphasize the importance of aromatic and polar residues in hantavirus–cell membrane fusion.

INTRODUCTION

Andes virus (ANDV) belongs to the genus Hantavirus and causes hantavirus pulmonary syndrome in humans, resulting in mortality rates of up to 40% in Chile and Argentina. Hantaviruses are spread worldwide and represent one of the five genera within the family Bunyaviridae. Members of this family are characterized by a tripartite ssRNA(−) genome (Elliott, 1990). Bunyavirus virions contain a lipid envelope membrane which is acquired through viral budding during cell exit (Booth et al., 1991; Goldsmith et al., 1995; Kuismanen et al., 1982; Pettersson & Melin, 1996; Rwambo et al., 1996; Salanueva et al., 2003; Smith & Pifat, 1982). This envelope membrane anchors two glycoproteins, Gn and Gc, that originate from a glycoprotein precursor (GPC) through cleavage in the endoplasmic reticulum (Elliott, 1996; Kuismanen, 1984; Lober et al., 2001; Sanchez et al., 2002). Envelope glycoproteins are likely to be responsible for the viral cell entry process as has been shown for many other animal viruses. Cell entry is initiated by cell attachment (Choi et al., 2008; Gavrilovskaya et al., 1998; Krautkramer & Zeier, 2008; Pekosz et al., 1995) and subsequent receptor-mediated endocytosis (Hacker & Hardy, 1997; Jin et al., 2002; Lozach et al., 2010; Pekosz & Gonzalez-Scarano, 1996; Smith & Helenius, 2004). A recent report indicates entry of a bunyavirus into early Rab5a+ and endosomes and even Rab7a+ and Lamp-1+ late endosomes (Lozach et al., 2010). From within endosomes, the ribonucleocapsids of bunyaviruses have to be released into the cell cytoplasm in order to initiate viral replication. This key step in infection is thought to occur through fusion between the viral and the endosomal membrane at low pH (Arikawa et al., 1985; Lozach et al., 2010).
In general, virus–cell membrane fusion is mediated by viral fusion proteins which have been assigned to three different classes based on their molecular structures. Class I fusion proteins are principally composed of α-helices, class II of β-sheets and class III combines structural features of class I and class II (Harrison, 2008; White et al., 2008). Independent of their molecular structures, all viral fusion proteins contain a transmembrane region which anchors them at the viral membrane. At the opposite end of the molecule, activated fusion proteins expose a fusion peptide which drives insertion into the target membrane, thereby connecting viral and cellular membranes. Once inserted in membranes, fusion peptides are thought to destabilize target membranes in order to promote the fusion process (Epand, 2003; Nieva & Agirre, 2003). When merging of membranes has concluded, viral fusion proteins have reached a hairpin structure in which the fusion peptide and the transmembrane region are in a juxtapose position inserted in the same membrane (Kielian & Rey, 2006).

Fusion peptides have been identified for a wide range of viruses through membrane-binding assays of analogous peptides (Harter et al., 1989; Kliger et al., 1997; Lear & DeGrado, 1987; Martin et al., 1993; Melo et al., 2009; Stegmann et al., 1991) and site-directed mutagenesis studies in which mutants display decreased fusion activity, while they conserve other wild-type (wt) functions (Allison et al., 2001; Hamah et al., 2007; Hernandez & White, 1998; Horvath & Lamb, 1992; Ito et al., 1999; Kadlec et al., 2008; Levy-Mintz & Kielian, 1991; Sun et al., 2008b). Fusion peptides are located N-terminally (class I) or internally as a fusion loop (classes II and III) at the tip of activated fusion proteins (Harrison, 2008; White et al., 2008). As active centres of fusion proteins, they tend to be composed of short, conserved and amphipathic sequences enriched in glycine and bulky apolar residues (Earp et al., 2005; Epand, 2003; Hernandez et al., 1996; Nieva & Agirre, 2003; Tamm & Han, 2000).

