Inactivation of hantaviruses by $N$-ethylmaleimide preserves virion integrity

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Thiol groups of cysteine residues are crucial for the infectivity of various enveloped viruses, but their role in the infectivity of viruses of the family Bunyaviridae has thus far not been studied. This report shows that thiol groups are essential to the infectivity of hantaviruses. Alkylation of the thiol functional groups using the membrane-permeable compound $N$-ethylmaleimide (NEM) and membrane-impermeable compound 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) showed NEM to be a highly effective inactivator of Puumala and Tula hantaviruses. The NEM-inactivated hantavirus maintained the buoyant density of the wild-type virus. Furthermore, the antigenicity of glycoproteins and the cell attachment capacity of virions were retained at NEM concentrations that totally abolished virus infectivity. These results signified preservation of virion integrity following inactivation with NEM, making chemically inactivated virions valuable research antigens. It was demonstrated with biotin-conjugated maleimide, a mechanistic analogue of NEM, that all the structural proteins of hantavirus were sensitive towards thiol alkylation. In contrast to hantaviruses, NEM did not abolish Uukuniemi phlebovirus infectivity to the same extent. This indicates differences in the use of free thiols in virus entry among members of the family Bunyaviridae.

INTRODUCTION

Hantaviruses (genus Hantavirus, family Bunyaviridae) are zoonotic viruses that chronically infect rodents and insectivores, causing no apparent disease, but when transmitted to man are linked to two major clinical symptoms: haemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas. Depending on the virus, HFRS manifests as a mild [Puumala virus (PUUV)], moderate (Seoul virus) or severe (Hantaan virus) disease, and some hantaviruses, including Tula virus (TULV) are of low virulence (Klempa et al., 2003; Plyusnin et al., 1994; Schmaljohn & Hjelle, 1997; Vapalahti et al., 2003). Hantaviruses are negative-sense ssRNA viruses with a tripartite genome of large, medium and small segments encoding the RNA-dependent RNA polymerase (L protein), envelope precursor protein of two glycoproteins, Gn and Gc, and nucleocapsid (N) protein (Plyusnin et al., 1996).

Fusion of enveloped viruses with cellular membranes is mediated by viral glycoproteins, divided into class I, II and III fusion proteins (Weissenhorn et al., 2007). Frequently, thiol groups mediate the formation and breakdown of disulfide bonds of the viral fusion protein under catalysis either by the cellular protein disulfide isomerase (PDI) family of proteins (Appenzeller-Herzog & Ellgaard, 2008; Sanders, 2000) or by virally encoded PDIs. The infectivity of many viruses is blocked by thiol-alkylating reagents, as described at least for herpesviruses (Mirazimi et al., 1999), retroviruses (Ryser et al., 1994), alphaviruses (Abell & Brown, 1993), paramyxoviruses (Jain et al., 2007), hepatitis B virus (Abou-Jaoude & Sureau, 2007) and coronaviruses (Gallagher, 1996). The active site of the PDI is formed of a conserved Cys-X-X-Cys motif (Freedman et al., 1994). Recent studies have suggested that Gc of hantaviruses is a class II fusion protein (Garry & Garry, 2004; Hepojoki et al., 2010a; Tischler et al., 2005), and it contains a conserved Cys-X-X-Cys that partially overlaps the proposed fusion protein loop. In the case of retroviruses, inactivation of the virion can also be achieved by covalent modification of intraviral thiol groups, which form a conserved zinc finger (ZF) domain required for virus infectivity. Importantly, this mode of inactivation preserves virion antigenicity (Chertova et al., 2003; Lifson et al., 2004; Morcock et al., 2005; Rossio et al., 1998). The possible role of PDI activity and the potential importance of thiol groups for hantavirus infectivity are so far unresolved.
Hantaviruses contain many conserved cysteine residues (Plyusnin et al., 1996), the majority of which reside in the virion external domains (20 in both Gn and Gc). The intraviral domain of Gn contains seven and the Gc membrane protrusion tail one preserved cysteine (Hepojoki et al., 2010a, b; Huiskonen et al., 2010). Recently, it was shown that the intraviral domain of Gn, with the conserved cysteine residues, forms a tandem ZF domain of an unknown function (Estrada et al., 2009). Three to four conserved cysteines are found dispersed over the hantavirus N protein, and 22 in the L protein. Our aim in this study was to elucidate the overall importance of thiol groups in virion proteins for infectivity of hantaviruses. We blocked the cysteine residues with thiol-alkylating reagents, and showed that thiols are essential for the infectivity of hantaviruses. Following this, we showed that thiol alkylation retained the integrity of hantavirus particles, as well as the conformational epitopes of the envelope glycoproteins.

