Analysis of the humoral immune response against the envelope glycoprotein Gc of Schmallenberg virus reveals a domain located at the amino terminus targeted by mAbs with neutralizing activity

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Orthobunyaviruses are enveloped viruses that are arthropod-transmitted and cause disease in humans and livestock. Viral attachment and entry are mediated by the envelope glycoproteins Gn and Gc, and the major glycoprotein, Gc, of certain orthobunyaviruses is targeted by neutralizing antibodies. The domains in which the epitopes of such antibodies are located on the glycoproteins of the animal orthobunyavirus Schmallenberg virus (SBV) have not been identified. Here, we analysed the reactivity of a set of mAbs and antisera against recombinant SBV glycoproteins. The M-segment-encoded proteins Gn and Gc of SBV were expressed as full-length proteins, and Gc was also produced as two truncated forms, which consisted of its amino-terminal third and carboxyl-terminal two-thirds. The sera from convalescent animals reacted only against the full-length Gc and its subdomains and not against the SBV glycoprotein Gn. Interestingly, the amino-terminal domain of SBV-Gc was targeted not only by polyclonal sera but also by the majority of murine mAbs with a neutralizing activity. Furthermore, the newly defined amino-terminal domain of about 230 aa of the SBV Gc protein could be affinity-purified and further characterized. This major neutralizing domain might be relevant for the development of prophylactic, diagnostic and therapeutic approaches for SBV and other orthobunyaviruses.

INTRODUCTION

Schmallenberg virus (SBV) is an arthropod-borne virus of the genus Orthobunyavirus of the family Bunyaviridae that has recently been isolated from infected cattle in Europe (Hoffmann et al., 2012). This genus is subdivided into 18 serogroups (Calisher, 1996; Elliott, 2011); SBV belongs to the Simbu serogroup, which comprises teratogenic viruses that infect ruminants, mainly in Africa, Asia and Australia (Calisher, 1996). The clinical signs of SBV infection in cattle can be increased body temperature, reduced milk yield and diarrhoea, and, like other members of this group, i.e. Akabane virus, the virus also infects the embryo or fetus of ruminants and can cause severe malformation (Bilk et al., 2012; Hoffmann et al., 2012; Wernike et al., 2014).

The orthobunyaviruses are enveloped and possess a tripartite genome composed of three negative-sense ssRNA molecules of different sizes, designated small (S), medium (M) and large (L), respectively. The L segment encodes the viral polymerase, whereas the S fragment encodes the nucleocapsid protein N, and an internal ORF leads to the synthesis of the non-structural protein S (Elliott, 2011). The single ORF of the M segment encodes the surface proteins, which according to their order in the precursor are referred to as Gn and Gc, and, in between, the NSm, whose function remains unknown (Eshita & Bishop, 1984; Fazakerley et al., 1988). Once the polyprotein of the Bunyamwera virus (BUNV) is proteolytically processed, the Golgi localization signal of Gc targets the Gc hetero-multimers to this organelle (Bupp et al., 1996; Shi et al., 2004), where virion assembly occurs (Salanueva et al., 2003, reviewed by Elliott, 2011).
Studies carried out with La Crosse virus (LAC) of the California serogroup shed light on the role of Gc in viral entry. Thus, the LAC Gc protein mediates virus attachment to the cell membrane (Pekosz et al., 1995) and is the exclusive target of anti-LAC neutralizing antibodies (Gonzalez-Scarano et al., 1982; Grady et al., 1983). Besides that, the LAC Gc protein is involved in virus–cell or cell–cell fusion (Gonzalez-Scarano et al., 1985; Plassmeyer et al., 2005, 2007). The humoral immune response against the glycoproteins of SBV has not been analysed yet.

Therefore, the present study was aimed at identifying the domains of the glycoproteins targeted by antibodies from naturally and experimentally infected animals as well as mAbs.

RESULTS
The humoral immune response against the glycoproteins of the recently described orthobunyavirus SBV has not, we believe, been studied before. Here, the SBV glycoproteins were expressed as full-length or truncated proteins or protein domains, and their reactivity with sera from infected animals as well as mAbs was analysed.