For hantaviruses, the membrane fusion activity has been experimentally proven to be conferred by the viral glycoproteins (Ogino et al., 2004) and has been associated by us with Gc through the identification of an internal candidate fusion peptide (Tischler et al., 2005). This study was based on in silico sequence analysis and on in vitro peptide-liposome-binding assays. For other bunyaviruses, mutagenesis studies of Gc from the genus Orthobunyavirus identified an equivalent region as the candidate fusion peptide for La Crosse virus (LACV) and bunyamwera virus (Plassmeyer et al., 2007; Shi et al., 2009), indicating that members of the family Bunyaviridae may share common fusion protein characteristics. Further, in our previous work a three-dimensional comparative molecular model based on crystallographic data of tick-borne encephalitis virus (TBEV) E protein (Rey et al., 1995) was derived for the ANDV Gc ectodomain supporting a feasible class II fusion-protein fold. The model structure fulfilled stereo-chemical and dynamic stability parameters and together with the in vitro results lead us to associate hantavirus Gc with class II fusion proteins (Tischler et al., 2005).

To provide mutational evidence for the existence of key residues within the candidate fusion peptide of ANDV, in this paper we designed several amino acid substitutions within this region and report on the characterization of mutants in terms of: (i) their proper folding and transport to the plasma membrane, (ii) cell–cell fusion activity, as well as (iii) cell binding and infectivity of their respective Gn/Gc-pseudotyped particles. We found that the candidate fusion peptide contains essential residues for membrane fusion activity including aromatic and polar residues and discuss how these residues may be involved in membrane binding and fusion loop stabilization.

**RESULTS**

**Selection of Gc residues for mutagenesis**

Logo representations of multiple sequence alignments including the Gc candidate fusion peptide from different genera of the family Bunyaviridae are shown in Fig. 1(a). As phleboviruses have a shorter candidate fusion peptide sequence, they were not included in this comparison. Residues selected for their substitution were located within the ANDV Gc candidate fusion peptide loop (residues 115–121, Fig. 1b) and accomplished the criteria for sequence conservation within the genus Hantavirus (N118 and D121) and family Bunyaviridae (W115) (Fig. 1a). Residues were not substituted when they were likely to be involved in loop structure formation through the disruption of secondary structures (P119) and in loop stabilization through the formation of disulphide bridges (C117). Although G128 is located outside the predicted fusion loop (Fig. 1b), this residue was also selected for mutagenesis based on its striking conservation throughout the family Bunyaviridae (Fig. 1a). Control amino acid substitutions included the non-conserved residue G116 (Fig. 1a). Mutations, including deletions or substitutions of the entire candidate fusion peptide sequence, were not produced since it was shown that such mutations cause difficulties for protein folding in other fusion proteins (Levy-Mintz & Kielian, 1991; Plassmeyer et al., 2007). Residues were either substituted by alanine as this residue is known to produce minor structural alterations, or alternatively, by more similar (W115F and D121N) or dissimilar residues (G116D; Fig. 1b).

**Synthesis of wt and mutant Gc proteins and transport to the cell surface**

A crucial condition for the functionality of cell–cell fusion assays and the production of Gn/Gc-pseudotyped particles is the localization of fusion proteins on the plasma membrane of transfected cells. Furthermore, the presence of mutant Gc on the cell surface represents a qualitative measure for proper protein folding. For these reasons, initial experiments aimed to analyse the cellular distribution of each Gc mutant. To this end, proteins on the cell
surface were biotinylated and the fractions corresponding to intracellular and surface proteins separated for subsequent Western blot analysis.

As seen in the intracellular fraction of cell lysates, wt Gc migrated as a prominent band at 55 kDa (Fig. 2a). To a minor degree, multimers were detected, corresponding most probably to Gn/Gc heteromultimers and misfolded higher order multimers. All Gc mutants, except G128A, migrated identically to wt Gc (Fig. 2a, top). The mutants W115A and W115F were produced in similar amounts as wt Gc, while minor amounts were found of mutants G116A, G116D, N118A and D121N. Mutant G128A was hardly detected and migrated only in higher order aggregates (Fig. 2a). It is likely that the substitution of G128A considerably affected the folding of the protein. To compare the amount of loaded proteins in each lane, β-actin was detected as a control (Fig. 2a, bottom).

When the surface fractions were analysed for the presence of Gc, the wt and all mutants except G128A were found as monomeric bands of 55 kDa (Fig. 2b, top). The amounts of Gc detected on the cell surface coincided with the efficiency of synthesis of each protein (compare with Fig. 2a, top). To ensure that no membrane leakage and subsequent exposure of intracellular proteins to the biotinylation reagent occurred during the biotinylation process, a control immunoblot was performed of the same cell lysates and tested for the presence of β-actin, a protein which is exclusively present in intracellular fractions of cells. In all cases, β-actin was not found in the biotinylated fractions, confirming that wt and mutant Gc were located on the cell surface (Fig. 2b, bottom). In this experimental design, protein overexpression may have affected Gc maturation through the possible saturation of the post-translational cellular machinery. However, these alterations would have affected wt and mutant Gc.

