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Mutations in the Schmallenberg virus Gc Glycoprotein Facilitate Cellular Protein Synthesis Shutoff and Restore Pathogenicity of NSs Deletion Mutants in Mice.

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Abstract

Serial passage of viruses in cell culture has been traditionally used to attenuate virulence and identify determinants of viral pathogenesis. In a previous study, we found that a strain of Schmallenberg virus (SBV) serially passaged in tissue culture (termed SBVp32) unexpectedly displayed increased pathogenicity in suckling mice compared to wild type SBV. In this study, we mapped the determinants of SBVp32 virulence to the viral genome M segment. SBVp32 virulence is associated with the capacity of this virus to reach higher titers in the brains of experimentally infected suckling mice. We also found that the Gc glycoprotein, encoded by the M segment of SBVp32, facilitates host cell protein shutoff *in vitro*. Interestingly, while the M segment of SBVp32 is a virulence factor, we found that the S segment of the same virus confers by itself an attenuated phenotype to wild type SBV as has lost the ability to block the innate immune system of the host. Single mutations present in the Gc glycoprotein of SBVp32 are sufficient to compensate both the attenuated phenotype of the SBVp32 S segment and the attenuated phenotype of NSs deletion mutants. Our data also indicate that the SBVp32 M segment does not act as an IFN antagonist. Therefore SBV mutants can retain pathogenicity even when they are unable to fully control the production of IFN by the infected cells. Overall, this study suggests that the viral glycoprotein of orthobunyaviruses can compensate, at least in part, the function of NSs. In addition, we also provide evidence that the induction of total cellular protein shutoff by SBV is determined by multiple viral proteins while the ability to control the production of IFN maps to the NSs protein.

Importance

The identification of viral determinants of pathogenesis is key to the development of prophylactic and interventions measures. In this study we found that the bunyavirus Gc glycoprotein is a virulence factor. Importantly, we show that mutations in the Gc glycoprotein can restore pathogenicity of attenuated mutants resulting from deletions or mutations in the non-structural protein NSs. Our findings highlight the fact that careful consideration should be taken when

designing live attenuated vaccines based on deletions of non-structural proteins since single mutations in the viral glycoproteins appear to revert attenuated mutants to virulent phenotypes.

Introduction

The B*unyaviridae* is one of the largest families of RNA viruses comprising pathogens of importance for both human and veterinary medicine. More than 170 viruses transmitted by arthropods form the O*rthobunyavirus* genus. Schmallenberg virus (SBV) is an orthobunyavirus of ruminants that emerged in central Europe in the summer of 2011 and spread very quickly throughout the rest of the continent (1). Although SBV genomes and antibodies have been detected in wild ruminants, camelids and a dog, so far only infections of ruminants have been associated with the disease (2-4). SBV has been detected in various *Culicoides* species and it is assumed that these insects provide the main route of transmission for this virus (5, 6).

Infection of adult animals with SBV results in unspecific and mild clinical signs, while infection during gestation can result in stillbirths, abortions and malformations similar to infections with related viruses of the Simbu serogroup like Akabane virus (AKAV), Sathuperi virus (SATV) and Shamonda virus (SHAV) (7, 8). SBV was not detected in archived brain samples and no evidence of antibodies towards this virus was found in sera collected before 2010 in ruminants (9, 10). Hence, it is believed that the virus emerged for the first time in Europe in 2011. However, there is little information on the viral genetic characteristics and ecological conditions driving SBV emergence.

Like other orthobunyaviruses, the SBV genome comprises three RNA segments of negative polarity referred to as small (S), medium (M) and large (L). The S segment encodes for the viral nucleocapsid and the non-structural protein NSs in an overlapping reading frame. The M segment encodes for the viral glycoproteins Gn and Gc, decorating the viral lipid bilayers, in addition to the NSm glycoprotein, a second non-structural protein of poorly defined characteristics. The L segment encodes for the viral RNA-dependent RNA polymerase (RdRp).