Generation and characterization of the SBV glycoproteins
The expression and purification of viral glycoproteins from transiently transfected human embryo kidney (HEK) cells allows the recovery of significant protein amounts through affinity purification.

Different DNA constructs were generated to express the SBV glycoproteins or subdomains under the control of a strong eukaryotic promoter, with a tag at the carboxyl terminus to control the expression and for downstream affinity purification (Fig. 1a).

Gc was expressed as full-length protein in the constructs Gc-WT, Gc-L-Gc and Gc, respectively. In all these plasmids, the affinity tag substituted the cytoplasmic tail and the transmembrane domain of Gc (Fig. 1a).

In the construct Gc-WT, the polypeptide is expressed without the cytoplasmic tail and transmembrane domain of Gc (Fig. 1a). In the plasmid Gc-L-Gc, the authentic signal sequence was substituted by that of the IgG kappa light chain, and a serine–glycine-rich linker was included at the position of the putative transmembrane domain and cytoplasmic tail of the Gn as well as the NSm protein. Furthermore, the putative ectodomain of Gc was expressed downstream of a signal sequence in the plasmid ‘Gc’ (Fig. 1a). The ectodomain of the Gc was expressed in the plasmid ‘Gn’ using a similar strategy as for the Gc ectodomain (Fig. 1a).

The Gc amino-terminal third, plasmid ‘Gc Amino’, and the Gc without this domain, plasmid ‘Gc AminoΔ’, were expressed independently (Fig. 1a). Each domain is preceded by the signal sequence of the IgG kappa light chain. All generated constructs (Figs 1a and 2c) could be expressed successfully, but only the proteins Gc-L-Gc, Gc Amino and Gc AminoΔ were affinity-purified in amounts allowing further characterization by indirect ELISA. The other proteins were difficult to solubilize from the cell lysates (data not shown), and immunofluorescence staining with bovine SBV antiserum was not possible owing to a strong background reaction with the mammalian cell culture (data not shown).

The proteins Gc-L-Gc and Gc AminoΔ were not secreted, and were isolated from the cell lysates after transfection of the expression plasmids. When purified Gc-L-Gc was analysed without reduction of disulfide bonds, besides the band corresponding to the monomer, two bands of a higher molecular mass were observed (Fig. 1b) that were not detectable under reducing conditions. Western blotting (Fig. 1c) with an anti-tag antibody confirmed the identity of the proteins and also revealed the presence of a product of lower molecular mass.

Gc AminoΔ migrated almost exclusively as a disulfide-linked oligomer that was completely reduced to the molecular mass of the monomer (Fig. 1b) in the presence of a reducing reagent. A protein of similar size was also present in the preparation, which was not related to Gc AminoΔ as it was not recognized by the anti-tag antibody (Fig. 1c).

Gc Amino could be isolated from the supernatant, and migrated as a double band, also after disulfide bond reduction (Fig. 1b, c) or deglycosylation (data not shown).

Reactivity of sera from naturally SBV-infected and non-infected cattle with SBV glycoproteins
The reaction of the sera was tested in an indirect ELISA using the purified proteins Gc-L-Gc, Gc Amino and Gc AminoΔ as antigens. A positive reaction was observed with the three antigens in the indirect ELISA (Fig. 2a), which was more obvious with the antigens Gc-L-Gc and Gc Amino. In the case of the Gc AminoΔ, two of the positive sera had values close to the reactivity of the negative control sera.