**Essential Gc residues for cell–cell fusion activity**

All Gc mutants which localized at the cell surface were analysed for their fusion activity in a cell–cell fusion assay. This assay employs three different fluorophores to stain the cell nuclei, cell cytoplasm and viral Gc, simplifying the identification of syncytia. For this, Vero E6 cells were transfected with expression plasmids coding for Gn and wt or mutant Gc. At 48 h post-transfection, cells were exposed to low pH media in order to activate Gc accumulated on the plasma membrane. When cells were transfected with the plasmid coding for the wt glycoproteins, syncytia with between five and 20 nuclei were produced upon low pH activation (Fig. 3). Syncytia formation was not detected when the pH of cells expressing wt glycoproteins was maintained at 7.0 or when cells were transfected with the empty expression plasmid. When cells expressed Gc mutants W115A, W115F, N118A or D121N, syncytia were not detected at any tested pH, indicating the loss of fusion functionality of these mutants. Conversely, the capability to produce syncytia was maintained by the mutants G116A and G116D at similar levels as wt Gc (Fig. 3). When the fusion indices of all Gc mutants were calculated (see Fig. 4), values above 0.5 were reached by mutants G116A and G116D, similar to wt Gc, while mutants W115A, W115F, N118A and D121N showed fusion indices below 0.1 throughout the pH range 4.0–7.0. The fusion protein density provided by the lower amounts of Gc at the cell surface was enough to promote syncytia formation since
High fusion indices were reached with Gc mutants G116A and G116D (compare Figs 2b and 4). Inversely, no fusion activity was detected for mutants W115A and W115F which were present in high amounts on the cell surface (Fig. 2b). To test whether the fusion activity is influenced by the amount of Gc on the cell surface, a titration experiment was performed in which cells were transfected with increasing amounts of the wt Gc/Gc-coding plasmid. Equivalent fusion indices were obtained within a range as wide as 0.3–0.7 µg DNA per well, suggesting that the Gc fusion activity did not vary over a wide range of amounts of Gc (Supplementary Fig. S1, available in JGV Online). Overall, from these results it can be concluded that the substitutions W115A, W115F, N118A and D121N affected Gc-mediated cell–cell fusion, while the substitutions G116A and G116D sustained fusion.

In order to determine the activation pH of wt and fusion-active mutant Gc, a fine screening of pH was performed. The pH threshold for fusion activation was found to be pH 5.8 for Gc wt and mutants G116A and G116D (Supplementary Fig. S1). From these results it can be concluded that substitutions of G116 by apolar and acidic residues were well tolerated by Gc.

**Essential Gc residues for infectivity of ANDV-Gn/Gc-pseudotyped simian immunodeficiency virus (SIV) particles**

As the cell–cell fusion assay does not represent all steps required for cell entry to occur, nor reflect viral fusion in vivo where the fusion protein is assembled on the viral envelope, a cell entry assay was used to characterize ANDV Gc mutants. This assay was previously established by us (Cifuentes-Muñoz et al., 2010), based on the transduction of cells by SIV particles pseudotyped with the ANDV wt Gc and wt or mutant Gc.

In order to assess the incorporation of ANDV glycoproteins on to pseudotyped SIV particles, the purified supernatant of producer cells was analysed for the presence of ANDV-Gn/Gc and the structural protein p28 derived from SIV Gag. In supernatants containing particles pseudotyped with Gc wt or mutant W115A, W115F, G116A, G116D or N118A, equivalent amounts of glycoproteins were detected (Fig. 5a). In contrast, in supernatants containing mock-pseudotyped particles (prepared with the empty pl.18 plasmid) or pseudotyped with Gc mutant D121N, none of the glycoproteins were detected (Fig. 5a). The p28 protein of SIV was found in all purified supernatants (Fig. 5a, bottom). The presence of ANDV Gn/Gc together with SIV p28 in supernatants of producer cells strongly suggests the incorporation of ANDV Gn/Gc wt and mutant W115A, W115F, G116A, G116D or N118A on to SIV-pseudotyped particles. Similar amounts of glycoproteins were found in the purified supernatant, indicating that the amount of ANDV glycoproteins at the plasma membrane was not a limiting factor for Gn/Gc-pseudotyped particle formation. However, Gc mutant D121N proteins were not integrated on to pseudotyped particles although being present on the cell surface (Fig. 2b) and hence could not be further characterized.