**RESULTS**

**Hantaviruses are effectively inactivated by thiol-blocking reagents**

We used the thiol-reacting N-ethylmaleimide (NEM) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) to study the role of thiols in the infectivity of hantaviruses. NEM is a membrane-permeable compound that is known to block a post-entry step in human immunodeficiency virus type 1 (HIV-1) infection. In contrast, DTNB is a membrane-impermeable compound commonly used to block both viral and cellular PDI activities. We analysed the effect of thiol-reacting chemicals on TULV and PUUV, the respective models for low-virulent and pathogenic hantaviruses used in our research. Virus-containing conditioned Vero E6 cell-culture supernatants, commonly used as virus inocula, were reacted with the chemicals for 1 h, and the residual reagent activity of NEM was quenched with reduced glutathione (GSH). The DTNB-mediated alkylation of cysteines is reduction-labile and accordingly was not quenched. Both quantitative and qualitative differences in susceptibility to the thiol-blocking agents were observed (Fig. 1; Table 1). The titres, monitored as f.f.u., showed a potent inactivation of TULV (Fig. 1a) and PUUV (Fig. 1b) by NEM and to lesser extent by DTNB. The IC_{50} value of NEM (~0.03 pmol f.f.u.^{-1}) for both viruses was two orders of magnitude lower compared with that of DTNB (Table 1). The NEM-mediated inactivation obeyed an exponential inhibition curve, resulting in the need for relatively high concentrations of reagent to reach complete virus inactivation (Fig. 1). In these experiments, we used virus-containing cell-culture supernatants containing large amounts of non-viral proteins, which probably reacted with the applied thiol-reacting reagents. To verify this assumption, we purified the TULV from the cell-culture supernatant by pelleting the virus through a sucrose cushion. To avoid working with high concentrations of live pathogenic virus, these experiments were not performed with PUUV. The reactivity of thiol-blocking compounds compared with the cell-culture supernatant TULV (Fig. 1a, c) showed a considerable decrease (in the order of 10^6) in the IC_{50} of NEM after virus purification (0.014 amol f.f.u.^{-1}). This result supported the hypothesis that proteins present in the cell-culture supernatant hamper the reactivity of virions towards thiol-blocking chemicals. The reagent-quenching effect of the conditioned cell-culture medium was found to be even greater for DTNB (11-fold) compared with NEM, indicating a relatively high reactivity of non-viral proteins with this chemical (Table 1).

To determine that the effect of NEM was indeed due to its thiol reactivity, we applied pre-quenched NEM (1 mM NEM quenched with GSH) to virus preparations for 1 h (Fig. 1d). The pre-quenched NEM (or plain GSH) had no effect on the infectivity of TULV and PUUV supernatants. In contrast, the infectivity of purified TULV was reduced to approximately 1%; however, a similar inactivation was seen with purified TULV treated with plain GSH for the same time period. This demonstrated that the cell-culture supernatant contained substances, most likely proteins, that protected the virions from the inactivating effects of GSH. Nevertheless, based on these results, the inactivating effect of NEM seen in Fig. 1(a–c) was due to its ability to react with free thiols. In conclusion, both of the tested chemicals significantly inhibited TULV and PUUV infectivity, but NEM was clearly the more virucidal. A 99.99% inactivation of hantavirus was reached at a concentration of NEM that gave only around 50% inhibition of infection with DTNB (Fig. 1).