Humoral immune response against the SBV glycoproteins in experimentally infected mice
The IFN-α/β receptor knockout mouse is susceptible to SBV infection, and is thus considered an alternative in vivo model of SBV infection (Wernike et al., 2012). Nevertheless, the serological reactivity of these mice against SBV Gc has not to our knowledge been assessed before. Therefore, we also decided to analyse the humoral immune response against SBV Gc in the course of an SBV infection in this model. The sera of SBV-infected mice were analysed in an indirect ELISA, and the
Fig. 1. Schematic representation and analysis of the different SBV glycoprotein constructs (not to scale). (a) A construct was generated in which the Gc putative transmembrane domain (TMD) and the cytoplasmic tail (CT) were substituted by a twin Strep-tag (Strep-tag) (Gn-Gc-WT). The Gn-L-Gc construct expresses the ectodomains of the Gn and Gc proteins flanked by the signal sequence of the IgG kappa light chain (Ig-κ-SS) and a Strep-tag, with a glycine-rich linker (SG-linker) placed at the position of the Gn transmembrane domain (Gn-TMD) and cytoplasmic tail (Gn-CT) as well as the NSm protein. The putative Gn and Gc ectodomains were expressed individually with an Ig-κ-SS and a Strep-tag. In the Gc Amino construct, the first 234 residues (Gc 234 aa) are followed by the enterokinase cleavage site (EK site). Gc AminoΔ encodes the carboxy-terminal 628 residues of the mature Gc. The position of each residue is given based on the deposited sequence for the SBV M fragment (NCBI GenBank accession number HE649913) (Hoffmann et al., 2012). All proteins were expressed in HEK-293T cells and the Gn-L-Gc, Gc Amino and Gc AminoΔ proteins were affinity-purified. The estimated molecular mass of each protein including the affinity tag is 123.8, 30.3 and 72.8 kDa for Gn-L-Gc, Gc Amino and Gc AminoΔ, respectively. (b) Two micrograms of Gn-L-Gc and GcAmino and 0.5 µg Gc AminoΔ were analysed by SDS-PAGE before and after reduction with DTT, as indicated, followed by staining, and tested with an anti-Strep-tag specific mAb by Western blotting (c). The asterisks indicate the bands corresponding to either a Gc AminoΔ multimer (**) or the monomer (*). The protein mass markers are shown on the left.
serological pattern resembled that of the bovine sera (Fig. 2b). However, there were no evident differences in the reaction with the two subdomains. The murine sera were also analysed by indirect immunofluorescence staining. The sera from all infected mice reacted against the three full-length variants of the Gc as well as its two truncated forms (Fig. 2c). The reactivity with Gc Amino was weaker in this test, which may be related to the fast transport of the protein through the secretory pathway. On the other hand, the fixation might also influence the reactivity with the serum antibodies, contrary to the ELISA test.

Interestingly, the expressed Gn protein was not recognized by any of the sera in the different assay systems.

Determination of the target of anti-SBV murine mAbs with neutralizing activity

A panel of mAbs against SBV virions has been recently characterized (Wernike et al., 2015). The reaction pattern of the anti-Gc specific mAbs was tested with all different Gc-derived constructs by using indirect immunofluorescence (Fig. 3a; Table 1). The majority of the mAbs, namely eight of ten, reacted with the full-length Gc and with Gc Amino, displaying a reactivity pattern similar to mAb 2G10. mAb 5F8 was the only antibody recognizing an epitope located in Gc AminoΔ. On the other hand, mAb 2H11 reacted with the full-length Gc only. These results were confirmed when the mAbs were tested with
Fig. 3. Reactivity of mAbs against Gc and the Gc domains. (a) The reactivity of hybridoma supernatants from SBV Gc-specific mAbs (Wernike et al., 2015) with all Gc-encoding constructs was tested in an indirect immunofluorescence assay. Eight of the mAbs, with the exception of mAb 2H11 and mAb 5F8, showed a reaction pattern similar to that of mAb 2G10. The expression of each construct was controlled with an anti-Strep-tag mAb. Bars, 100 μm. Pictures of mAbs 2G10, 2H11 and 5F8 were taken with a ×20/0.45 objective and pictures of the anti-Strep-tag mAb were taken with a ×10/0.30 objective. (b) The reaction pattern observed in the indirect immunofluorescence assay was also confirmed in an indirect ELISA. The proteins were coated onto the ELISA plate and tested in duplicate with the respective mAb. The bars represent the mean OD$_{450}$ values. The anti-Strep-tag mAb (Strep) and a non-related mAb (Control) were used as positive and negative control, respectively. The experiment was performed once. The neutralizing activity of each mAb is indicated (Neut.) (Wernike et al., 2015). NA, not applicable. (c) The reactivity of each mAb was analysed with Gc Amino in its native and reduced forms as well as with an unrelated Strep-tagged protein in an indirect ELISA. The proteins were immobilized onto Strep-Tactin-coated plates, and tested in triplicate with the corresponding mAb. The bars represent the mean, and the error bars indicate SD. The experiment was performed once. Gc Amino, Native Gc Amino; Gc Amino red, Gc Amino after treatment with a reducing agent; (−) Control, unrelated Strep-tagged protein.
recombinant proteins in an indirect ELISA (Fig. 3b). All mAbs displayed a stronger reaction with the full-length Gc than with the subdomains, which was less evident for mAb 5F8. This mAb gave the strongest signal in the test.