In order to test that the wt and mutant Gc/Gc-pseudotyped particles were able to attach specifically to Vero E6 cells, a binding assay was developed. For this, Gc/Gc-pseudotyped particles were incubated with Vero E6 cells at 4 °C for 2 h. Subsequently, cells were washed and cell lysates analysed for the presence of Gc by immunoblotting. As a positive control for specific Vero E6 cell binding, wt Gc/Gc-pseudotyped particles were used (Fig. 5b). Conversely, HeLa cells which are not susceptible for transduction with wt Gc/Gc-pseudotyped particles (Cifuentes-Muñoz et al., 2010) were used as a negative control.
control (data not shown). When cell binding was tested for Gn/Gc mutant W115A-, W115F- or N118A-pseudotyped particles, the Gc protein was detected in all Vero E6 cell lysates (Fig. 5b), while Gc was not detected in HeLa cell lysates (data not shown). To estimate the amount of bound pseudotyped particles, dilutions of pseudotyped particles were incubated with cells. Minor amounts of wt and mutant Gc were detected at the dilution 1:2 and no proteins were detectable at the dilution 1:10. These results indicate that wt and mutant ANDV-pseudotyped particles

![Image of fluorescence microscopy images](image.png)

**Fig. 3.** Cell–cell fusion activity of wt and mutant Gc. Vero E6 cells expressing wt or mutant Gc were treated for 5 min with low pH and fixed 4 h later. Cell cytoplasm was labelled with 5-chloromethylfluorescein diacetate (CMFDA; green fluorescence) and nuclei with DAPI (blue fluorescence). Gc was detected with anti-Gc antibody (Alexa555; red fluorescence). Syncytia formation was analysed by fluorescence microscopy (∗200 magnification).
bound to Vero E6 cells and suggest that cell binding was not impaired by the amino acid substitutions introduced in Gc.

With the aim of testing the infectivity of wt and mutant Gn/Gc-pseudotyped particles, their ability to infect Vero E6 cells was assessed. When wt Gn/Gc-pseudotyped particles were used for cell transduction, a significant number of cells expressing GFP were observed in the FACS plots (Fig. 6 as an example). As controls, cells were incubated in the absence of pseudotyped particles (mock) or incubated with mock-pseudotyped particles (p1.18). In both negative controls, a very low number of fluorescent cells were detected (Fig. 6), with at least five times lower mean fluorescence intensity (data not shown). Based on the similarity of the fluorescence intensity of non-treated cells and cells treated with mock-pseudotyped particles, we assumed that the small fluorescent cell population represents autofluorescent cells. When Vero E6 cells were transduced with particles pseudotyped with Gn/Gc mutant W115A, W115F or N118A, again a very low number of fluorescent cells were detected with low fluorescence intensity, similar to the level of the negative controls (Fig. 6). Even when the amount of particles pseudotyped with Gn/Gc mutant W115A, W115F or N118A was increased sixfold, the number of fluorescent cells did not rise over the background level (data not shown). Conversely, when cells were transduced with particles pseudotyped with Gn/Gc mutant G116A or G116D, higher numbers of cells expressed GFP (Fig. 6), with mean fluorescence as high as in cells transduced with wt Gn/Gc-pseudotyped particles (data not shown). For the calculation of transduction titres, the mean value of three independent experiments obtained for cell infection was calculated and the mean value obtained with mock-pseudotyped particles (data not shown). In this context, the transduction titres of pseudotyped particles produced with Gn/Gc wt and mutant G116A or G116D amounted to values ranging from $5.8 \times 10^3$ to $5.5 \times 10^4$ transducing units ml$^{-1}$, while pseudotyped particles produced with Gn/Gc mutant W115A, W115F or N118A did not display titres over the background level (Fig. 7).
Based on the fact that particles pseudotyped with Gn/Gc mutant bound with similar efficiencies to Vero E6 cells as particles pseudotyped with wt Gn/Gc, the results suggest that the infectivity of particles pseudotyped with Gn/Gc mutants W115A, W115F and N118A was abrogated at the level of viral particle–cell membrane fusion. Further, the data revealed by the pseudotyped-particle entry assay is consistent with results obtained from cell–cell fusion assays, highlighting the requirement of Gc residues W115 and N118 in the fusion activity of ANDV.