**The virucidal effect of NEM is not a general phenomenon of viruses of the family Bunyaviridae**

The observation that thiol-blocking reagents, especially NEM, are very potent inactivators of hantaviruses was obtained by measuring virus titres, as described previously (Kallo et al., 2006). We wanted to study the effects of these chemicals in a conventional infection assay wherein the chemically treated virus was adsorbed to cell monolayers and protein expression was monitored after infection. Furthermore, this assay format enabled us to compare the effects of DTNB and NEM on the infectivity of Uukuniemi virus (UUKV; genus Phlebovirus, family Bunyaviridae), which lacks both the conserved Cys-X-X-Cys motif of the PDI found in the fusion loop of hantaviruses (Garry & Garry, 2004) and the ZF domain present in the Gn cytoplasmic tail of hantaviruses (Estrada et al., 2009). TULV and UUKV in cell-culture supernatant were treated with thiol-reactive chemicals as above (Fig. 1), and infection of Vero E6 cell monolayers was monitored at 4 days post-infection (p.i.) by Western blotting of the viral N proteins (Fig. 2a, left panels). The levels of N protein expression in DTNB-treated virus inocula compared with
non-treated virus inocula remained unaltered for UUKV and TULV. In contrast, N protein levels were reduced in infections with NEM-treated virus, showing that NEM inactivates both hantaviruses and phleboviruses. However, NEM totally abolished the replication of TULV at concentrations that had a negligible effect on the replication of UUKV (Fig. 2a, left panels). To gain a better insight into the extent of NEM inactivation, we infected the cells with 1:10 and 1:100 dilutions of the original m.o.i. and analysed the virus replication as above (Fig. 2a, right panels). By comparing the effects of NEM and virus dilutions on N expression, this assay indicated that

![Fig. 1. Hantaviruses are efficiently inactivated by thiol-reacting chemicals. (a–c) The effects of thiol-blocking compounds on hantavirus infectivity were analysed for TULV (a) and PUUV (b) in Vero E6 cell-culture supernatants and for TULV (c) after purification through a sucrose cushion. Viruses were exposed for 1 h at room temperature to increasing amounts of NEM or DTNB and, in the case of NEM, residual reagent activity was quenched with 5 mM GSH for 10 min at room temperature. Virus titres of treated and untreated samples were determined as focus-forming units (f.f.u.). The percentage of infectious virus after treatment with thiol-blocking reagents is plotted against specific concentration of the reagent as pmol f.f.u.\(^{-1}\) (a, b) or as amol f.f.u.\(^{-1}\) (c). The x-axis at the top indicates molar reagent concentrations used. (d) Virus preparations were treated with pre-quenched NEM (1 mM NEM plus 5 mM GSH) for 1 h at room temperature. In parallel, viruses were exposed to 5 mM GSH alone. Error bars for virus titre measurements represent the SD from the results of four individual wells. The titres of untreated virus preparations were \(\approx 10^5\) f.f.u. ml\(^{-1}\) for TULV and PUUV in supernatants (Sup.) and \(\approx 2.5 \times 10^{11}\) f.f.u. ml\(^{-1}\) for purified (Pur.) TULV.

