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Sin Nombre virus glycoprotein trafficking

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Abstract

Sin Nombre virus (SNV) is a major representative of the New World hantaviruses and the most common cause of hantavirus pulmonary syndrome (HPS) with high mortality in North America. Unlike other members of the family *Bunyaviridae* which mature in the Golgi complex, New World hantaviruses have been previously reported to mature at the cell surface. For family *Bunyaviridae* viruses, retention of the viral glycoproteins at the Golgi complex is thought to be responsible for their Golgi maturation. In our studies, the majority of SNV glycoproteins, G1 and G2, was localized in the Golgi complex when expressed from a full-length GPC clone or in SNV-infected cells, in agreement with data for other members of the family *Bunyaviridae*, including the Old World hantaviruses. However, the SNV glycoproteins could also be detected at the cell surface at advanced posttransfection or postinfection time points. G1 expressed in the absence of G2 did not accumulate in the Golgi, but remained predominantly associated with the endoplasmic reticulum (ER). Overexpressed amounts of apparently misfolded G1 were aggregated in a subcellular compartment likely to represent the aggresome. Unexpectedly, an additional major pool of G1 was detected intracellularly in SNV-infected and GPC-expressing transfected cells, by using a SNV G1-specific Fab antibody. This pool of G1 is predominantly localized in late endosomes–lysosomes.

Introduction

Hantaviruses, family *Bunyaviridae*, are rodent-borne human pathogens. Members of the *Hantavirus* genus are broadly split into the Old World hantaviruses, which cause hemorrhagic fever with renal syndrome (HFRS), and the more recently discovered New World hantaviruses, which cause hantavirus pulmonary syndrome (HPS) (Nichol, 2001). The prototype strain for the Old World hantaviruses is Hantaan virus (HTNV). Sin Nombre virus (SNV) is the major representative of the New World hantaviruses in North America and the most common etiologic agent of HPS with high mortality (Nichol et al., 1993). SNV is an enveloped virus with a tripartite negative-sense RNA genome. The viral genome encodes the nucleocapsid N protein, the glycoprotein precursor GPC (which is cotranslationally cleaved by cellular endopeptidases into the two viral glycoproteins, G1 and G2), and the RNA polymerase L protein (Lober et al., 2001; Schmaljohn and Hooper, 2001; Schmaljohn et al., 1987).

One of the hallmarks of family *Bunyaviridae* members is the unusual property of their maturation at the Golgi apparatus (Jantti et al., 1997; Pettersson and Melin, 1996; Schmaljohn and Hooper, 2001). The Golgi maturation is thought to be due to the retention and accumulation of the two viral glycoproteins G1 and G2 in the Golgi complex (Pettersson and Melin, 1996). As a consequence, a number of studies have been done to determine the characteristics of the glycoproteins that contribute to Golgi retention (Andersson et al., 1997a,b; Matsuoka et al., 1994, 1996, 1988). The glycoprotein closest to the N-terminus of the GPC precursor

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is thought to possess the signal for Golgi localization (Chen et al., 1991; Pettersson and Melin, 1996). The N-terminal glycoproteins of two genus *phlebovirus* members, Punta Toro and Uukuniemi viruses, are among the most extensively studied. Less is known about the trafficking and maturation of the glycoproteins of hantaviruses, and studies to date have been limited to HTNV. In one of those studies, the HTNV N-terminal glycoprotein G1 was reported to accumulate in the Golgi complex when expressed alone, while the G2 protein expressed alone was retained in the endoplasmic reticulum (ER) (Pensiero and Hay, 1992), whereas in another, either HTNV G1 or G2 expressed alone was reported to be retained in the ER (Ruusala et al., 1992). However, both of these studies reported Golgi localization of G1 and G2 proteins when the two proteins were coexpressed. No detection of expressed hantavirus glycoproteins at the cell surface have been so far reported.

Earlier studies of hantavirus ultrastructure and maturation based on electron microscopy (EM) also focused on HTNV (Hung et al., 1985). In those studies, intracellular maturation of hantaviruses could be observed in cells infected with HTNV, but not as easily as with some other members of the family *Bunyaviridae* (e.g., Rift Valley fever and Uukuniemi viruses) (Schmaljohn and Hooper, 2001). Recent EM studies of HTNV-infected patient tissues have also revealed intracellular virus maturation (Wang et al., 1997).

It was only recently, with the discovery of the New World hantaviruses, that the intracellular maturation as a general feature of the hantaviruses has been questioned. EM studies on SNV and Black Creek Canal virus (BCCV) have suggested that their maturation did not occur intracellularly in the Golgi compartment but at the cell surface (Goldsmith et al., 1995; Ravkov et al., 1997). The suggestion of cell-surface maturation of the New World hantaviruses was based on the observation that most of the virus particles were present extracellularly, with only rare virus-like particles observed inside the cell (Goldsmith et al., 1995).

These reports, supporting cell-surface maturation of the New World hantaviruses contrary to the intracellular maturation of the Old World hantaviruses, and the presence of conflicting reports in the literature on the HTNV G1 localization, prompted the initiation of this study. The main objectives were to clearly understand the trafficking of SNV glycoproteins and to gain insights into the virus maturation process. If the intracellular localization of the glycoproteins defines the site of virus maturation in the family *Bunyaviridae*, it was of interest to determine where SNV-expressed glycoproteins were localized. In addition, if the glycoproteins expressed from the GPC precursor colocalize within the Golgi complex, what is the fate of individually expressed N-terminal glycoprotein (G1)? Finally, how does the localization of the expressed glycoproteins correlate with that observed in SNV-infected cells?

Results

Detection of the G1 and G2 glycoproteins expressed from individual clones and from the full-length GPC clone

For the expression experiments, HeLa-T4 cells were infected with vTF7-3 and transfected with pGEM-G1, pGEM-G2, or IRES-GPC constructs. At 12-h posttransfection, cells were radiolabeled, chased, and immunoprecipitated. Results of the immunoprecipitation experiments are shown in Fig. 1. The expressed G1 with apparent molecular weight of 75 kDa was precipitated by using the affinity-purified G1-specific antipeptide antibody (Fig. 1A, lane 2) and the anti-SNV polyclonal sera (lane 5) and comigrated with authentic G1 found in SNV-infected Vero-E6 cell lysates (lane 7). Similarly, expressed G2 with an apparent molecular weight of 55 kDa was immunoprecipitated by affinity-purified G2-specific antipeptide antibody (lane 3) and anti-SNV polyclonal sera (lane 4) and comigrated with authentic G2 found in SNV-infected Vero-E6 cell lysates (lane 7). The constructed SNV full-length GPC clone was successfully expressed and correctly processed to G1 and G2 proteins (Fig. 1B). The expressed glycoproteins from the GPC clone were detected by using the anti-G2 antipeptide antibody (lane 4), anti-G1 antipeptide antibody (lane 5), and anti-SNV sera (lane 6). The estimated molecular weights of the processed G1 and G2 expressed from GPC (lane 7) were in good accord with those of the G1 and G2 proteins immunoprecipitated from SNV-infected Vero-E6 cells (lane 8). In both cases, however, more G2 than G1 was apparently immunoprecipitated. This was not due to differences in cysteine (the amino acid used to radiolabel the proteins) content of the two proteins as there are 31 and 26 Cysteine residues in G1 and G2, respectively, and similar relative amounts of G1 and G2 are also seen when [^{35}S]Cysteine-Methionine mixtures are used as radioactive label (data not shown).

One might expect to detect equimolar amounts of G1 and G2 based on their processing from the GPC precursor. Several explanations are possible for the excess of G2 detected in immunoprecipitation experiments. First, the SNV polyclonal sera may preferentially precipitate G2. Second, the possibility exists that in addition to the G2 processed from the GPC molecule, additional G2 could be synthesized as a result of leaky scanning initiation of G2, as suggested previously for HTNV (Kamrud and Schmaljohn, 1994). Third, the observed results could also be due in part to formation of G1 multimers, which have a slower mobility than does the monomeric from, thus shifting much of the G1 protein to a higher position in the gel, away from the position of the monomeric band. Additional high molecular weight bands are apparent (Fig. 1B, lane 7) and could represent G1 or G2 homo- or heterooligomers forming in the absence of the virus N protein. Such multimers could also be seen in immunoprecipitation experiments of the HTNV-expressed G1 (Antic et al., 1992; Hooper et al., 2001; Pensiero and Hay, 1992). Finally, a preferential deg-