**DISCUSSION**

The virus–cell membrane fusion process is a fundamental step during the infection of cells by enveloped viruses. We have previously provided evidence that hantavirus fusion activity is associated with Gc and identified a sequence which includes the candidate fusion peptide. Direct interaction of the candidate fusion peptide of ANDV with membranes was demonstrated using in vitro binding assays of analogue peptides with liposomes (Tischler *et al.*, 2005). As a continuation of this earlier work, here we found that amino acid substitutions at positions W115 and N118 within the candidate fusion peptide impaired cell–cell fusion and ANDV-pseudotyped particle infectivity. By using a novel cell-binding assay, it was corroborated that ANDV wt or mutant Gc-pseudotyped particles bound to cells. From these results we conclude that the cell-attachment step preceding membrane fusion was not abrogated; instead amino acid substitutions directly affected virus–cell membrane fusion (see summary Table 1).

Among class II fusion proteins, highly conserved tryptophans are present in their fusion peptide loops (Bressanelli *et al.*, 2004; Gibbons *et al.*, 2004; Lescar *et al.*, 2001; Modis *et al.*, 2003, 2004; Rey *et al.*, 1995). In line with class II fusion proteins, the candidate fusion peptide of Gc from members of the family Bunyaviridae contains a strikingly conserved tryptophan residue (Plassmeyer *et al.*, 2007; Tischler *et al.*, 2005). For LACV Gc, this residue corresponds to W1066 and its substitution to alanine abolishes cell–cell fusion activity, cell entry by pseudotyped particles and gene transfer by virus-like particles (Plassmeyer *et al.*, 2007; Soldan *et al.*, 2010). Here, we found for ANDV Gc that the substitution by either alanine or phenylalanine of the highly conserved residue W115 of the candidate fusion peptide also produced complete loss of the fusion activity.

As measured by the free energy of transfer from water into membrane interfaces, the highest potential of amino acids to partition into membranes is presented by tryptophan (MacCallum *et al.*, 2007; Wimley & White, 1996; Yau *et al.*, 1998). Different approaches show that the indole ring of tryptophan is usually found in the vicinity of the glycerophosphocholine group of the phospholipid interface, with little penetration into the hydrocarbon core (Esbjorn & Nymeyer, 2006; Sun *et al.*, 2008a; Yau *et al.*, 1998). In comparison, the location of alanine and phenylalanine among bilayers is known to be more equilibrated among the polar and apolar phases of

**Fig. 6.** Infectivity of wt and mutant ANDV Gn/Gc-pseudotyped particles. Vero E6 cells were transduced with pseudotyped particles and 72 h post-transduction analysed for GFP expression by flow cytometry. Representative plots are shown, counting in each condition 100 000 cells.

**Fig. 7.** Quantification of cell infection by wt and mutant ANDV Gn/Gc-pseudotyped particles. Viral titres of ANDV-pseudotyped particles were calculated from three independent experiments, counting in each condition 100 000 cells. The mean value of three independent experiments obtained for cell infection with mock-pseudotyped particles was deducted from all samples.
membranes (MacCallum et al., 2007; Norman & Nymeyer, 2006). Hence, the loss of fusion activity by substitutions of ANDV Gc W115 by alanine or phenylalanine may be explained by their lower tendency of transfer from water into membrane bilayers and by their non-differential distribution within membranes. Altogether these data indicate a main role of Gc W115 in fusion and suggest a preferred interaction of the ANDV fusion peptide with the polar phospholipid-head groups of membranes.