Interestingly, with the exception of mAb 5F8, all seven mAbs that were able to neutralize SBV in vitro recognized their epitopes in the Gc domain ‘Gc Amino’ (Fig. 3b). Therefore, the role of disulfide bonds in the formation of the epitopes recognized by the Gc Amino-specific mAbs was also assessed with the Gc Amino protein after treatment with reducing agents (Fig. 3c). The signal of all Gc-specific antibodies was strongly affected by disruption of these disulfide bonds.

**DISCUSSION**

The envelope glycoproteins of orthobunyaviruses are targeted by the humoral immune response and Gc is recognized by neutralizing mAbs (Gonzalez-Scarano et al., 1982; Kingsford & Hill, 1983; Kingsford et al., 1983). However, the humoral immunity against the glycoproteins or their subdomains of the orthobunyavirus SBV has not been assessed. Such studies aiming to identify the viral domains responsible for inducing an immune response would be valuable, for example, for vaccine development or the use of diagnostics.

In the present study, SBV antigens were expressed in eukaryotic cells that were functional in order to characterize the humoral immune response against the Gc protein in two animal species.

The positive SBV antisera reacted against the Gc protein either as full-length protein or when truncated in two subdomains, indicating the presence of epitopes resembling the ones present on the viral particles in a shorter subdomain. Even though the Gc expressed without its amino-terminal third only folded as a disulfide-linked oligomer, epitopes targeted by the polyclonal sera were still present and could bind the specific antibodies.

The presence of intermolecular disulfide bonds in the orthobunyaviral glycoproteins has not been observed (Bowden et al., 2013). One explanation might be the absence of Gn in these constructs, as it plays a role by translocating Gc to the Golgi complex and assists the correct folding of Gc (Shi et al., 2004, 2009). However, the Gc protein expressed without Gn (Fig. 1a, Table 1) was still reactive with all mAbs that recognized conformational epitopes. Hence, the SBV Gc protein seems to mature to a certain extent without Gn. Alternatively, the amino terminus of SBV Gc could support a folding in which the fusion peptide is masked, and in its absence the protein might undergo conformational changes that lead to aggregation, as was hypothesized in a study where N-terminal residues of the BUNV Gc were removed (Shi et al., 2009).

Interestingly, no positive reaction against Gn was observed with the different antisera. Hence the SBV Gc protein is most likely the major antigen for neutralizing activity. In mice actively infected with orthobunyaviruses of the California serogroup, the humoral immune response also targeted the Gc protein (Gonzalez-Scarano et al., 1982).

The low immunogenicity of SBV Gn might be reflected by the lack of hybridomas secreting anti-Gn antibodies, since the donor mouse was immunized with viral particles (Wernike et al., 2015).

The ultrastructure of BUNV has been published recently (Bowden et al., 2013), and the glycoproteins were shown to build a tripodal spike with Gn at its base. It is very likely that the SBV particle resembles that of BUNV and that, similarly to the BUNV Gc, the SBV Gc is well exposed at the tip of the spikes, whereas Gn remains less accessible to the immune system. Altogether, these results indicate that the SBV virion-associated Gc protein, but not the Gn protein, is strongly immunogenic, generating an immune response against both tested subdomains.

In this study, differently to previous reports, we purified the Gc amino-terminal domain of an orthobunyavirus via affinity chromatography, and analysed its reactivity with polyclonal sera and mAbs.