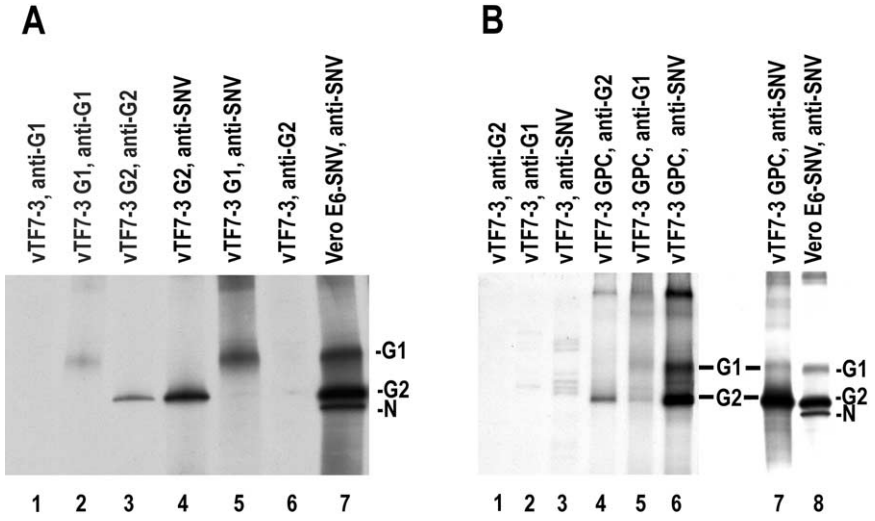

Fig. 1. Expression of full-length clones of G1, G2, and GPC in the vTF7-3 system. HeLa-T4 cells were infected with vTF7-3 and transfected with pGEM-G1, pGEM-G2, and IRES-GPC. Cells were labeled with [^{35}S]Cys at 12 h posttransfection for 1 h, chased for 1 h, and lysed. Protein samples were immunoprecipitated and analyzed by SDS–PAGE. (A) Lysates from cells transfected with pGEM-G1 were immunoprecipitated using affinity-purified G1 antipeptide antibody (lane 2) or anti-SNV-specific polyclonal sera (lane 5). Also, lysates from cells transfected with pGEM-G2 were immunoprecipitated using affinity-purified G2 antipeptide antibody (lane 3), or SNV-specific polyclonal rabbit serum (lane 4). Expressed G1 and G2 proteins comigrated with authentic G1 and G2 synthesized in infected Vero-E6 cells and immunoprecipitated using anti-SNV-specific polyclonal serum (lane 7). Lanes 1 and 6 represent vTF7-3 lysates immunoprecipitated by using anti-G1 and anti-G2 antipeptide antibodies, respectively. (B) Lysates from cells transfected with IRES-GPC following immunoprecipitation using G2 antipeptide antibody (lane 4), G1 antipeptide antibody (lane 5), and anti-SNV polyclonal sera (lane 6). Lanes 1, 2, and 3 represent vTF7-3-infected cell lysates, immunoprecipitated using anti-G2, anti-G1, and anti-SNV sera, respectively. Lane 7 shows the GPC clone expressed and processed to G1 and G2 glycoproteins that coimmigrate with the authentic G1 and G2 immunoprecipitated from SNV-infected Vero-E6 cells (lane 8), using the anti-SNV rabbit serum.

radation of G1 in lysosomes could occur, as will be discussed later (see below).

The above results demonstrate that each glycoprotein can be expressed either individually or from the GPC clone and yield products of the expected size which can be recognized by antibodies specific for SNV.

Cellular localization of G1 and G2 proteins expressed from the GPC clone

The intracellular maturation of the Old World hantaviruses and other members of the family *Bunyaviridae* has been attributed to the accumulation of the viral glycoproteins in the Golgi complex. To investigate the localization of the glycoproteins in the New World hantaviruses, we expressed G1 and G2 glycoproteins from the IRES-GPC clone, initially using the vTF7-3 expression system and double-labeled indirect immunofluorescence microscopy. As shown in Fig. 2A, polyclonal anti- SNV sera clearly labeled the perinuclear Golgi-like region in transfected GPC expressing NRK cells (left panel). Double staining with the anti-SNV polyclonal sera and a monoclonal antibody against mannosidase II, a Golgi marker, clearly show the presence and retention of the expressed glycoproteins in this region of the Golgi (right panel). In these experiments, even after prolonged cycloheximide treatment (5 h), no cell-surface staining was detected. To eliminate any possible interference from the vaccinia infection, G1 and G2 proteins were also expressed in the absence of vaccinia virus by using BHK cells constitutively expressing T7 polymerase. To enhance the stability of GPC mRNA, a polio-IRES was placed in the construct between the T7 promoter and the GPC open reading frame (IRES-GPC). Double-immunofluorescence labeling using anti-SNV sera and anti-syntaxin-6 (a *trans*-Golgi marker) confirmed the Golgi localization of the expressed glycoproteins (Fig. 2B). One of the main advantages of using the BHK-T7 expression system over the vaccinia-T7 transient expression system was the lack of vaccinia-induced (CPE), which limits the extent of time course possible. The transfected BHK-T7 cells remain intact, allowing effects at later time points to be examined. In cell-surface staining experiments using the BHK-T7 cells transfected with IRES-GPC, accumulation of punctate staining at the cell surface was detected at 24–36 h post-transfection (Fig. 2C, left and middle panel). This staining pattern was similar to that seen in the SNV-infected cells at the later stages of the infection (Fig. 2C, right panel). Of note was the formation of filopodia on the surface of cells expressing the G1 and G2 (Fig. 2C, middle panel), indicating a possible involvement of actin (Laakkonen et al., 1998).

These results suggest that the majority of the viral glycoproteins of both the New and the Old World hantaviruses, expressed from full-length GPC clones, are retained in the Golgi complex (Pensiero and Hay, 1992; Ruusala et al., 1992). However, the SNV-expressed glycoproteins reached the cell surface after a prolonged period. It is unclear if

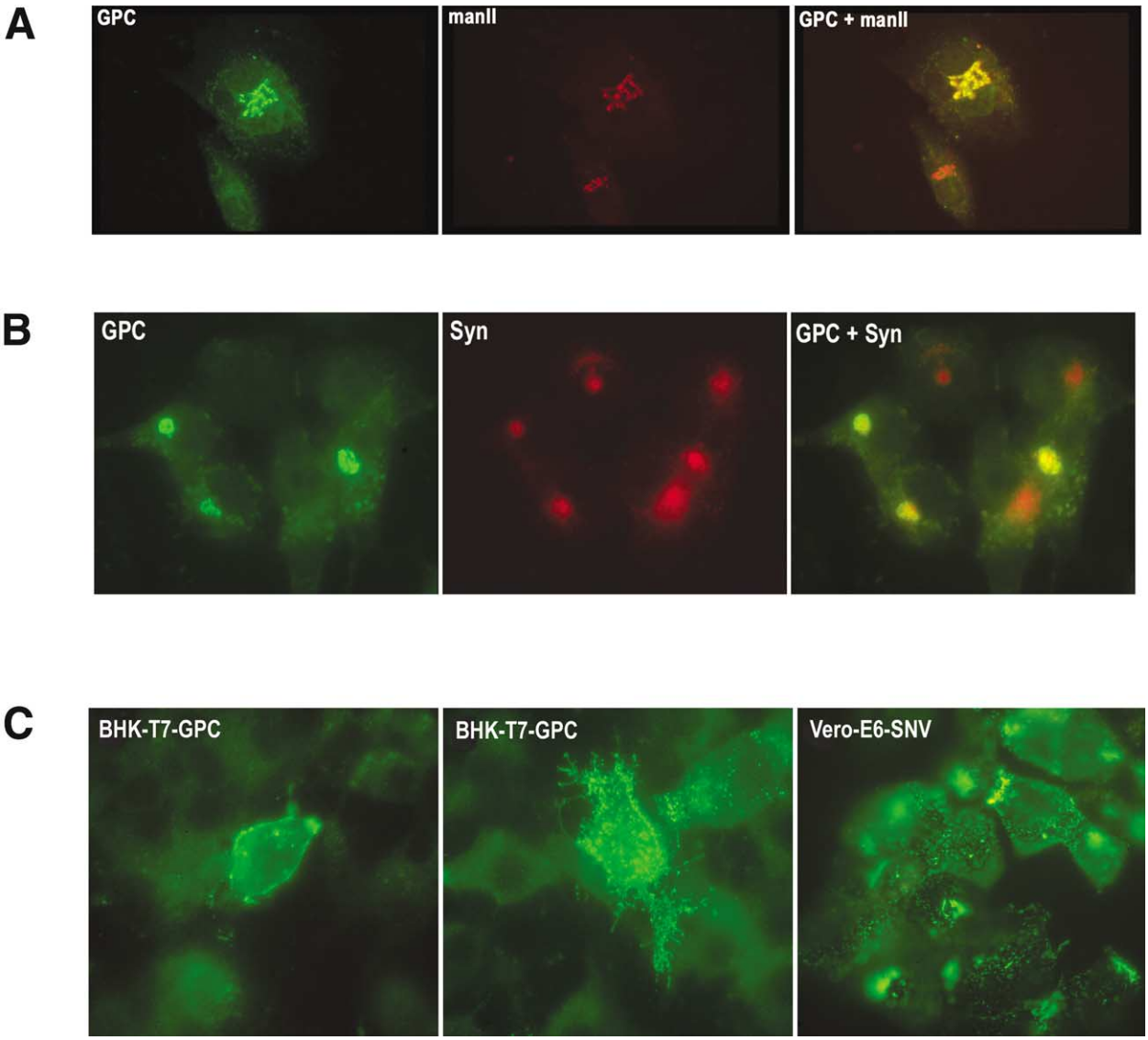

Fig. 2. Cellular localization of SNV G1 and G2 glycoproteins expressed from the full-length GPC clone. (A) Colocalization of G1 and G2 expressed from the GPC clone with mannosidase II by immunofluorescence microscopy. NRK cells were infected with vTF7-3 and transfected with IRES-GPC. At 12 h posttransfection, cycloheximide (50 μg/ml) was added to the medium, and the cells were incubated at 37°C for 5 h and then were double stained with rabbit anti-SNV sera (left) and monoclonal antimannosidase II (middle). The right panel shows the merge image of left and middle panel, in which yellow represents overlap between red and green staining patterns. (B) Colocalization of G1 and G2 with syntaxin-6 in BHK-T7 cell line transfected with IRES-GPC construct. At 36 h posttransfection, cells were fixed, permeabilized, and double stained using rabbit anti-SNV sera (left), and monoclonal anti-syntaxin-6 (middle). The right panel shows the merge image of left and middle panels, and yellow represents overlap between red and green staining. (C) Cell-surface staining of the expressed GPC at late stages posttransfection (left and middle panels). Vero E6 cells infected with SNV and labeled for surface staining at day 5 postinfection are shown in the right panel.

cell-surface localization of the expressed glycoproteins is also occurring in the Old World hantaviruses and other members the family *Bunyaviridae*, as most studies with these viruses have been limited to short time courses post-transfection due to the cytopathic nature of the viral expression systems used.