Using reverse genetics, we and others have previously shown that the SBV NSs protein is a determinant of pathogenesis (11-13). Deletion of the SBV NSs protein results in attenuation of pathogenicity in a suckling mice model of infection. *In vitro*, NSs deletion (SBV-ΔNSs) leads to: i) impaired virus replication in interferon (IFN) competent cells; ii) inability to inhibit IFN synthesis in

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infected cells; and iii) inability to induce cellular protein synthesis shutoff. The defects possessed by SBV-ΔNSs are the result, at least in part, of the incapacity of the mutated virus to induce the degradation/dephosphorylation of the cellular RNA polymerase II as means of inhibiting cellular transcription and the IFN response of the host (12). Regulatory authorities in some European countries have granted provisional marketing authorization for an inactivated SBV vaccine (14). In addition, a double NSs-NSm deletion SBV mutant has been proposed as a vaccine candidate as it provides full protection in cattle upon wild type virus challenge and does not induce viremia nor clinical signs in cattle (15).

In an attempt to identify other viral determinants of pathogenicity, we serially passaged SBV in sheep IFN incompetent cells (CPT-Tert) and the resulting virus, referred to as SBVp32, was unexpectedly more virulent in 3 and 7 day-old NIH-Swiss mice inoculated intracerebrally (11). Although SBVp32 displayed similar replication kinetics to wild type SBV in CPT-Tert cells, it spread and induced pathological changes faster in the brains of suckling mice. We found SBVp32 to possess nucleotide substitutions (most of which non-synonymous) in all the viral genes compared to wild type SBV (11). The objective of this study was to identify the viral proteins associated with increased virulence of SBVp32 in order to better understand orthobunyavirus pathogenesis. We found that the Gc glycoprotein of SBVp32 is a virulence determinant and facilitates cellular protein synthesis shutoff. In addition, the SBVp32 Gc can compensate the attenuated phenotype of NSs deletion mutants.

Materials and Methods

Cell lines. BSR-T7/5 cells (kindly provided by Karl Conzelmann) stably expressing the T7 polymerase were grown in Glasgow modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 10% tryptose phosphate broth and G418 at a final concentration of 1 mg/ml. Sheep choroid plexus cells (CPT-Tert) (16) were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS. A549-ISRE-GFP expressing GFP under the control of an ISRE promoter (provided by R. Randall) (17) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. All cell lines were cultured at 37°C in a 5% CO_2 and 95% humidified atmosphere and were supplemented with penicillin and streptomycin.

Isolation of primary fibroblasts. Ovine fibroblasts were isolated from sheep ear skin. Briefly, the dermal layer was removed, cut into explants and plated into dry tissue culture plates, dermis side up. The explants were incubated without media for 1 hour and then DMEM culture media containing 10% FBS, penicillin and streptomycin and 1% nystatin was added. After sufficient outgrowth, the explants were discarded and the remaining cells cultured using standard methods.

Antibodies. Antisera used in this study included a rabbit polyclonal antiserum against the SBV N protein (Proteintech) (11). Antibodies against γ tubulin were obtained from Sigma while antibodies against puromycin from Millipore (used at 1:3,000 dilution). Peroxidase labelled secondary antibodies against rabbit and mouse were purchased from GE Healthcare Life Sciences and Cell Signalling respectively.

Viruses. All viruses used in this study were rescued by reverse genetics in BSR-T7/5 cells as previously described (11). Titers were determined by standard plaque assays performed in CPT-Tert cells. The reassortants derived in this study were named by including the segment corresponding to the SBVp32 strain. For example SBV-S32 contains the S segment from SBVp32 and the L and M segments from wild type SBV. SBVp32 refers to SBV passaged 32 times in cell culture. SBV-SML32 contains all the segments from SBVp32 but was derived by reverse genetics. A deletion mutant of

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the NSm protein was constructed by mutagenesis of the SBV M segment by deletion of amino acid residues 338 to 448 (Shi et al. unpublished results).

Labelling of nascent proteins with puromycin. Monolayers of CPT-Tert cells at 30-40% confluency were infected with the indicated viruses at a multiplicity of infection (MOI) of 1. 16 hr post infection, the media was replaced with IMDM supplemented with 10ug/ml puromycin dihydrochloride (Sigma) for 12 min. Cells were then washed with PBS and lysed using 1x Laemmli buffer. SDS-PAGE and western blotting were performed from total cell lysates as previously described (18). For quantitative western blotting, primary antibodies were detected using peroxidase labelled secondary antibodies using a ChemiDoc XRS+ scanner and quantified with Image Lab™ software (Biorad). γ-tubulin was used to normalize loading. All experiments were performed independently at least three times. For each virus, at least two independent virus preparations were used.