Mutants of an orthobunyavirus that lacked the domain analogue to SBV Gc Amino still replicated in cell culture (Pollitt et al., 2006), and further analysis of the BUNV Gc function (Shi et al., 2009) corroborated these results. Besides that, recombinant BUNVs have been generated that lacked almost half of the Gc domain and tolerated the insertion of fluorescent proteins at this position (Shi et al., 2010). Nevertheless, certain mutations resulted in viruses with an attenuated phenotype and reduced virus-like particle formation (Shi et al., 2009).

A few studies with other bunyaviruses have indicated the presence of a domain at the amino terminus of Gc recognized by polyclonal sera and mAbs. A domain of the Gc of the snowshoe hare bunyavirus located at a position analogous to the SBV Gc Amino was cleaved by trypsin, and reacted with mAbs (Fazakerley et al., 1988).

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**Table 1. Reactivity pattern of the anti-SBV mAbs in the indirect immunofluorescence assay using cells transfected with the respective expression plasmids**

<table>
<thead>
<tr>
<th></th>
<th>Gn-Gc-WT</th>
<th>Gn-L-Gc</th>
<th>Gc</th>
<th>Gc Amino</th>
<th>Gc AminoA</th>
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<tbody>
<tr>
<td>mAb 2G10</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>mAb 4D9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>mAb 2H11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>mAb 4E5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>mAb 5F8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>mAb 1C11</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>mAb 1F4</td>
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<td>+</td>
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<td>–</td>
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<tr>
<td>mAb 4B6</td>
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<tr>
<td>mAb 1C1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>mAb 3A5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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</table>
Biochemical analysis of proteolytically processed virions of LAC showed that neutralizing mAbs bind to an amino-terminal domain of Gc, which is removed by trypsin treatment (Gonzalez-Scarano et al., 1982; Kingsford & Hill, 1983; Kingsford et al., 1983). The virions were still infectious and resistant to neutralization when treated with antibodies that target the virion-associated Gc domain.

Since bunyaviruses lacking this domain do not occur naturally, these observations underscore the plasticity of the Gc protein for viral growth in vitro, and it can be hypothesized that the Gc amino terminus plays a role at the entry step. Nevertheless, further experiments are needed to investigate the function of this domain during entry. If the recently determined structure for BUNV virions (Bowden et al., 2013) holds true for SBV, the Gc Amino domain might be located at the tip of the Gc trimer. The most exposed surfaces of such a structural arrangement potently stimulate B-cells via interaction with receptors, resulting in immunogenic domains (reviewed by Schneider-Ohrum & Ross, 2012). Interestingly, a high frequency of mutations in immunogenic domains (reviewed by Schneider-Ohrum & Ross, 2012). Additionally, these observations underscore the plasticity of the Gc domain.

In order to explain the neutralization of virus infection by mAbs targeting the Gc Amino domain at a mechanistic level, the critical residues for the binding of mAbs with neutralizing activity are being determined by site-directed mutagenesis. Considering similarities within the genus Orthobunyavirus at structural level to occur, our study should support approaches that aim at identifying viral determinants involved in invasion of the host cell. Hence, this information could be helpful for the development of vaccines and therapeutic strategies against orthobunyaviruses.

METHODS

Cell lines. Baby hamster kidney cells [BHK-21, clone BRSS, L194 Collection of Cell lines in Veterinary Medicine (CCLV)] and human embryo kidney cells (HEK-293T, L1018 CCLV) were provided by the cell culture collection of the Friedrich-Loeffler-Institut, Greifswald – Insel Riems, Germany.

Virus. SBV strain BH 80/11-04 was obtained from the virus collection of the Friedrich-Loeffler-Institut, Greifswald – Insel Riems, Germany (Hoffmann et al., 2012).

Sera. A total of ten bovine sera were tested (five positive and five negative sera). Also, one bovine hyperimmune serum and one negative serum were used as controls (Wernike et al., 2013). The status of each serum was verified by virus neutralization test as described below and with a commercial ELISA (IDEXX Schmallenberg Ab; IDEXX). In addition, ten SBV-positive and two SBV-negative sera from type I IFN-receptor-deficient mice experimentally infected with SBV (K. Wernike, unpublished data) were analysed. One of these positive sera served as control and its status was determined with a commercial ELISA (ID Screen Schmallenberg Virus Competition; ID-Vet) following the instructions of the manufacturer.