Cellular localization of G1

After the confirmation of the Golgi localization of the SNV glycoproteins expressed from the GPC clone, it was of interest to see if the N-terminal glycoprotein (G1 for the hantaviruses) was responsible for Golgi retention as has been seen with other members of the family *Bunyaviridae*. It was also hoped that these studies would shed light on the earlier conflicting results obtained for localization of expressed HTNV G1 protein (Pensiero and Hay, 1992; Ruusala et al., 1992). However, the initial results obtained regarding the localization of SNV G1 expressed in the absence of G2 were also puzzling. Immunofluorescence experiments using the vaccinia vTF7-3 system to express

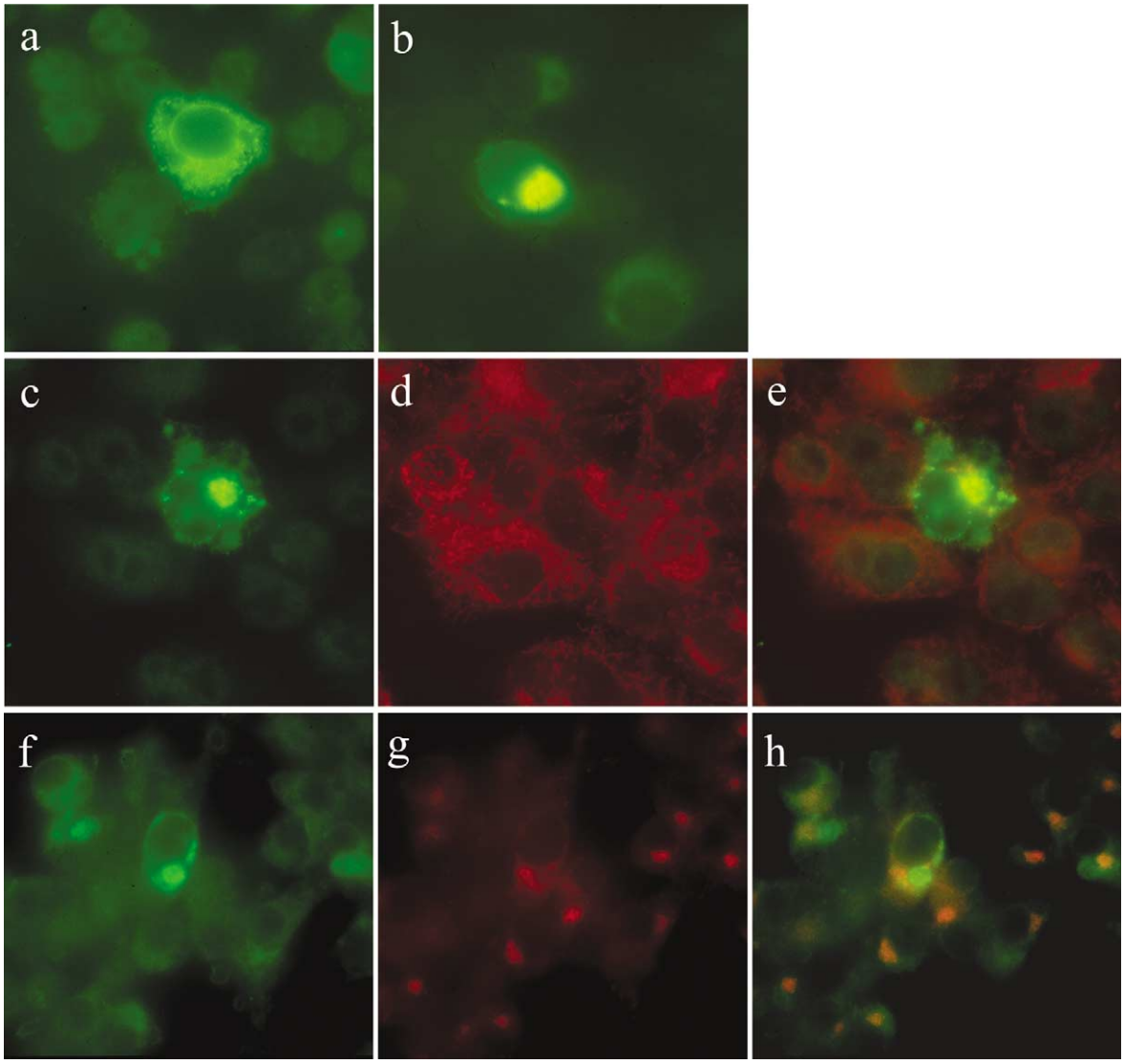

Fig. 3. The SNV G1 protein expressed alone is not localized in the Golgi complex. HeLa-T4 cells were infected with vTF7-3 and transfected with a pGEM-G1 plasmid as described under Materials and methods. At 6 and 12 h posttransfection, panels a and b, respectively, cells were treated with 50 μg of cycloheximide and chased for 5 h. Cells were then fixed, permeabilized with Triton X-100, and stained with rabbit anti-G1 sera (a, b). Possible aggresome formation of G1 expressed in BHK-T7 cell line. Cells were transfected with IRES-G1 construct and at 24 h posttransfection were fixed, permeabilized, and double stained using rabbit anti-G1 sera (c and f) and ER marker anticalnexin (d) or Golgi marker antisyntaxin-6 (g) as primary antibodies. Panels e and h show the merge image of panels (c and d) and (f and g), respectively.

G1 showed an ER localization for G1 at early time points posttransfection (Fig. 3a). At later times posttransfection, the majority of the cells expressing G1 showed a prominent juxtanuclear fluorescent pattern (Fig. 3b). Colocalization experiments using double-immunofluorescence staining of G1 and markers of the *cis*-, *medial*-, and *trans*-Golgi failed to identify the G1 as a Golgi protein, and brefeldin A treatment also failed to disrupt this Golgi-like staining (data not shown). On the basis of these results, it appeared that the majority of G1 expressed in the absence of G2 was localized in the ER. However, at later time points when G1 was overexpressed in cells, the G1- specific antipeptide antibody detected G1 predominantly accumulating in defined structures in the proximity of the Golgi complex. Such structures have been recently identified and called aggresomes (Garcia-Mata et al., 1999; Kopito, 2000). Consistent with these structures representing aggresomes, immunofluorescence staining of an intermediate filament protein, vimentin, shows its redistribution from filaments to ring-like formations in cells overexpressing G1 (data not shown). Vimentin collapse such as this is a characteristic of aggresome formation (Johnston et al., 1998).

To rule out possible influence of the vaccinia infection on the aggresome formation, G1 was also expressed using the IRES-G1 construct and the BHK-T7 cell line. The localization results however were the same as in vaccinia vTF7-3 expression system. Anti-SNV polyclonal sera staining of the expressed G1 again showed a diffuse ER staining

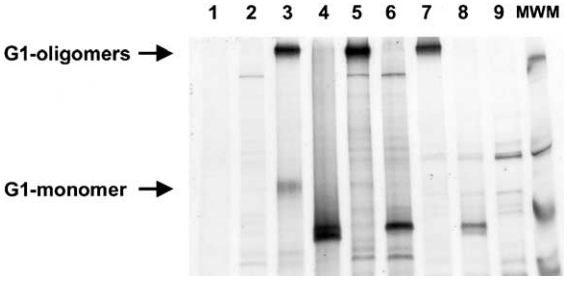

Fig. 4. Time course of expression of G1 and G2 in HeLa-T4 cells using the vTF7-3 transient system. HeLa-T4 cells were infected with vTF7-3 and transfected with the pGEM-G1 and pGEM-G2 plasmids. At 5, 12, or 16 h posttransfection, the cells were labeled with [^{35}S]Cys for 1 h, chased for another 5 h, and immunoprecipitated using the rabbit anti-SNV sera. Lanes 3, 5, and 7 show the immunoprecipitation of the expressed G1 at time points 5, 12, and 16 h. Lanes 4, 6, and 8 show the immunoprecipitated G2 at the time points 5, 12, and 16 h, respectively. Lanes 1, 2, and 9 represent the vTF7-3 controls at the equivalent time points. MWM, high molecular weight markers.

(data not shown). G1 antipeptide sera preferentially showed a juxtanuclear fluorescent pattern of the expressed G1 which was next to, but did not colocalize with, markers of ER (calnexin) (Fig. 3e), or Golgi (syntaxin-6) (Fig. 3h). Unlike the G1 expressed from the GPC precursor, G1 expressed in the absence of G2 showed no convincing cell-surface staining even at late posttransfection time points (data not shown). SNV G2 localizes to the ER when expressed in the absence of G1, as expected based on previous HTNV studies (Pensiero and Hay, 1992; Ruusala et al., 1992), and even when G1 and G2 are coexpressed but from separate plasmids (data not shown). This result further emphasizes the importance of G1 and G2 expression from a full-length GPC ORF to obtain authentic protein folding and trafficking.

In parallel with the above immunofluorescence experiments, immunoprecipitation experiments were also performed at different times (5, 12, and 16 h) posttransfection, followed by a chase period of 5 h. The G1 protein expressed in the absence of G2 was detected in both monomeric and multimeric forms at the 5 h time point (Fig. 4, lane 3), a time corresponding to G1 being localized in the ER (Fig. 3a), whereas only multimeric forms were seen at later time points (Fig. 4, lanes 5, and 7), which may correspond with the G1 protein being located in the aggresomes (Fig. 3b). The oligomeric high molecular forms of G1 are stable, consistent with previous reports which demonstrated the stability of proteins within aggresomes (Johnston et al., 1998). In comparison, a time course of the expression of the G2 (expressed in the absence of G1) was also included in this experiment. G2 was detected as a doublet band (Fig. 4, lane 4), representing the unglycosylated and glycosylated forms of the protein (Spiropoulou, 2001). The G2 protein predominantly shifts to the glycosylated form (the upper band) by the 12 and 16h time points (Fig. 4, lanes 6 and 8). In contrast to the G1 protein, no G2 protein multimers were detected.