Virus replication assays. Virus replication kinetics were determined by infection of the indicated cell lines at a multiplicity of infection (MOI) of 0.001, unless stated otherwise, followed by titration of supernatants collected at different time points post infection by limiting dilution assays in BSR cells. Titers are expressed as the tissue culture infectious dose 50 ($TCID_{50}$; determined by the Reed-Muench method). Each experiment was performed in triplicate and repeated three times using two different virus preparations.

Immunofluorescence and confocal microscopy. The indicated cell lines were infected with the indicated viruses and fixed with 5% formaldehyde followed by immunofluorescence using a polyclonal antiserum towards the SBV N protein as previously described (19, 20). Slides were analysed by using a Leica GMIR2 confocal microscope.

IFN bioassays. Primary sheep fibroblasts were infected with the indicated reassortants (MOI 1 or 0.5) and 16 h post infection the supernatants collected and clarified. UV-inactivated supernatants were used to stimulate A549-ISRE-GFP for 24 hs when cells were fixed and the number of GFP positive cells was determined by FACS. The amount of IFN present in each sample was estimated based on an IFN standard of known concentration and expressed relative to SBV-ΔNSs.

146 **Quantification of viral mRNA.** Viral RNA was reverse transcribed using a SBV specific primer (5'-
147 TTCGGCCCCAGGTGCAAATC-3') with AccuScript HF reverse transcriptase following manufacturer's
148 instructions using 200 µg of total RNA. Two µl of cDNA were used for qRT-PCR using the Brilliant III
149 Ultra Fast QPCR master mix as suggested by the manufacturer. Primers and probe used are the
150 following: SBV-S-FW (TCAGATTGTCATGCCCCTTGC); SBV-S-RW (TTCGGC CCCAGG TGCAAATC); and
151 SBV-S-FAM (TTAAGGGATGCACCTGGGCCGATGGC). Reactions were cycled on a Stratagene Mx3005
152 qPCR System (Agilent Technologies) and data was analysed with the Mx3000P software.
153 **Histopathology and *In situ* hybridization.** Organ samples were fixed in formalin and embedded in
154 paraffin using standard histological techniques. Slides were stained with hematoxylin eosin (HE).
155 *In situ hybridization* (ISH) to detect SBV mRNA was performed on all sections as described before
156 (21). Briefly, paraffin sections were dewaxed, hydrated, and washed in DEPC-treated water. After
157 proteolytic digestion, postfixation, acetylation, and prehybridization, sections were hybridized
158 overnight with a digoxigenin-labeled probe (88 base pairs, 100 ng/ml) directed against the SBV
159 nucleoprotein (21). Hybridized probes were detected using an anti-DIG antibody conjugated with
160 alkaline phosphatase and the substrates nitrobluetetrazoliumchloride (NBT) and 5-bromo-4-chloro-
161 3-indolyl phosphate (BCIP, X-phosphate). SBV-positive and negative animals, as well as sections
162 incubated only with hybridization buffer were included as controls.
163 ***In vivo* experiments**. Animal experiments were carried out at the Istituto Zooprofilattico
164 Sperimentale dell'Abruzzo e del Molise G. Caporale (Teramo, Italy) in accordance with locally and
165 nationally approved protocols regulating animal experimental use (Protocol number 5383/2012). For
166 survival studies, suckling NIH-Swiss mice (n=10-15 per each group) were inoculated intracerebrally
167 with 400 PFU of the indicated reassortants/mutants and monitored daily for signs of disease for a
168 period of 14 days. In order to test virus spread in the brain, 5 day old NIH-Swiss mice (n=3 per virus
169 and timepoint) were inoculated intracranially with SBV, SBV-SML32 and SBV-M32 and euthanized at
170 8, 24, 48 and 72h post-infection. SBV and SBVp32 pathogenicity was also compared in adult IFNAR$^{(-/-}$
171 $^{)}$ mice where groups of 5 mice were inoculated intraperitoneally (1,000 PFU) and the weight

recorded over a 15-day period. For histology and ISH, IFNAR$^{(-/-)}$ mice (n=2 per virus) were infected with either SBV, SBVp32 or uninfected cell culture media and animals were euthanized 3 days post-infection. Organs were then collected for histological examination and ISH.