Table 1. Specific IC\(_{50}\) values of thiol-blocking reagents of TULV and PUUV in an f.f.u. assay

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supernatant TULV</th>
<th>Supernatant PUUV</th>
<th>Purified TULV</th>
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<tr>
<td></td>
<td>pmol f.f.u.(^{-1})</td>
<td>(\mu)M</td>
<td>pmol f.f.u.(^{-1})</td>
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<tr>
<td>DTNB</td>
<td>9.69</td>
<td>969.2</td>
<td>0.72</td>
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<tr>
<td>NEM</td>
<td>0.03</td>
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treatment of TULV with 0.1 mM NEM reduced its infectivity to less than 1% (N protein was still visible after a 1:100 dilution of TULV) of the original infectivity. This was expected based on the titration experiment (Fig. 1). In contrast to TULV, by comparing the effects of NEM and virus dilutions on the N protein expression of UUKV, infectivity was reduced to approximately 10% with 1 mM NEM (similar amounts of N protein were expressed after treatment with 1 mM NEM compared with a 10% dilution of virus). However, a 0.1 mM concentration had no measurable effect on its infectivity. We also analysed the infectivity of chemically treated UUKV in its more widely studied host cells, baby hamster kidney (BHK-21) cells, as carried out in Vero E6 cells (Fig. 2b). In these cells, no measurable reduction in UUKV infectivity was seen, even with 1 mM NEM, probably reflecting the faster growth kinetics of UUKV in these cells (Pettersson, 1974). Overall, these results indicated that the virucidal effect of NEM is far more pronounced for hantaviruses than phleboviruses and is therefore not a general phenomenon of the family Bunyaviridae. Having established that NEM was overwhelmingly the most potent inactivator of hantaviruses (Figs 1 and 2), we decided to focus on studying the mechanism of NEM inactivation further.

**NEM inactivation of TULV retains the virion structure**

Next, we were interested in knowing whether the NEM-inactivated virus particle retained its native structure by analysing its sedimentation characteristics in a sucrose density gradient. TULV was either left untreated or treated with 500 µM NEM in cell-culture supernatant and subjected to ultracentrifugation in a sucrose density gradient. The fractions collected from the bottom of the gradient were separated by SDS-PAGE, the gels were silver stained and the viral proteins were identified according to their molecular size. We observed that both NEM-treated and control virions sedimented at the same buoyant density, indicating that NEM treatment did not affect the integrity of the virions (Fig. 3). The calculated buoyant density of ~1.19 g cm⁻³ was slightly higher than that reported for members of the family Bunyaviridae in sucrose (1.16–1.18 g cm⁻³) (ICTV Universal Virus Database, version 4, http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdb). We used TULV as our model hantavirus over PUUV in this assay because of our previous experience in gradient ultracentrifugation of TULV (Huiskonen et al., 2010). Gradient ultracentrifugation requires high loading concentrations of virus, which is more readily achieved with TULV because of its favourable growth kinetics (Strandin et al., 2008) and low virulence.

**NEM inactivation of TULV retains its cell-binding capacity**

To elucidate further the effect of NEM on the integrity of hantaviruses, we studied whether NEM-inactivated hantavirus was still capable of attaching to a monolayer of Vero E6 cells. To enhance the sensitivity of detection, virus binding to the host cells was carried out with radiolabelled TULV concentrated by pelleting the virus through a sucrose cushion. We adsorbed Vero E6 monolayers with untreated, NEM-treated (10 mM) and heat-inactivated TULV as our model hantavirus over PUUV in this assay. As assay controls, we performed virus adsorption in the presence of heparin and in the absence of cell monolayer. After incubation, the cells were lysed and ³⁵S-labelled viral proteins were separated by SDS-PAGE. The virus adsorption assay showed (by the presence of all structural proteins) that NEM-inactivated TULV bound to cells at levels comparable to those of untreated virus (Fig. 4). The fact that NEM-inactivated virions were able to bind host cells suggested that the integrity of the receptor-binding glycoproteins was preserved after NEM treatment. The observed blocking effect of heparin on cell binding of
TULV may reflect competition of ionic interactions upon cell attachment. Heparin can concentrate viruses on the cell surface and function as an attachment factor (Marsh & Helenius, 2006). However, as heparin has been reported not to prevent Prospect Hill or New York-1 hantavirus infections (Gavrilovskaya et al., 1998), further evidence is required for a putative role for heparin in hantavirus attachment.