It appears, then, that SNV G1 expressed in the absence of G2 could be detected intracellularly in the ER, as has been shown in one of the previous studies for HTNV G1 (Ruusala et al., 1992). However, G1 has the tendency to form SDS stable oligomers and it can be found in what seems to be aggresome formations at later time points when overexpressed. These structures are most clearly seen when staining with the G1-specific antipeptide antibody and structures such as these could have been misidentified as the Golgi complex in earlier studies (Kopito, 2000).

Localization of G1 and G2 in SNV-infected cells

Endothelial cells play an important role in SNV-infected patients. During infection the majority of virus antigen is expressed in these cells, and endothelial cell leakage is a prominent feature of HPS (Zaki et al., 1995). For this reason, we included both primary lung endothelial cells (HMVEC) and Vero E6 cells (the cell line most commonly used to propagate hantaviruses) in studies to examine the correlation between SNV glycoprotein trafficking in infected cells and that observed in our studies using plasmid expression systems. Vero E6 and HMVEC cell lines were infected with SNV, and a double-label indirect-immunofluorescence assay was performed using the anti-SNV polyclonal antibody and antibodies specific for the ER and Golgi. The observed staining with anti-SNV polyclonal sera was well defined in the Golgi region (Fig. 5). Although the anti-SNV sera recognized the N protein in addition to the G1 and G2 proteins, the observed Golgi pattern represents mainly the G1 and G2 proteins, as a completely different pattern is observed when an N-specific monoclonal antibody is used (see Fig. 9B, panel a). A punctate staining throughout the cytoplasm could be seen at late time points, but these did not costain with any Golgi markers and therefore did not represent dispersed Golgi elements. The late punctate cytoplasmic staining could represent sites of increased expression of N protein. Although results are shown only for the primary endothelial cell line, similar results were observed with the Vero E6 cells (data not shown). In addition, cell-surface staining was also detectable late in infection in these endothelial cells, similar to that observed in SNV-infected Vero E6 cells (Fig. 2C, right panel).

On the basis of the above results, it appears that the G1 and G2 proteins accumulate initially in the Golgi apparatus when expressed in both natural SNV infections and in transient expression systems using GPC encoding plasmids. At late stage post-SNV infection or GPC transfection, the glycoproteins can be detected on the cell surface.

An additional pool of G1 exists outside the Golgi complex in SNV-infected cells

Recently, a number of Fabs were generated from a phage display library constructed by using RNA extracted from bone marrow of an SNV-infected convalescent-phase pa-

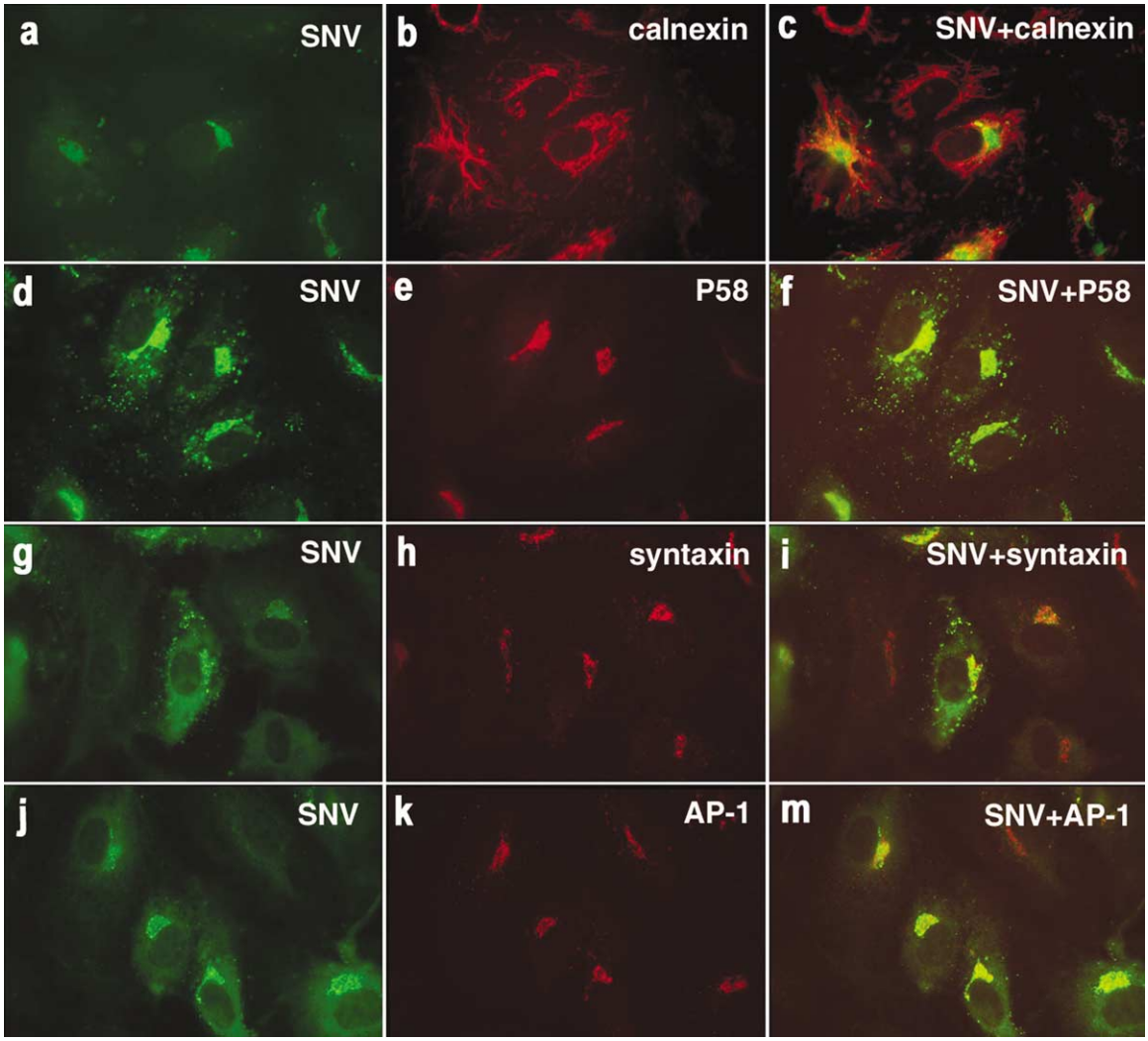

Fig. 5. Accumulation of the virus structural proteins in the Golgi compartment. Primary lung endothelial cells grown on glass coverslips were infected with SNV and fixed at day 1 (a, b, c) or day 5 (d, e, f, g, h, i, j, k, m) postinfection. On the left, panels a, d, g, and j were stained using anti-SNV polyclonal sera. In the middle, various cellular compartments were stained with the following antibodies: ER, anti-calnexin (b); *cis*-Golgi, anti-P58 (e); Golgi, anti- syntaxin-6 (h); TGN, anti-AP-1 (k). On the right, panels c, f, i, and m are the merge images of (a, b), (d, e), (g, h), and (j, k), respectively, and yellow represents overlap between red and green staining.

tient (P.W. Parren and D.R. Burton, unpublished data). The panning of the Fab library was done by using SNV-infected Vero E6 cells as the antigen source. The Fab used in this study, SNV-26, was also engineered to a complete human IgG1. Immunofluorescence studies using Fab SNV-26 show that it does not react with uninfected cells (Fig. 6B, panel a), but specifically recognizes the G1 glycoprotein as shown by immunoprecipitation experiments, using SNV-infected cell lysates (Fig. 6A). Interestingly, the G1 localization seen in immunofluorescence experiments using the Fab SNV-26 (Fig. 6B, panel b) was quite different to that seen previously when using anti-SNV polyclonal sera (Fig. 5). G1 stained with Fab SNV-26 did not localize with the Golgi in SNV-infected cells (Fig. 7A) or GPC expressing transfected BHK-T7 cells (Fig. 7B). From the punctate staining pattern observed with Fab SNV-26, it was apparent that a population of G1 also accumulates in an additional intracellular compartment distinct from the Golgi complex.

To examine in more detail the interaction of SNV-26 antibody with G1, we tested the ability of the Fab to recognize hantaviruses other than SNV. Surprisingly, SNV-26 also cross-reacted with other New World hantaviruses and Old World hantaviruses, including Andes, Black Creek Canal, Hantaan, Seoul, Dobrava, and Prospect Hill viruses (data not shown). Analysis of the amino acid sequences of the G1 glycoprotein revealed that one of the most highly conserved areas was a 12 amino acid region of the ectodomain adjacent to the transmembrane domain. To map the recognition domain, a series of plasmids encoding SNV G1 deletion mutants was constructed (Fig. 8A). The deleted forms of G1 were shown to be efficiently expressed from each of the plasmids (Fig. 8B). SNV-26 was found to still