Statistical analysis. Statistical analysis was performed using GraphPad Prism. All graphs display data average and standard deviations.

Results

The M segment is a major determinant of SBVp32 virulence. Our first goal was to identify the viral genomic segment/s conferring higher virulence to SBVp32. To this end, we rescued by reverse genetics SBV, SBVp32 and the reassortants between the two viruses mentioned below as previously described (Fig. 1A) (11). Table 1 shows the mutations present in the viruses used in this study.

We inoculated 11-day old NIH-Swiss mice intracerebrally (400 PFU) with all the reassortants and survival was monitored for 15 days. All the reassortants carrying the M segment of SBVp32 were more pathogenic than wild type SBV ($P \leq 0.01$ for SBV-SML32 and $P \leq 0.0001$ for SBV-M32, SBV-SM32 and SBM-SL32). These data suggest that the pathogenicity of SBVp32 maps to the M segment. A reassortant carrying the L segment of SBVp32 (SBV-L32) displayed the same pathogenicity of wild type SBV, however somewhat surprisingly a reassortant carrying the S segment of SBVp32 (SBV-S32) was attenuated (Fig. 1B). It is interesting to note that the attenuation conferred by the S segment of SBVp32 could be compensated by the M segment of SBVp32, since a SBV-SM32 mutant was more pathogenic than wild type ($P \leq 0.0001$).

We previously showed that SBVp32 spreads faster in the brains of infected mice (11). To understand the possible mechanisms behind the extra pathogenicity conferred by the M segment of SBVp32 we compared the amount of virus found in brains of 6-day old NIH-Swiss mice infected intracranially with wild type SBV, SBV-SML32 and SBV-M32. For each virus, groups of 3 mice were killed at different times post infection (8, 24, 48 and 72h) and total RNA was extracted from brains followed by qRT-PCR to quantify the number of SBV genome equivalents. We found that both SBV-SML32 and SBV-M32 reached higher number of viral genomes compared to wild type SBV in the brains of infected mice (Fig. 2)

SBV-S32 attenuation maps to a single nucleotide change within the S segment. We first investigated the molecular determinants of the SBV-S32 attenuated phenotype. We derived 4 single mutant viruses (SBV-S-C66T, SBV-S-G124A, SBV-S-A167G and SBV-S-A319G) where nucleotides in positions 66, 124, 167 or 319 of the SBV S segment were mutated with the corresponding nucleotide

found in SBVp32 while the M and L segments were identical to SBV wild type. These mutants were used to inoculate suckling mice. All the mutants killed 100% of mice similarly to wild type SBV except for mutant SBV-S-A167G (carrying a G to A mutation corresponding to nucleotide 167 of the viral nucleocapsid gene and nucleotide 142 of the NSs gene) that was markedly attenuated ($P \leq 0.05$; Fig.3A-B).

While all the reassortants had similar replication kinetics as wild type SBV in IFN incompetent cells (CPT-Tert cells), replication of SBV-S-A167G mutant was impaired in IFN competent primary sheep fibroblasts (Fig. 3C). This data suggests that the attenuation of the S segment of SBVp32 due to mutation at position 167/142 (N/NSs) relates to the inability of this virus to inhibit the production of IFN. To confirm these data we measured the ability of each mutant to induce IFN synthesis using an IFN bioassay. Primary sheep fibroblasts were infected with the different mutants, supernatants collected 16h post infection and the amount of IFN present was estimated as described in Materials and Methods. As expected no IFN was produced by cells infected with wild type SBV, while cells infected with SBV-S32 and SBV-S-A167G induced the release of IFN into supernatants (Fig. 4A).

We have previously shown that the NSs protein of SBV counteracts the antiviral response of the host by inducing shutoff of host cell protein synthesis in line with other orthobunyaviruses (11-13, 22). To test if the attenuation of SBV-S32 relates to its inability to block cellular protein synthesis, we labelled nascent protein synthesis with puromycin in cells infected with the mutants described above and compared them by western blotting. Cells infected with SBV-ΔNSs were used as control. SBV, SBV-S-C66T, SBV-S-G124A and SBV-S-A319G induced host cell proteins shutoff as expected. On the other hand, we found that SBV-S32 and SBV-S32-A167G were unable to induce total cellular protein shutoff similarly to SBV lacking the NSs protein (SBV-ΔNSs) (Fig. 4B).