Plasmid DNA purification. All plasmids were purified with Plasmid Midi or steja kits (Qiagen), following the instructions of the manufacturer.

Murine mAbs. Hybridoma supernatant from 11 anti-SBV mAbs (ten against the glycoprotein and one against the capsid) was used (Wernike et al., 2015).

Indirect immunofluorescence of transfected cells. HEK-293T cells were transfected with the corresponding plasmid in a six-well plate (Corning) as explained below (see Expression and purification of proteins). On the next day the cells were distributed in a 96-well plate (Corning) and 24 h later fixed by heating for 2 h at 80 °C. Diluted supernatant (1 : 25) or mouse serum (1 : 100) of each antibody was tested with transfected and non-transfected cells. The protein expression was controlled with an anti-Strep-tag mAb (StrepMAB-classic; IBA).

After a washing step, a second incubation period with an FITC-labelled anti-species conjugate (anti-mouse immunoglobulins FITC; Dako) was performed. Subsequently, the monolayer was washed repeatedly, embedded with DABCO fluorescence conservation buffer (DABCO Sigma 2.5% w/v; glycerol (Roth) 90%v/v), and analysed under an inverted fluorescence microscope (IX50; Olympus).

ELISA

(i) ELISA with recombinant proteins. Each serum or hybridoma supernatant was evaluated in duplicate with the three antigens and without protein as background control in an indirect ELISA. The ELISA was performed using the ELISA Starter Accessory Kit II (Bethyl Laboratories). Briefly, the ELISA plates (Microlon) were incubated with the respective antigen overnight at 4 °C followed by a blocking step. The sera and hybridoma supernatant were diluted 1 : 20 and 1 : 5, respectively, and the reaction was revealed with the corresponding anti-species HRP conjugate (anti-bovine IgG or anti-mouse IgG; Sigma). The plates were washed and substrate was added, the reaction was stopped and the OD560 was measured. For data analysis of the sera, but not the mAbs, the OD560 of the blank well was subtracted from the one obtained with each antigen. The presence of the proteins was controlled with an anti-Strep-tag mAb (StrepMAB-classic; IBA). The results for the sera were expressed as the percentage of reaction of the sample relative to that of the positive serum [(mean sample OD560/mean positive control OD560) × 100], whereas the mean OD560 values for the mAbs were plotted directly.

<table>
<thead>
<tr>
<th>Table 2. Final plasmids generated for protein expression</th>
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<tbody>
<tr>
<td><strong>Plasmid</strong></td>
</tr>
<tr>
<td>pGRS-2</td>
</tr>
<tr>
<td>pGRS-15</td>
</tr>
<tr>
<td>pGRS-17</td>
</tr>
<tr>
<td>pGRS-11</td>
</tr>
<tr>
<td>pGRS-12.1</td>
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<tr>
<td>pGRS-13</td>
</tr>
</tbody>
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*The schemes are provided in Fig. 1(a).
(ii) Indirect ELISA to assess the reactivity of the mAbs after reduction of the disulfide bonds. The Gc Amino protein was subjected to treatment with 10 mM DTT, 0.2 % Triton X-100 (v/v) in buffer W (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) at 95 °C for 5 min, and the cysteines were alkylated with 10 mg ml⁻¹ iodoacetamide for 15 min at 37 °C. The volume was then adjusted with buffer W-Tx-100 (buffer W, 0.2 % Triton X-100 (v/v)). The non-reduced Gc Amino protein and the unreacted protein [control protein with Strep-Tag II (IBA)] were diluted in buffer W-Tx-100. Fifty nanograms of each protein mixture was applied into a Strep-Tactin-coated microtitre plate (IBA) and the mAbs were incubated with each protein in triplicate for 1 h at 37 °C. The wells were washed three times with PBS–Tween 20 (v/v), 0.05 %. The plate was then incubated with an anti-mouse HRP conjugate (Sigma) for 1 h at 37 °C. The plate was washed as described above and ready-to-use enzyme substrate 3,3',5,5'-tetramethylbenzidine/1 % hydrogen peroxide (IDEXX) was applied to each well. After 10 min at room temperature the reaction was stopped and the OD₄₅₀ was determined. The mean OD₄₅₀ values with the standard deviation were plotted.