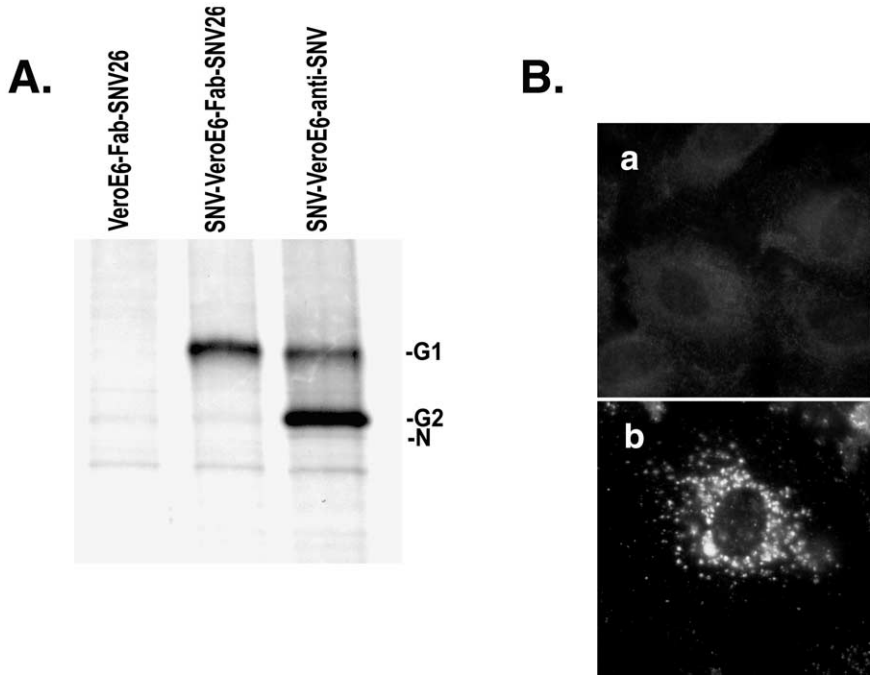

Fig. 6. Fab SNV-26 recognizes the G1 glycoprotein. (A) SNV-infected Vero E6 cells were labeled for 3 h with [^{35}S]Cys at day 5 postinfection, lysed, immunoprecipitated, and analyzed by SDS–PAGE. Cell lysates were immunoprecipitated using SNV-26 IgG1 (middle lane) and anti-SNV polyclonal sera (right lane). The control in left lane represents uninfected Vero E6 cell lysates immunoprecipitated with anti-SNV-26 IgG1. (B) Primary lung endothelial cells were used as an uninfected control (a) or were infected with SNV (b). The cells were fixed at day 5 postinfection and indirect immunofluorescence assay performed using SNV-26 IgG1 as the primary antibody.

react with the form of G1 which represented the amino-terminal 455 amino acids and included deletion of the cytoplasmic tail and most the transmembrane domain (see G1–455, Fig. 8C). However, further deletion of amino acids 443 to 455 resulted in loss of recognition (see G1–442, Fig. 8C). As a positive control, the various truncated forms of G1 remained detectable with a G1 antipeptide antibody which recognizes the amino-terminus of G1 (Fig. 8C). The region, amino acids 443 to 455, contains the highly conserved amino acids adjacent to the transmembrane domain. Thus, the results of the mapping experiments were consistent with SNV-26 cross-reactivity with other hantaviruses and G1 amino acid conservation analysis.

A population of G1 is colocalized with Lamp-1, a late endosomal-lysosomal resident protein

On the basis of the punctuate immunofluorescent pattern of the G1 detected by the Fab antibody, additional double-immunofluorescence experiments were performed with SNV-infected endothelial cells, this time using endosomal–lysosomal markers and antibodies to the SNV N protein. No convincing evidence of colocalization of G1 with EEA1, an early endosomal marker (Fig. 9A, panels a, b, and c), or the N protein (Fig. 9B, panels a, b, and c) was found. In contrast, an extensive overlap of the G1 staining with that of Lamp-1, a late endosomal-lysosomal marker, was detected (Fig. 9A, panels d, e, and f). To clearly show that G1 colocalizes with Lamp-1, EM studies were performed on SNV-infected endothelial cells (Fig. 10). Immunogold labeling using the SNV-26 IgG1 demonstrated that G1 was present in intracellular dense vesicles. Double-immunogold labeling using both SNV-26 IgG1 and Lamp-1 specific antibody showed that G1 is contained within intracellular vesicles that possess Lamp-1 in their membranes. These vesicles represent late endosomal–early lysosomal structures.

Discussion

The initial objectives of these studies were to attempt to express the SNV G1 and G2 glycoproteins in a mammalian expression system, to clarify their site of cellular localization, and to compare their trafficking with that of G1 and G2 synthesized during SNV infection.

The first step of the project, the cloning of the full-length GPC of SNV, was quite challenging, which was not unexpected given the history of constructing different hantavirus M segment clones (Bharadwaj et al., 1999; Hooper et al., 2001; Spiropoulou, 2001). To date, reports of expression of the two SNV glycoproteins involved only fragments of GPC (Bharadwaj et al., 1999; Jenison et al., 1994). By studying the localization of the G1 and G2 expressed from the GPC clone, it was expected that an indirect answer might be obtained for the site of virus maturation, since Golgi mat-

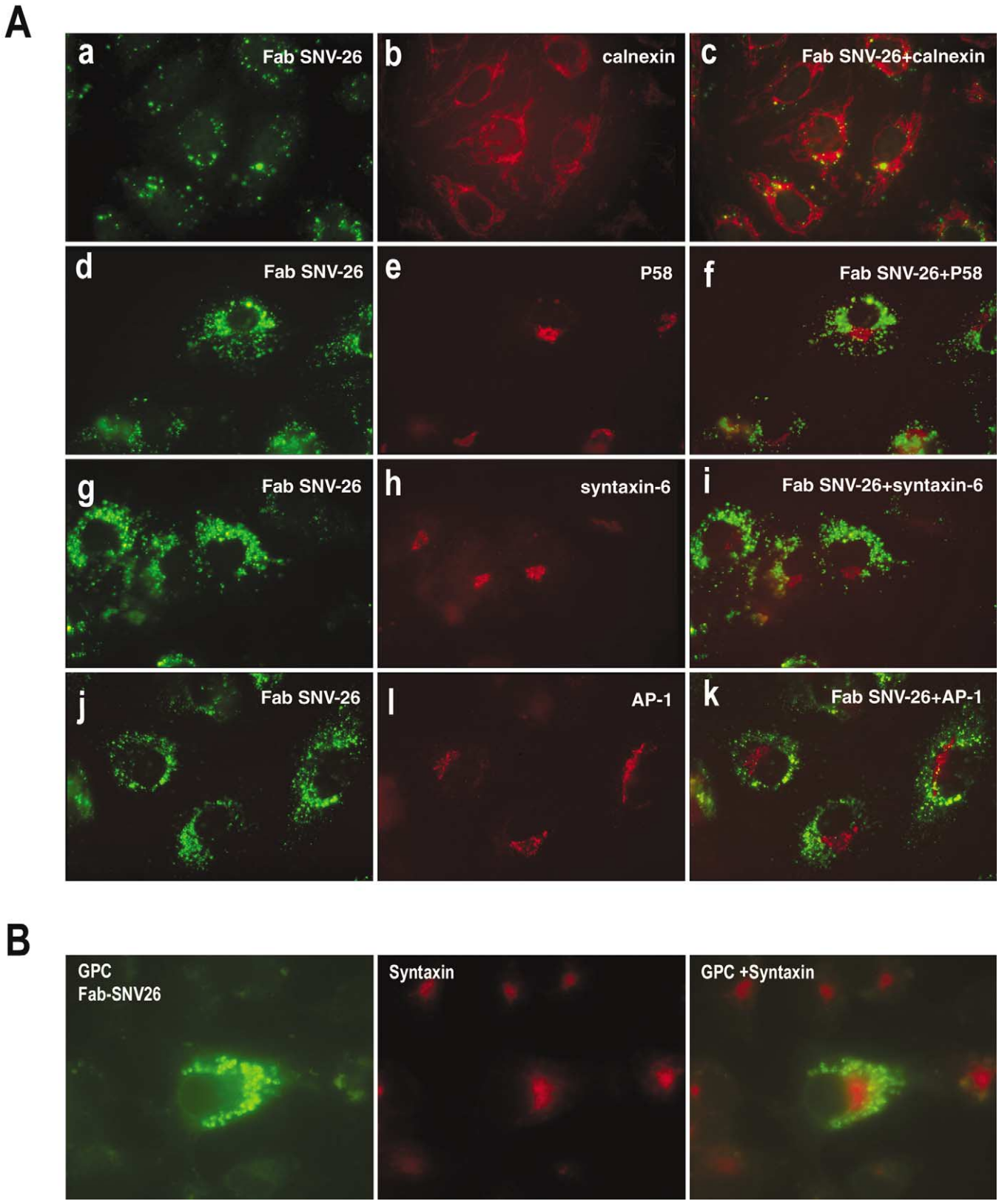

Fig. 7. Absence of colocalization of the G1 detected with Fab SNV-26 with Golgi markers. (A) Primary lung endothelial cells grown on glass coverslips were infected with SNV and fixed at day 1 (panels a, b, c,) or day 5 (panels d, e, f, g, h, i, j, l, k) postinfection. On the left, panels a, d, g, j were stained using Fab SNV-26. In the middle, various cellular compartments were stained with the following antibodies: ER, anti-calnexin (b); *cis*-Golgi, anti-P58 (e); Golgi, antisyntaxin- 6 (h); TGN, anti-AP-1 (l). On the right, panels c, f, i, and k are the merge images of (a, b), (d, e), (g, h), and (j, l), respectively. (B) G1 expressed from the GPC clone and detected with Fab SNV- 26 does not localize in the Golgi. BHK-T7 cells were transfected with IRES-GPC construct. At 24 h posttransfection cells were fixed, permeabilized, and double stained using Fab SNV-26 (left) and antisyntaxin-6 (middle). Right panel shows the merge image of the left and middle panels.