NEM inactivation of PUUV retains the structure and function of the viral envelope glycoproteins

The results with TULV indicated that NEM treatment preserved the buoyant density and cell-binding capacity of the virus, suggesting unaltered glycoprotein integrity. To analyse further the effect of NEM on hantavirus glycoproteins, we performed immunoprecipitations with conformation-dependent neutralizing mAbs directed against Gn (5A2) and Gc (4G2) of PUUV (Lundkvist & Niklasson, 1992), which were shown previously to precipitate a complex formed from Gn and Gc along with the viral ribonucleoprotein (Hepojoki et al., 2010a, b). These antibodies are strictly specific for PUUV envelope proteins and therefore TULV was not used in this assay. Radioactively labelled PUUV pelleted through a sucrose cushion was inactivated at a high NEM concentration (full inactivation with 4 mM NEM was verified in an f.f.u. assay) and lysed for immunoprecipitation. As shown in Fig. 5, NEM inactivation of PUUV did not decrease but rather increased the amount of immunoprecipitated glycoproteins. This supported the preservation of conformational epitopes of both glycoproteins. It is possible that the observed increased amount of viral glycoproteins by NEM treatment was due to improved protein migration during
SDS-PAGE by prevention of spontaneous disulfide bridge formation, as described previously for NEM-treated TULV (Hepojoki et al., 2010a). Furthermore, the interaction between Gn and Gc was sustained in the NEM-inactivated virus as shown by co-immunoprecipitation of Gn with Gc and vice versa (Fig. 5), thus further indicating sustained integrity of the glycoproteins. Both antibodies also co-immunoprecipitated the N protein, showing that the previously described interaction between the intraviral domains of the glycoproteins and the ribonucleoprotein was also retained (Hepojoki et al., 2010b; Wang et al., 2010).

**All structural proteins of hantavirus harbour free thiols**

To determine which of the viral proteins harbour the most susceptible thiols for alkylation by the maleimide group (the reactive group in NEM), we made use of a biotin-conjugated maleimide (B-mal) chemical mechanistically analogous to NEM. This reagent enabled us to detect the viral proteins that reacted with maleimide using the infrared dye IRDye800-conjugated streptavidin. To begin with, we observed that B-mal was dramatically (10–20-fold) less efficient than NEM in inactivation of TULV (IC_{50} for B-mal ~0.5 pmol f.f.u.^{-1} vs NEM ~0.03 pmol f.f.u.^{-1}) when applied to cell-culture supernatant virus (Fig. 6a). This result implied that the thiols critical for virus infectivity were inaccessible to the biotin-conjugated reagent. To analyse the reactivity of B-mal further, we applied the treatment to wild-type or NEM-inactivated TULV that had been purified through a sucrose gradient as in Fig. 3. Furthermore, to gain insight into the membrane permeability of B-mal, the viruses were either lysed or kept intact. The B-mal-treated samples were separated by SDS-PAGE, blotted onto membrane and probed first with IRDye800-conjugated streptavidin and then stripped and reprobed sequentially with anti-N, anti-Gc and anti-Gn antibodies (Fig. 6b). As shown in Fig. 6(b, left panel), B-mal reacted with all structural proteins of the intact and non-inactivated TULV. This indicated that the Gn, Gc and N proteins all had free thiols that were accessible for thiol alkylation. As expected, the preceding NEM treatment prevented B-mal alkylation of the virus. B-mal was to a certain degree able to penetrate the viral membrane, as judged by its ability to react with the N protein. However, membrane lysis exposed free thiols in N and Gg for reaction with B-mal. These results showed that all viral structural proteins were reactive towards NEM, but no direct indication was obtained of those thiols or proteins that are responsible for the loss of hantavirus infectivity in response to this chemical.