The substitution of the conserved residue Gc N118 in the centre of the fusion loop model structure (Fig. 1) by alanine also produced the loss of syncitia formation and infectivity of ANDV-pseudotyped particles. Among class II fusion proteins, crystallographic information shows that a conserved asparagine or glutamine residue can be found in an equivalent position of the fusion loop of dengue virus (DENV), TBEV and Sindbis virus (Bressanelli et al., 2004; Modis et al., 2003, 2004; Rey et al., 1995). Among most of these available crystal structures, a salt bridge can be observed which is formed between these asparagines or glutamine residues with a charged residue contained in the flexible fusion loop model structure (Fig. 1) by glutamine residues with a charged residue contained in the flanking β-sheet (Modis et al., 2004). However, the orientation of residues contained in the flexible fusion loops may differ among crystal structures and structures in solution in the presence of model membranes as demonstrated by nuclear magnetic resonance studies for L107 of the DENV fusion peptide (Melo et al., 2009). Interestingly, asparagine is also present in the middle of the class I fusion peptide of influenza virus at the tip of the boomerang-shaped structure formed in lipid membranes at low pH (Han et al., 2001). This residue (N12) is positioned exactly at the level of the lipid phosphate groups of model membranes; presumably it participates in directing the immersion depth of the fusion peptide (Han et al., 2001; Vaccaro et al., 2005). In this context, the requirement of ANDV Gc N118 for fusion may be interpreted in two different ways: N118 may play an important structural role in fusion loop stabilization through intramolecular salt bridges; or alternatively, this residue may be required in establishing contacts with polar phospholipid-head groups of the membrane during the fusion process. Further studies are required to define the exact role of N118 during the ANDV membrane fusion process.

The candidate fusion peptide of members of the family Bunyaviridae further contains highly conserved, charged residues such as glutamic acids (genera Orthobunyavirus and Tospovirus), arginine (genus Nairovirus) or aspartic acid (genus Hantavirus) (Garry & Garry, 2004; Plassmeyer et al., 2007; Tischler et al., 2005) (compare with Fig. 1a). When we substituted the conserved residue D121 of ANDV Gc with asparagine, the cell–cell membrane fusion activity was completely abolished. In spite of the accumulation of this mutant on the plasma membrane of transfected cells, wt Gc and Gc mutant D121N were not incorporated on to ANDV-pseudotyped particles. Intra- or intermolecular salt bridges or hydrogen bonding may have been affected by the D121N substitution resulting in altered quaternary conformations of Gc required for particle incorporation. Further studies to assess the role of this residue in Gc fusion activity are under way.

In order to study the role of non-conserved residues within the Gc candidate fusion peptide, we also substituted G116. When this small polar residue was changed to an apolar methyl group or even to the bulkier, charged aspartic acid residue, no fusion and little cell entry impairment could be observed, indicating that this amino acid may fulfil a minor role. This notion may be extended to other members of the family Bunyaviridae, since the substitution by alanine of G1067 located at an equivalent position in the candidate fusion peptide of LACV displayed little effect on cell–cell fusion and infection by pseudotyped particles (Plassmeyer et al., 2007).

The activation pH for cell–cell fusion for Gc wt and mutants G116A and G116D was found to occur at ~5.8 and below. This pH was found to be slightly lower than reported previously for Hantaan virus (pH ~6.3; Arikawa et al., 1985). For ANDV it has been recently reported that Gn/Gc incorporated into pseudotyped particles suffer irreversible conformational changes at pH ~6.0 and below.

### Table 1. Characterization of ANDV Gc mutants

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(Ray et al., 2010). Our data indicate that the pH at which ANDV fusion occurs is more similar to that of phlebo- and orthobunyaviruses (pH ~5.4–5.8; Hacker & Hardy, 1997; Lozach et al., 2010), indicating that ANDV may correspond to late-penetrating viruses which escape from maturing endosomes or even later compartments. The characterization of the detailed compartments used by hantaviruses to penetrate into the host-cell cytoplasm remains to be described in future work.

In conclusion, the present work strongly suggests a role of ANDV residues 115–121 as the fusion peptide and hence fully affirms our previous prediction for a role of Gc as the viral fusion protein. The candidate Gc fusion sequence contains five highly conserved residues, three of which were substituted. The residual two correspond to conserved cysteine and proline residues which are likely to affect the fusion loop structure. As this sequence contains a low hydrophobic residue content, we hypothesize that the candidate ANDV fusion peptide may establish preferential interactions with the polar head groups of membranes and the membrane interface. The complete loss of fusion activity by substitutions of W115 by either alanine or phenylalanine indicates a key role of this residue in fusion and suggests that W115 may provide the driving force to insert the fusion peptide into the target membrane. Other residues may stabilize the fusion loop structure through hydrogen bonds and salt or disulphide bridges. Alternatively, some of the polar residues may also establish ionic interactions with the membrane head groups. In this context, we suggest that aromatic as well as polar residues within the candidate fusion peptide region are crucial in ANDV membrane fusion and cell entry.