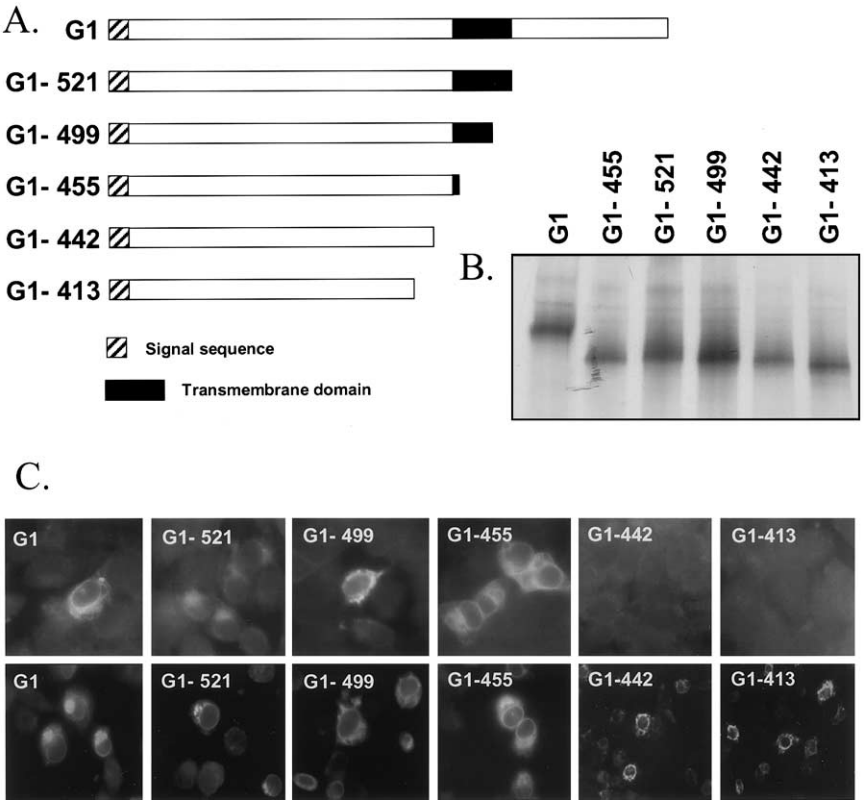

Fig. 8. Detection of the G1 region recognized by Fab SNV-26. (A) Schematic representation of the constructs encoding the full-length G1 and truncated G1 mutants (numbers on the G1 truncation mutants refer to the amino acids included). (B) Expression of the G1 truncation mutants in the vTF7-3 system. HeLa-T4 cells were infected with vTF7-3 and transfected with plasmids expressing G1 and mutants G1–445, G1–521, G1–499, G1–442, G1–413. At 12 h posttransfection, the cells were labeled with [^{35}S]Cys for 1 h, chased for another 1 h, lysed, and immunoprecipitated using the G1-specific antipeptide antibody as described under Materials and methods. (C) HeLa-T4 cells were infected with vTF7-3 and transfected with G1 and G1 truncation mutant expressing plasmids. At 12 h posttransfection, cells were fixed, permeabilized, and stained using Fab SNV-26 antibody (top) or G1 antipeptide antibody (bottom). G1 truncation mutants G1–442 and G1–413 show staining with control G1 antipeptide antibody, but not with SNV-26.

uration for viruses of the family *Bunyaviridae* has been thought to be due to the retention and accumulation of the two viral glycoproteins G1 and G2 in the Golgi complex (Pettersson and Melin, 1996). For the hantaviruses in particular, it had been previously shown for HTNV (which the literature suggests matures in the Golgi complex) that GPC-expressed G1 and G2 glycoproteins are localized in the Golgi and do not reach the cell surface (Pensiero and Hay, 1992; Ruusala et al., 1992).

As expected on the basis of these earlier HTNV GPC studies, in our SNV glycoprotein studies using the vTF7-3 system in two different cell lines (Hela-T4 and NRK) we were also able to show that G1 and G2 proteins expressed from the GPC clone accumulated in the Golgi complex and were not detected at the cell surface. However, expression of the IRES-GPC clone in the BHK-T7 cell line had the advantage of expressing the glycoproteins in intact cells for a prolonged period. In this expression system, cell-surface localization of the viral glycoproteins was detected at late time points. These results may be interpreted as SNV and HTNV glycoproteins trafficking differently, which is possible given the low extent of amino acid identity between these glycoproteins, 68% for the G1 and 80% for the G2 (Spiropoulou et al., 1994). However, the results may simply reflect the difference in the expression systems used. Because the HTNV studies were done only using cytopathic viral expression vectors, it will be interesting to see if cell-surface localization of the expressed HTNV glycoproteins is observable at later time points using noncytopathic expression systems.

Contrary to our initial expectations, our results clearly demonstrated that the majority of SNV G1 expressed in the absence of G2 accumulated in the ER and did not transit to the Golgi. With the G1 encoding plasmid, at later time points posttransfection when G1 is overexpressed, G1 also accumulated into structures likely to represent aggresome formations. G1 possesses extensive hydrophobic regions that normally should be embedded in the lipid bilayer or interact with the G2 protein (Spiropoulou, 2001). It is possible that exposure of these hydrophobic regions to the cytosolic environment may lead to nonnative conformations and to the accumulation of misfolded G1 aggregates in the aggresome. Consistent with this, G1 expressed in the absence of G2 was found to form stable SDS-resistant multimers at all time points analyzed. The region of G1 responsible for the multimer formation has been mapped on the

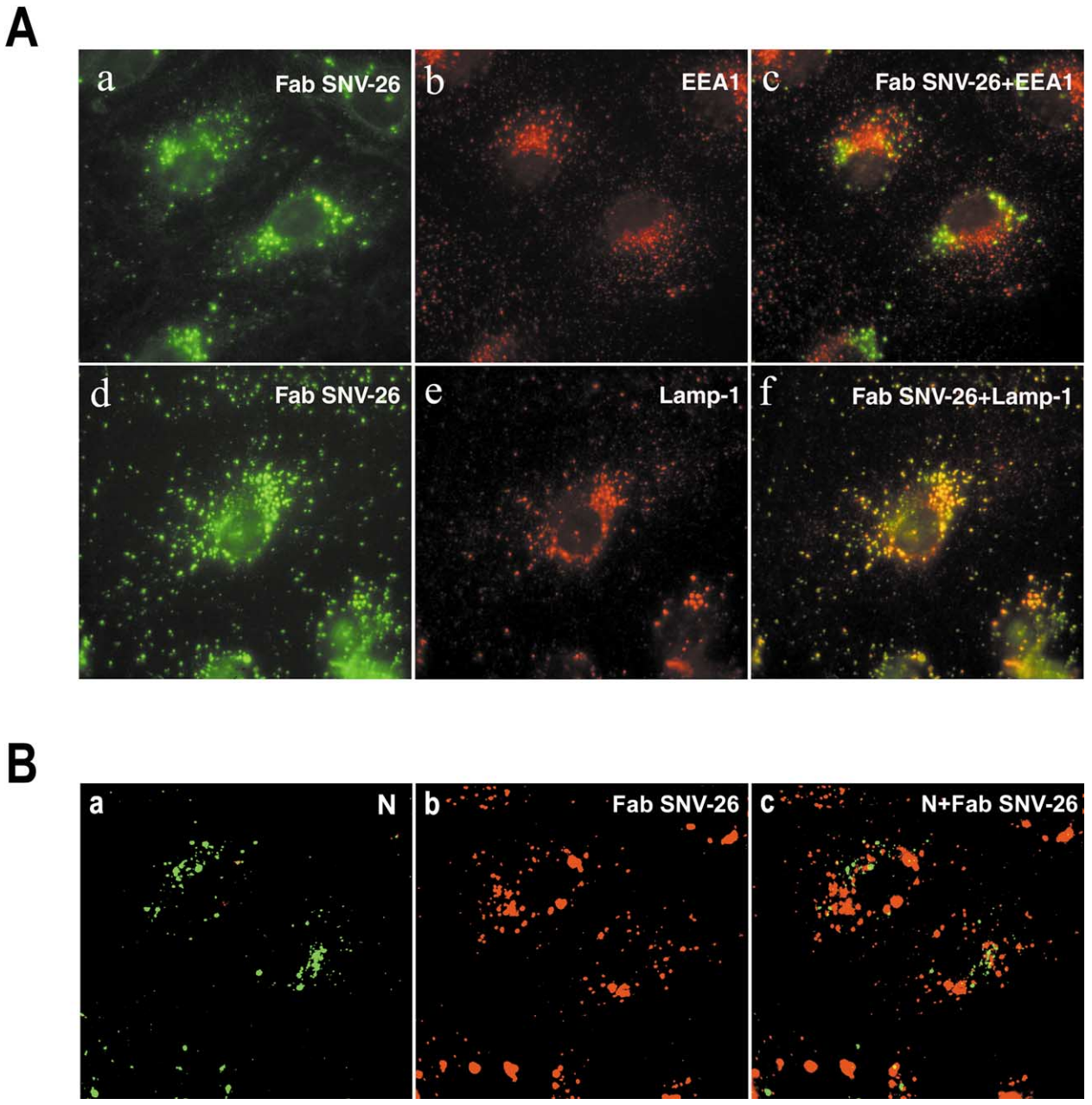

Fig. 9. Colocalization of G1 detected by the Fab SNV-26 with Lamp-1 a late endosomal lysosomal marker. (A) Primary lung endothelial cells were infected with SNV and fixed at day 5 postinfection. Panels a and d show immunostaining of G1 using as primary antibody Fab SNV-26. Panels b and e show immunostaining using markers of the endocytotic pathway: anti-EEA1 (b) and anti-Lamp- 1 (e). Panels c and f are the merge image of (a, b) and (d, e), respectively. Yellow represents overlap between red and green staining patterns. (B) Absence of colocalization of G1 detected by the Fab SNV-26 with N protein. Double-immunofluorescence staining was performed as described previously. Panel a shows immunostaining of N using as primary antibody the N-specific monoclonal GB04-BF07; panel b shows immunostaining of G1 using as primary antibody Fab SNV-26 and panel c shows double staining using both antibodies. Note the distinctive pattern of punctuated staining of N protein in the infected cells.

second part of the hydrophobic domain at amino acids 500–520 (C.F. Spiropoulou et al., unpublished data). The accumulation of SNV G1 in the aggresome may have no biological significance, since it is only seen when G1 is expressed in the absence of G2 and is not seen in SNV-infected cells or when G1 is expressed via the GPC precursor. However, it may be important for explaining earlier reported discrepancies relative to HTNV G1 trafficking.