**DISCUSSION**

In this study, we elucidated the importance of thiol groups to the infectivity of hantaviruses. We used thiol-blocking reagents (membrane-permeable NEM and membrane-impermeable DTNB) to evaluate the importance of cysteine residues to the infectivity of hantaviruses. Initially, we observed that, even though both of these reagents affected the infectivity, NEM was clearly the more potent inactivator of hantaviruses. We showed that NEM treatment of hantaviruses not only retained virion integrity, as shown by unaltered virion mobility in density gradient ultracentrifugation, but also the conformation and structure of the surface glycoproteins, as judged by the sustained ability to bind to cells and recognition by neutralizing antibodies.

Viruses exploit thiols in their entry into host cells in at least two well-described strategies: to facilitate thiol–disulfide exchange required for conformational change of viral glycoproteins in virus–host cell membrane fusion as described for a variety of viruses (Sanders, 2000) and by aiding in the folding of a ZF domain required to initiate transcription as a post-entry step of retroviruses (Guo et al., 2000; Johnson et al., 2000; Rein et al., 1998). Whilst the results presented in this paper clearly show the indispensable nature of free thiols in hantavirus infectivity, they do not distinctly argue for any specific mechanism of free thiol usage in virus entry. The ability of membrane-impermeable DTNB to markedly inhibit virus infectivity in the virus titre assay (Fig. 1) suggested that hantaviruses could, at least to some extent, exploit a PDI. Another likely target of thiol-mediated inactivation is located at the conserved ZF fold in the cytoplasmic domain of the hantaviral Gn protein (Estrada et al., 2009). In the case of
retroviruses and arenaviruses, thiol-reacting reagents inhibit virus infectivity by destroying their respective ZF folds (Garcia et al., 2000; McDonnell et al., 1997; Rice et al., 1993, 1995; Tummino et al., 1997). The nucleocapsid protein of HIV-1 (Ncp7), like hantavirus Gn, contains tandem Cys-Cys-His-Cys-type ZFs, which play a crucial role in post-entry reverse transcription of the viral RNA to dsDNA (Guo et al., 2000; Johnson et al., 2000). In the case of arenaviruses, thiol-reacting reagents act specifically towards a viral RING-finger domain (Garcia et al., 2006), another type of ZF closely related to that found in Gn of hantaviruses. It is thus intriguing to hypothesize that the ZF fold in the intraviral domain of Gn in hantaviruses may be essential for infectivity either by being involved in viral fusion or by facilitating post-fusion transport of the viral genome to the site of replication. Among members of the family Bunyaviridae, phleboviruses are the only members that do not contain a predictable ZF domain in their genome-encoded proteins or the Cys-X-X-Cys motif in the predicted fusion peptide. The fact that NEM was unable to block the infectivity of UUKV (Fig. 2b) to the same extent as that seen for TULV is in accordance with the hypothesis that either one or both of the mechanisms of free thiol usage in virus entry presented here are exploited by hantaviruses.

The observed differences in the efficiency of alkylation between NEM, B-mal and DTNB can be explained by differences in their reaction rates and pH dependence, as well as by accessibility and selectivity of the reactive cysteines to the compounds. The membrane-permeable NEM was superior in inhibition of hantaviruses, and treatment at a concentration that totally abolished virus infectivity retained discontinuous glycoprotein neutralizing epitopes, virus sedimentation velocity and cell-binding activity. Therefore, the mechanism of hantavirus inactivation by NEM probably lies in a post-attachment step of virus entry. We found using B-mal that all structural proteins of hantavirus are reactive towards maleimides. In the L protein, no B-mal alkylation was observed, which could of course be due to limiting amounts of the protein in the virion. All in all, with B-mal, we were unable to determine unambiguously the protein that carries free thiols that are critical to infectivity.