**Virus neutralization test.** The test was performed as previously described (Wernike et al., 2013).

**Construction of plasmids.** The residue position is given according to the published sequence of the M protein (NCBI GenBank: HE649913) (Hoffmann et al., 2012). All constructs were controlled by DNA sequencing with the oligonucleotides pEXPR-fwrd and pEXPR-rev (Table 3). A codon-optimized synthetic gene encoding the M protein (333,555'-tetrathymethylbenzidine/1 % hydrogen peroxide (IDEXX)) was applied to each well. After 10 min at room temperature the reaction was stopped and the OD₄₅₀ was determined. The mean OD₄₅₀ values with the standard deviation were plotted.

**Table 3.** Oligonucleotides used to generate the expression constructs

<table>
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<th>Oligo</th>
<th>Sequence</th>
<th>Polarity</th>
<th>Restriction site</th>
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<tr>
<td>pEXPR-fwrd</td>
<td>gagaacccactgtctactgcg</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>pEXPR-rev</td>
<td>tagaaaccaagctggggga</td>
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<tr>
<td>O-GRS-1</td>
<td>aattatctagagccacatctgctgacatcgtg</td>
<td>+</td>
<td>XbaI</td>
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<tr>
<td>O-GRS-3</td>
<td>acttttttgtctgctctagatagctgc</td>
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<td>Half of AfeI restriction site</td>
</tr>
<tr>
<td>O-GRS-6</td>
<td>aattatttgtcgctctgactcaatcagcc</td>
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<td>SalI</td>
</tr>
<tr>
<td>O-GRS-7</td>
<td>gctactacgctgagttggtaggtc</td>
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<td>Half of AfeI restriction site</td>
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<tr>
<td>O-GRS-8</td>
<td>aattatttgtcagctctgaggcagactggac</td>
<td>+</td>
<td>SalI</td>
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<tr>
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<td>pMK-RQ rev</td>
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<td>−</td>
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</table>

Restriction site sequences are shown in bold.
ligated to the vector pEXPR-IBA103, also digested with these enzymes.

**Expression and purification of proteins.** HEK-293T cells were lysed a day before transfection. The monolayer was washed and kept in serum-free medium until transfection. The DNA was mixed with branched polyethylenimine (PEI; Sigma-Aldrich) at a PEI : DNA ratio of 3 : 1 in serum-free medium. After 15 min incubation at room temperature the transfection mixture was added, and the cells were incubated at 37 °C in a 0.5 % CO₂ atmosphere for 3 h before the medium was exchanged for complete medium containing 10 % FCS. Three days after transfection, the cell monolayer was scraped (Gn-L-Gc and Gc AminoA) in lysis buffer [100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1 % Triton X-100], supplemented with cOmplete Protease Inhibitor Cocktail (Roche)]) or the supernatant was collected (Gc Amino). The cell lysate was then clarified and the supernatant was concentrated [Vivatrap system, cassette with membrane of molecular mass cut-off (MWCO) 5000 Da; Sartorius Stedim Biotech]. Then the material was applied onto a column packed with Strep-Tactin Superflow high-capacity slurry (IBA) pre-equilibrated with buffer W (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA). The column was then washed with buffer W and the protein was eluted with buffer E (buffer W plus 2.5 mM desthiobiotin) and fractions were collected. The presence of the respective protein was verified by SDS-PAGE of each fraction (Laemmli, 1970) and staining with Instant Blue (Expedeon). The fractions containing the respective protein were pooled and concentrated (Amicon Ultra, 3000 Da MWCO membrane; Millipore) and the protein concentration was measured by the Bradford method (Quick Start Bradford Dye Reagent; Bio-Rad). The identity of the purified material as Strep-tagged protein was verified by Western blot analysis with an HRP-conjugated anti-Strep-tag mAb (StrepMAB-classic HRPO; IBA) following the instructions of the manufacturer.

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**REFERENCES**