The trafficking of the glycoproteins expressed from the GPC-encoding plasmid corresponded well with the trafficking of the glycoproteins in SNV-infected cells. In cell infections, we included human primary lung endothelial cells which are thought to be the target cells during natural virus infection, in addition to Vero E6 cells, which are the cell lines most commonly used for hantavirus studies (Zaki et al., 1995). The Golgi localization of the SNV glycoproteins

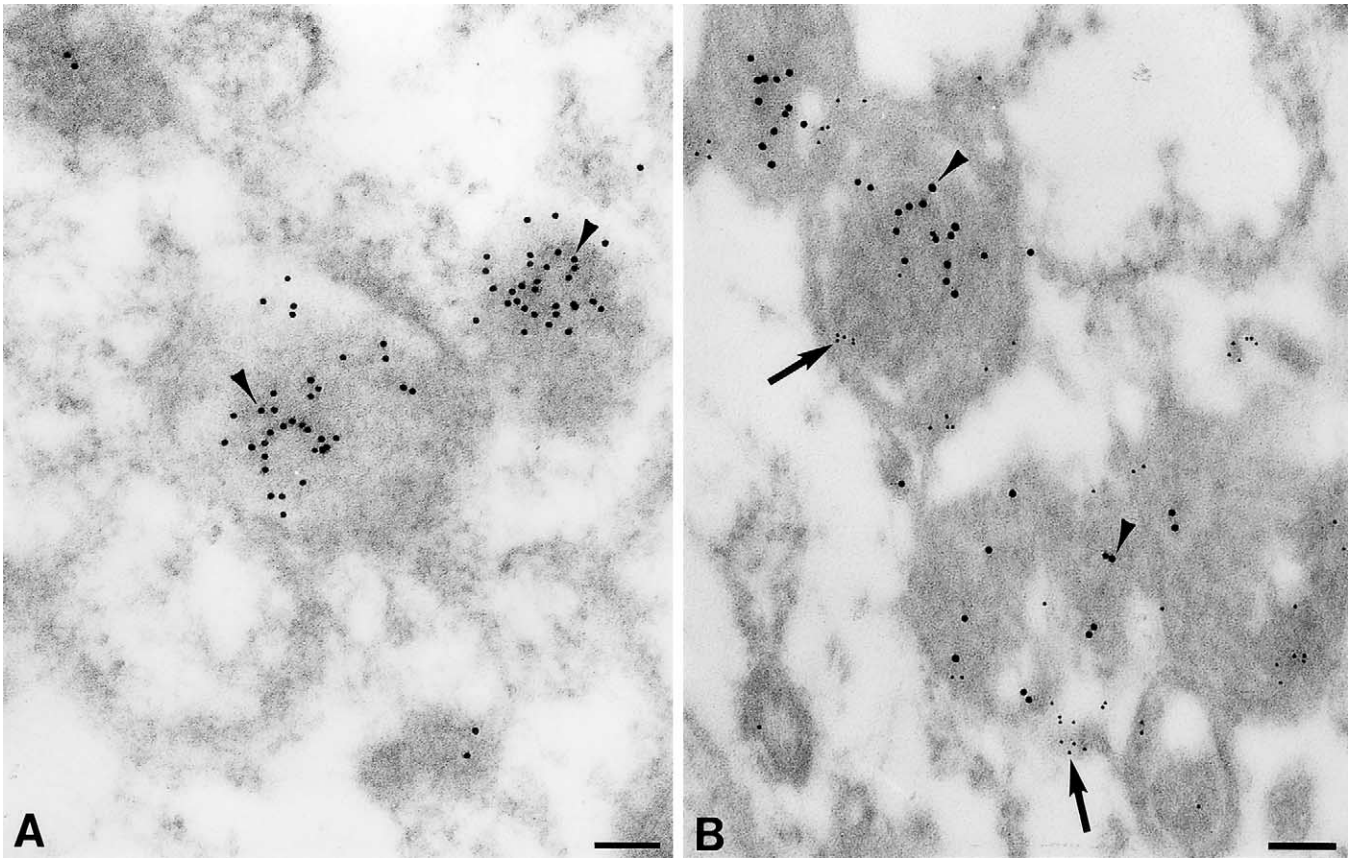

Fig. 10. Immunoelectron microscopic analysis of SNV G1 colocalization with Lamp- 1. EM labeling of SNV-infected primary lung endothelial cells at day 5 postinfection with SNV- 26 IgG1 and Lamp-1. (A) Structures that are labeled for G1 (12-nm gold, arrowheads). (B) Area with extensive label for G1 (12-nm gold, arrowheads) that is also labeled with Lamp-1 (6-nm gold, arrows). Bars, 100 nm.

was prominent during the course of the infection in both cell lines, and cell-surface expression of the glycoproteins, as in the BHK-T7 GPC expression system, was detectable late in infection.

These data obtained by using the GPC expression system provide some insight into the site of virus maturation. We clearly showed that G1 and G2 expressed in virus-infected cells or expressed from GPC-encoding plasmids predominantly accumulate in the Golgi. This finding, together with the fact that the N protein is also localized in the Golgi (Ravkov and Compans, 2001), suggests that the glycoproteins recruit viral cores to the Golgi where virus maturation takes place. On this matter it would be of interest to determine if the N protein in the Golgi is in the form of ribonucleocapsid by using virus N riboprobes in future in situ hybridization EM studies. However, we also showed that at late times following either virus infection or GPC transfection, virus glycoprotein is detectable at the cell surface. These results could be considered consistent with those of EM studies, which predominantly detect virus particles at the cell surface, but only rarely in the Golgi (Goldsmith et al., 1995). Further studies will be needed to resolve this issue. Such studies should include representatives of both Old and New World hantaviruses to firmly establish whether major differences exist among members of the hantavirus genus at the level of virus assembly and release.

The use of a SNV G1-specific Fab SNV-26 produced from a convalescent-phase SNV-infected HPS patient proved very interesting. This antibody was surprisingly cross-reactive with other New World and Old World hantaviruses. One of the most highly conserved regions of hantavirus G1 proteins is located in an approximately 12 amino acid region of the ectodomain adjacent to the transmembrane domain. Our mapping experiments confirmed that this is the region of SNV G1 recognized by SNV-26. No precise function has been ascribed to this region, but the high amino acid conservation seen among the diverse members of the hantavirus group would suggest this region plays a central role in the virus life cycle. SNV-26 detects a large fraction of G1 which colocalized with Lamp-1, a late endosomal–lysosomal membrane protein. Similar results were obtained whether G1 was expressed in the GPC-encoding plasmid transfection experiments or in SNV-infected endothelial or Vero E6 cells. This may represent Fab SNV-26 recognition of misfolded G1 accumulating in the lysosomes. However, Fab SNV- 26 may detect a conformational form of G1 that is induced at acidic pH, such as that found in lysosomal vesicles. Interestingly, Lamp-1 itself is known to

traffic to the *trans*-Golgi, from which the majority goes on to the lysosomes and a minority goes to the cell surface, and recycles between the *trans*-Golgi network and plasma membrane (Rohrer et al., 1996). The peptide motif contributing to the Lamp-1 trafficking to lysosomes is GYxxL at the end of its cytoplasmic tail (Gough et al., 1999). A tyrosine-based motif YxxL also exists at the end of the long cytoplasmic tail of SNV G1 at position 618Y. This motif is present, and highly conserved, in the G1 cytoplasmic tail of both New World and Old World Hantaviruses. As SNV G1 has an intrinsic tendency to aggregate, it is also possible that progressive accumulation of G1 in the *trans*-Golgi network (TGN) could result in its aggregation and delivery to the lysosomes for degradation (Wolins et al., 1997). Interestingly, early endosomes and lysosomes have also recently been implicated in the process of HTNV virus entry (Jin et al., 2002).

Presumably, the G1 that accumulates in the late endosomes–lysosomes is destined for degradation. Degradation of the lysosomal population of G1 could possibly explain the unequal amounts of G1 observed relative to G2 in immunoprecipitation experiments. It will be of interest in future studies to determine the turnover rate of G1 in the presence or absence of inhibitors of lysosomal proteolysis. As the YxxL motif has also been implicated in the internalization of surface glycoproteins (Ohno et al., 1995; Tirabassi and Enguist, 1999), this motif may contribute to recycling of the hantavirus glycoproteins between the cell membrane and TGN and would provide an explanation for the cell-surface expression of SNV glycoproteins seen late in virus infection or transfection with the GPC-encoding plasmid. Interestingly, the same motif is also present at the carboxy-end of the Crimean–Congo hemorrhagic fever virus G2 protein (equivalent to SNV G1), but not in the equivalent glycoproteins of Rift Valley fever, Uukuniemi, and Punta Toro viruses. Experiments are planned to examine potential recycling of the SNV glycoproteins expressed during virus infection or in transfection experiments in which GPC is expressed. The YxxL motif has been also reported to constitute part of the binding site for cellular proteins that may be involved in virus budding. For example, in the equine infectious anemia virus Gag protein, a YxxL motif was shown to mediate interaction with a component of the endocytotic pathway AP2 protein (Puffer et al., 1998). Therefore, it is possible that YxxL could also function in a similar way by recruiting the appropriate cellular proteins to the SNV budding site.

In summary, these results clearly suggest that the SNV G1 protein, when expressed from GPC, can follow at least two distinct pathways: one that leads to the formation of a noncovalent G1/G2 complex and its Golgi localization, and another that leads to the delivery of G1 glycoprotein to the lysosomes. It appears plausible that the first is the productive pathway which results in virion formation, whereas the second is a nonproductive pathway leading to degradation of the virus glycoproteins. This second pathway could represent a mechanism by which hantaviruses down regulate glycoprotein levels and virus maturation. One of the hallmarks of hantavirus maintenance in its primary rodent host species is the establishment of a persistent infection that lasts most of the life of the rodent (Yanagihara et al., 1985). It is possible that down regulation of virus glycoproteins and virus-particle formation could play a role in the mechanism of virus persistence in the rodent reservoir, in addition to other mechanisms previously suggested (Meyer and Schmaljohn, 2000).