For retroviruses, it is well established that thiol-reacting compounds do not alter the antigenic properties of the virus (Arthur et al., 1998; Chertova et al., 2003; Lifson et al., 2004; Morcock et al., 2005; Rossio et al., 1998), and many types of thiol-reacting compounds have been studied in the case of these RNA viruses for their potential in vaccine and antiviral drug development (De Clercq, 2002). These chemicals include azoic compounds, hydrazide derivatives and disulfide-based reagents, but the best-described compound for experimental and vaccine research purposes of HIV-1 in both animals and humans is 2,2'-dipyridyl disulfide [also known as aldrithiol-2 (AT-2)] (Lifson et al., 2004; Lu et al., 2004). AT-2, like NEM, is able to eject Zn(II) ions from the retroviral ZF domain. Interestingly, AT-2 was recently shown to prevent the replication of human respiratory syncytial virus (RSV; family Pneumoviridae). AT-2 is a known ZF-reactive compound and its target in RSV was shown, by protein cross-linking, to be the ZF protein M2-1, which functions...
as the polymerase processivity factor of the virus (Boukhvalova et al., 2010). Whatever the mechanism of inactivation in the case of hantaviruses, one could envisage that compounds effective towards retroviruses could also be used to inactivate hantaviruses for the preparation of vaccines against HFRS and HCPS. The so-called ZF-reactive compounds could also be effective drugs in the control of HFRS and HCPS infections. However, one of the major obstacles in the use of these thiol-reactive compounds in vivo is the potential inactivation of cellular proteins harbouring active cysteines (Potter et al., 2004).

METHODS

Hantavirus cultivation. TULV Moravia strain 5302 and PUUV Sotkamo strain were propagated in Vero E6 cells from which they were originally isolated, producing titres of $10^3$–$10^8$ f.f.u. ml$^{-1}$ in conditioned medium (Schmaljohn et al., 1985; Strandin et al., 2008; Vapalahti et al., 1996). Vero E6 cells (African green monkey kidney epithelial cell line; ATCC CRL-1586), grown at 37°C, were used to inactivate hantaviruses for the preparation of virus preparations were incubated with NEM, B-mal or DTNB for 1 h at room temperature. In the case of NEM, the residual reactivity was quenched with 5 mM GSH for 10 min at room temperature. Pre-quenched NEM, used as a control, was prepared by incubating 1 molar equivalent of NEM with 5 molar equivalents of GSH for 10 min at room temperature prior to incubation with virus preparations for 1 h at room temperature. Quenching of the chemicals was not applied if the thiol-reacting chemically treated virus preparation was subjected to purification through a sucrose gradient by ultracentrifugation.

Measurement of virus titre. Virus titres were determined as described previously (Kalio et al., 2006). Briefly, serially tenfold-diluted virus stocks were grown in Vero E6 cells on a ten-well microtitre plate, fluorescently stained at 3 days p.i. with antiserum to N protein and the foci enumerated. So was calculated from the results of four individual wells. The inhibition curves in f.f.u. ml$^{-1}$ versus thiol-blocking agent concentration (M) are also given for each thiol-reacting reagent in specific inhibitory concentrations (mol f.f.u.$^{-1}$). The curves were used to obtain the IC$_{50}$ values for the agent.

Analysis of virus infectivity on cell monolayers. To analyse the inhibition of virus infectivity on a cell–culture monolayer, TULV or UUKV supernatants treated with NEM or DTNB were used to infect semi-confluent Vero E6 cells in 25 cm$^2$ flasks at an m.o.i. of 0.1 for TULV or 1 for UUKV. In addition, semi-confluent BHK-21 cells were used as host cells for UUKV (m.o.i. of 0.001). Cells were collected at 4 days p.i. in RIPA buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40 and Protease Inhibitor Cocktail (Roche)] in which they were lysed for 15 min at room temperature. Laemmli sample buffer was added, the samples boiled and 20% of the total protein was separated by (10% acrylamide) SDS-PAGE followed by transfer to nitrocellulose for detection of the propagated virus by Western blotting using an N-specific antibody. Detection of actin was used as the Western blot protein loading control, and visualization was carried out using an Odyssey Infrared Detection System (LI-COR). The bands obtained were quantified by the Odyssey system, with N protein levels normalized with respect to actin protein levels and reported as percentages relative to infection with untreated virus.