**METHODS**

**Cells and antibodies.** Vero E6 cells (ATCC, CRL 1586) were maintained in Eagle’s minimal essential medium (E-MEM) supplemented with 10% FBS, 0.1 mM non-essential amino acids, 2 mM l-glutamine and 1 mM sodium pyruvate (Gibco). 293FT cells (Invitrogen) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS (Gibco). mAbs against ANDV antigens were developed previously in our laboratory and included anti-Gn 6B9/F5 (Cifuentes-Muñoz et al., 2010) and anti-Gc 2H4/F6 (Godoy et al., 2009). The anti-Gc mAb 5D11/G7, also generated in our laboratory, was used to corroborate all results.

**Cloning of the ANDV GPC-coding region and site-directed mutagenesis.** The GPC-coding region of the cDNA of ANDV M segment strain CHI-7913 (Tischer et al., 2003) was cloned into the mammalian expression plasmid pL18 (kindly provided by Jim Robertson from the National Institute for Biological Standards and Control, Hertfordshire, UK) as described previously (Cifuentes-Muñoz et al., 2010). Site-directed mutagenesis was performed using the QuikChange XL site-directed mutagenesis kit (Stratagene) as recommended by the manufacturer. The nucleotide substitutions were confirmed by sequence analysis (Sanger et al., 1977).

**Detection of surface proteins on transfected cells.** 293FT cells (3 x 10^6) grown in 100 mm plates were transfected with 8 µg of DNA using the calcium phosphate method (Graham & van der Eb, 1973). Cell surface proteins were biotinylated 48 h post-transfection, using a cell-surface protein isolation kit (Pierce). Gc and β-actin proteins were detected by Western blotting of the biotinylated (surface proteins) and non-biotinylated (intracellular proteins) fractions. Samples were resolved by 12.5% Tris-glycine polyacrylamide gel electrophoresis under denaturing conditions and transferred to a 0.45 µm pore-size nitrocellulose membrane. Gc was detected with mAb anti-Gc 2H4/F6 at a dilution 1:1 000. β-Actin was detected using the mAb anti-β-actin (Sigma) 1:5 000. Primary antibody was detected with anti-mouse immunoglobulin HRP conjugate (Sigma) 1:5 000. Detection of HRP was performed with a chemiluminescent substrate (SuperSignal WestPico; Pierce) following the manufacturer’s instructions.

**Production of pseudotyped particles.** ANDV Gn/Gc-pseudotyped SIV particles were prepared as previously described (Cifuentes-Muñoz et al., 2010) using the three plasmid co-transfection method (Soneoka et al., 1995). Briefly, 293FT cells were transfected with the following plasmids: pSIV3+ (Mangeot et al., 2000), pGAE1.0 (Mangeot et al., 2002) (kindly provided by Jean-Luc Darlix, INSERM, ENS-Lyon, France) and wt or the different mutant pI.18/GPC constructs. At 48 h post-transfection, pseudotyped particles released into supernatants were harvested and purified by ultracentrifugation at 100,000 g over a 25% sucrose cushion.

**Detection of structural proteins of pseudotyped particles.** Purified supernatants containing ANDV Gn/Gc-pseudotyped particles were loaded on 12.5% Tris-glycine polyacrylamide gels and Western blot analysis was performed as described above using the mAbs anti-Gn 6B9/F5 (1 : 1 000) and anti-Gc 2H4/F6 (1 : 1 000). SIV p2p protein derived from the SIV gag gene was detected with mAb against SIV p2p protein (Fitzgerald Industries) at a dilution of 1 : 2 000.

**Cell-binding assay.** Serial dilutions of ANDV-pseudotyped particles were incubated with Vero E6 cells seeded the day before into 24-well plates for 2 h at 4 °C. Next, cells were washed three times with PBS and subsequently lysed with 500 µl of lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, protease inhibitors). The whole lysate was transferred on to a 0.45 µm pore-size nitrocellulose membrane using a Slot Blot system (Minifold II; Whatman). ANDV Gc protein was detected with anti-Gc 2H4/F6 antibody as described above.
Transduction and titre calculation. Vero E6 cells were transduced as described previously (Cifuentes-Muñoz et al., 2010). At 72 h post-infection cells were trypsinized and analysed for GFP expression by flow cytometry (FACScan; Becton Dickinson). Transduction titres were calculated using the percentage of GFP positive cells, counting 100,000 cells for each condition.

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Essen residues of the Andes virus Gc fusion peptide