Material and methods

Cells, RNA, and viruses

HeLa-T4, Vero E6, CV-1, baby hamster kidney (BHK-21), and normal rat kidney (NRK) cells were maintained in monolayer culture in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Primary human pulmonary macrovascular endothelial cells (HMVEC-L), obtained from Clonetics (Walkersville, MD), were maintained in EGM-2MV growth media (Clonetics). BHK-T7, a cell line that constitutively expresses the T7 polymerase, was provided by K-Klaus Conzelmann, Max V. Pettenkofer-Institut Fur Virologie, Munich, Germany. Total RNA was extracted from virus-infected Vero E6 cells (for the completion of the GPC plasmid) or lung tissue of a HPS (H10) patient as described previously (Spiropoulou et al., 1994). The recombinant vaccinia virus vTF7-3 (Fuerst et al., 1986) was provided by B. Moss (National Institutes of Health, Bethesda, MD). The vTF7-3 was grown on HeLa T4 cells, and the virus titers were determined on CV-1 cells. SNV was grown and titered on Vero-E6 cells. All work with infectious SNV was performed in a biosafety level 3 laboratory.

Antibodies

Rabbit polyclonal antiserum against SNV-infected cells that recognizes the viral glycoproteins as well as the capsid protein was provided by T. G. Ksiazek. G1 expression was also detected with affinity-purified antipeptide antibody G1 (targeting amino acids 72–91, TTTQKYNQVDWTKKSSTTE). The target of the G1 antibody partially overlaps with a linear epitope detected in previous studies (amino acids 59 to 84) (Jenison et al., 1994). G2 expression was detected with affinity-purified antipeptide antibody G2 (targeting amino acids 690–704, YSYRRKLVNPANQEET). Human Fab SNV-26 and the whole human IgG1(SNV-26) were engineered by using a phage display library from RNA extracted from bone marrow of an HPS-convalescent patient and were provided by Paul Parren and Dennis Burton. Antibodies used as markers for different cellular compartments were monoclonal antimannosidase II (rat-specific) (BAbCO, Richmond, CA); monoclonal anti-syntaxin-6 and

monoclonal anti-Early Endosomal- Antigen 1 (EEA1), (BD Transduction Laboratories, Lexington, KY); monoclonal antiadaptin (AP1) clone 100/3, monoclonal antivimentin, and monoclonal anti-Golgi 58K protein (Sigma, St. Louis, MO); monoclonal anticalnexin (Affinity BioReagents, Golden, CO); and monoclonal anti-Lamp-1 (Research Diagnostics, Flanders, NJ). Secondary antibodies were fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, tetramethyl rhodamine isothiocyanate- (TRITC)-conjugated goat anti-mouse IgG, and FITC-conjugated anti-human $(Fab)_2$ (Sigma).

Construction of recombinant plasmids for expression studies

The SNV G1 gene fragment was generated as previously described (Ennis et al., 1997). The full-length G2 gene fragment potentially encoding the G2 glycoprotein was reverse transcribed and amplified by polymerase chain reaction (PCR) (Perkin–Elmer Cetus). The fragment was cloned using the oligonucleotide primers G1953(+) (with sequence 5′GGAATAGGATCCAGTATGGGAATTCTTTTAACAACTGAA 3′ having a *Bam*H1 site and the AUG introduced) and G3495(−) (with sequence 5′GGTGGCTGCAGTCTTGCTCACATATATATTCACTAATTAGC 3′). A series of G1 truncation mutants was generated by PCR amplification of the G1 plasmid, using the same 5′- primer as originally used for the G1 amplification (Ennis et al., 1997). A termination codon (boldface) and the *Eco*RI restriction site (underlined) were added to the 3′-prime end primers. The 3-end primer sequence for the G1–521 mutant was 5′AACGGAGAATTC**TTA**GAAAGTGAGCAACCTCAGGAT3′; G1–499 mutant was 5′AACGGAGAATTC**TTA**AAGGAGCCAGCCAAAGCAAAA3′; G1–455 mutant was 5′AACGGAGAATTC**TTA**AATGCATTGGCCTATGACCAG3′; G1–442 mutant was 5′AACGGAGAATTC**TTA**CTTTTGTCCATTACAATAGACAACT3′; and G1–413 mutant was 5′AACGGAGAATTC**TTA**TTGAACTTTATTCACTAGAC3′. The PCR products were purified using a Geneclean kit (Bio 101, La Jolla, CA), proteinase K digested, phenol-chloroform extracted, and ethanol precipitated. The products were then digested with the appropriate restriction enzymes and cloned into the plasmid pGEM-3Z (pGEM-G1 and pGEM-G2 encoding for the G1 and G2 protein, respectively) and pGEM-4Z (for the G1 mutants) using standard protocols.

A full-length cDNA for the GPC open reading frame was constructed initially in the pSINrep5 replicon (Invitrogen, Carlsbad, CA) by ligating the full-length repG1 with the *Sph*1-*Apa*1 fragment of G2 (G2 lacking 196 nt of the 5′ terminus). The plasmid for the GPC construct was completed by inserting a 771-bp fragment using the restriction sites *Spe* 1 (position 1432 nt on the M segment) and *Sph*1 (position 2203 nt). Full-length GPC was transferred into a pSP64 poly(A) vector (Promega, Madison, WI) containing a polio-IRES fragment (830 nt) downstream of a T7 promoter (T7-IRES was provided by Bert L. Semler, U.C. Irvine). In addition, the full-length G1 was also subcloned into the pSP64 poly(A) vector containing the polio-IRES. The plasmids were transformed and propagated in SURE-competent cells (Stratagene, La Jolla, CA) to minimize the possibility of recombination. Plasmid DNAs were then purified with maxi-prep Qiagen column and the correct orientation of the inserts and the precise nucleotide sequence were verified by dideoxynucleotide sequence analysis.

Expression of G1 and G2 constructs by using the vaccinia-T7 transient expression system

Subconfluent monolayers in 35-mm dishes of HelaT4 or NRK cells were infected with recombinant vaccinia virus expressing the T7 polymerase gene (vTF7-3) at a multiplicity of infection (m.o.i.) of 5 for 1 h and washed with serum-free medium Opti-MEM (Life Technologies, Gaithersburg, MD). Plasmid DNAs (5 μg) were mixed with 15 μl of lipofectin (Life Technologies) 700 μl Opti-MEM medium and added to the vaccinia-infected cells. After a 4 h incubation at 37°C, 300 μl of DMEM containing 10% FCS was added and the incubation was continued for 6 or 12 h posttransfection.

Cell radiolabeling and immunoprecipitation

Transfected HeLa T4 cells were labeled with 100 μCi/ml [^{35}S]Cys (G1 and G2 are cysteine-rich proteins having a total of 57 cysteines) in cysteine-free MEM for 1 h, at indicated times posttransfection. Cells were harvested in radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 1.0% Triton X-100, 1% deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 10 mM Tris, pH 8.0], 1% aprotinin, 2 mM phenylmethylsulfonylfluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml pepstatin. When antipeptide antibodies were used for immunoprecipitation, the cells were harvested in RIPA buffer containing 1% SDS and 1% 2-mercaptoethanol, and the cell lysate was diluted 1:10 before the addition of the antibody. The final amount of the cell lysate used in immunoprecipitations with the antipeptide antibodies was one-fourth of that used with the SNV polyclonal sera. The cell lysates were added to the antibody–protein A Sepharose (Sigma) complex for 3 h at 4°C and washed three times with RIPA buffer. The protein samples were heated at 37°C for 15 min in Laemmli loading buffer before analysis on 7 or 10% SDS–polyacrylamide gels (Nakitare and Elliott, 1993). Fluorography and autoradiogram exposure of dried gels were carried out by standard techniques.

Immunofluorescence

HeLa-T4 and NRK cells grown on coverslips were infected with vTF7-3 at an m.o.i. of 1 and were transfected with the recombinant plasmid DNA. At various times post-

transfection, the cells were treated with cycloheximide (50 μg/ml) for 5 h. BHK-T7 cells were transfected only with recombinant constructs IRES-GPC and IRES-G1. Vero E6 cells and primary endothelial cells were infected with SNV at an m.o.i. of 0.5. At certain times posttransfection or postinfection, cells were washed twice with phosphate-buffered saline (PBS) and fixed with 2% formaldehyde or acetone at room temperature for 10 min. After formaldehyde fixation, the cells were washed three times with PBS and either permeabilized with 0.1% Triton X-100 for intracellular fluorescence or left untreated for surface immunofluorescence. The primary antibody was added at a 1:200 dilution in 1% bovine serum albumin (BSA) in PBS for 30 min. The cells were then washed three times with PBS and incubated for 30 min with FITC- conjugated goat anti-rabbit IgG (Sigma), diluted 1:200 in 1% BSA in PBS. For double staining, cells were incubated with antibodies to viral proteins together with monoclonal antibodies to intracellular markers and stained with FITC-conjugated goat anti-rabbit IgG diluted 1:200, anti-human IgG(Fab-specific) FITC-conjugated diluted 1:200, and TRITC-conjugated goat anti-mouse IgG (Sigma) diluted 1:80 in 1% BSA in PBS. Multiple final washes were done and the cells were mounted on microscope slides and viewed by using a Zeiss microscope.

Immunogold EM

Endothelial cells (HMVEC) uninfected or infected at an m.o.i. of 1 were fixed at 5 days postinfection with phosphate-buffered 1.5% paraformaldehyde and 0.025% glutaraldehyde for 2 h. After fixation, the cells were irradiated with 2×10^6 rad of gamma from a cobalt source and then processed as described previously (Goldsmith et al., 1995). Antibodies used in the EM experiments were human Fab SNV-26 engineered to whole IgG1 molecule for detection of the virus and monoclonal anti-Lamp-1. Goat anti-mouse conjugated to 6 nm colloidal gold particles and goat anti-human conjugated to 12 nm colloidal gold particles (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibodies.

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