Phlebovirus cultivation. UUKV was propagated in BHK-21 cells grown in MEM supplemented with 2% heat-inactivated FCS, 2 mM glutamine, 100 IU penicillin ml$^{-1}$ and 100 g streptomycin ml$^{-1}$. Cell cultures in 75 cm$^2$ flasks were inoculated for 1 h at 37°C and conditioned medium was collected 7–10 days after infection with TULV and 12–21 days after infection with PUUV. Conditioned medium containing virus (stored in aliquots at −70°C) was used as inocula. For radio labelling of viral proteins, infected Vero E6 cell cultures were starved for 1 h at 37°C with medium depleted of methionine and cysteine, and propagated with a 1 mCi mixture of $^{35}$S)methionine (Wallac PerkinElmer) for 3 days at 37°C. For purification and concentration of viruses, cell–culture medium passed through a 0.22 μm filter (Millipore) was concentrated by pelleting through a 30% (w/v) sucrose cushion (27 000 r.p.m., 2 h, 4°C, in a Beckman SW28 rotor) and suspended in HBS buffer [10 mM HEPES (pH 7.4), 150 mM NaCl].

Sedimentation analysis of virus. Semi-confluent Vero E6 cell monolayers were infected with TULV, and at 3 days p.i. the conventional medium was changed to growth medium supplemented with 2.5% FCS (pre-filtered through a 100 kDa cut-off filter; Millipore). At 5 days p.i., the conditioned medium from six 75 cm$^2$ cell–culture flasks was collected, pooled and concentrated approximately 100-fold. Half of the concentrated virus suspension was treated with NEM (500 μM) and the other half was kept untreated. The NEM-treated and control samples were sedimented (25 000 r.p.m., 16 h, +4°C, in a Beckman SW28 rotor) in a 0–70% sucrose gradient [in 10 mM HEPES (pH 7.4), 100 mM NaCl] and the gradient was collected from the bottom in ~600 μl fractions. The protein composition of fractions was determined by non-reducing SDS-PAGE (10% acrylamide) with silver staining (PageSilver silver staining kit; Fermentas). The sucrose concentration of each fraction was determined at 22°C using a manual Zeiss Opton Abbe refractometer.

Immunoprecipitation. Immunoprecipitation of radiolabelled and NEM-inactivated PUUV was performed with PUUV-specific mAbs to Gn (5A2) and Gc (4G2) using 10 μg antibody per 5 μl virus preparation lysed with 1% Triton X-100 in HBS buffer. After overnight incubation in an end-over-end shaker at 4°C, pulldown of immunocomplexes was carried out for 2 h with 25 μl protein G–Sepharose beads (Amersham). Samples were washed twice with HBS, suspended in Laemmli gel loading buffer and boiled to elute the Sepharose-bound proteins. Proteins were separated by SDS-PAGE (10% acrylamide) followed by detection of the radioactive samples by autoradiography.
Virus–cell binding assay. To assess virus attachment to host cells, a confluent Vero E6 monolayer was incubated with 1 ml radiolabelled TULV (m.o.i. of 1000) in six-well plates for 30 min at 37°C. NEM-treated, untreated and heat-inactivated (95°C for 15 min) TULV preparations were allowed to attach to the cell monolayers and untreated TULV to culture wells devoid of a cell monolayer. In addition, an untreated virus sample was incubated with 50 μg heparin ml⁻¹ before attachment to the cell monolayer. Cells were washed twice with PBS and lysed in RIPA buffer for 15 min at room temperature. The cell-bound material was separated by SDS-PAGE (7% acrylamide) and the radioactively labelled viral proteins were detected by autoradiography.

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