The M segment of SBVpP32 facilitates virus-induced shutoff of host cell protein synthesis. The results obtained so far indicated that SBVp32 is more virulent than wild type SBV even though it harbours a defective S segment that is unable to counteract the IFN response of the host cell. However, the defect of the S segment can be compensated, at least in the experimental mouse

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model used in our studies, by the M segment of SBVp32. Hence, we measured the ability of reassortants carrying the S segment of SBVp32 in combination with L and M segments of SBVp32 to shutoff protein synthesis by puromycin labelling of nascent proteins. These experiments were designed in order to understand whether the M segment of SBVp32 compensates the relative inability of the SBVp32 S segment to block cellular protein synthesis. As expected, all the controls used in the experiment worked as expected: SBV induced host protein shutoff while SBV-ΔNSs or SBV-S32 were unable to do so (Fig. 5A). On the other hand, we found that any reassortant carrying the M segment of SBVp32 was able to induce global host protein shutoff, even in the presence of the attenuated S32 segment. In addition, SBV-L32 was also unable to block host protein synthesis, suggesting a role for the polymerase in this process.

We then performed the same assays with reassortants deleted of the NSs protein in combination with the M or L segments of SBVp32 (SBV-SΔNSs-M32, SBV-SΔNSs-L32). We found that the M segment of SBVp32 was also able to compensate the defect in host protein shutoff displayed by a NSs deletion mutant (11) (Fig. 5B).

The mutations that accumulated in the M segment of SBVp32 during serial passage map to both the NSm and the Gc glycoproteins (Fig. 6A and Table 1). To understand if the increased pathogenicity of SBVp32 maps to the NSm or the Gc glycoproteins, we mutated the NSm nucleotides within the SBV-M32 backbone into those present in wild type SBV and tested the pathogenicity of the resulting reassortant in our mouse model of infection. We found that a virus carrying a wild type NSm protein and a SBVp32 Gc protein in combination with L and S segments from wild type SBV (SBV-Gc32-NSmWT) was more pathogenic than wild type SBV in suckling mice ($P \leq 0.001$; Fig. 6B). In addition, the same reassortant in the context of a SBV S segment deleted of its NSs protein was still more pathogenic (SBV-SΔNSs-Gc32-NSmWT) than SBV wild type ($P \leq 0.001$).

Next, we checked the ability of these mutants to induce total cellular protein shutoff using puromycin labelling of nascent proteins. As expected, we found that all the reassortants carrying the Gc glycoprotein of SBVp32 induced total cellular shutoff even in combination with impaired S

segments (S32 and SΔNSs) (Fig. 6C). In addition, complete deletion of the NSm protein did not affect the ability of the Gc SBVp32 glycoprotein to shutoff total cellular protein production nor restore the impaired cellular protein shut down capability of NSs mutants (SBV-S32-Gc32-ΔNSm and SBV-ΔNSs-Gc32-ΔNSm) (Fig. 6D).

Next, we introduced the individual non-synonymous nucleotide changes identified in the Gc gene of SBVp32 by site directed mutagenesis into SBV and rescued each mutant within the context of a SBV wild type L segment and the S segment of SBVp32. The resulting mutants were called SBV-S32-MT1502C, SBV-S32-MA1894G, SBV-S32-MC2011G, SBV-S32-MA2236G, SBV-S32-MC2411T and SBV-S32-MT2506C). Unfortunately, SBV-S32-G2575A could not be successfully rescued despite several attempts. We then assessed the capacity of each mutant to shutoff total cellular protein production. We found that mutations at positions 1894, 2236 and 2411 were individually capable of rescuing the defect of the S segment of SBVp32 (Fig. 6E).

The M segment of SBVp32 is not an IFN antagonist. Next, we investigated if the M segment of SBVp32 could compensate the inability of the attenuated S32 segment to inhibit the production of IFN. To this end we firstly monitored the replication kinetics of a various SBVp32-SBV wild type reassortants in sheep IFN competent cells. We found that all reassortants carrying the S segment of SBVp32 (including those carrying M32) reached lower titers than wild type SBV (Fig. 7A). These data suggest that the S segment of SBV is by itself the major virus IFN-antagonist.

To support these results we measured the ability of each reassortant to block the synthesis of IFN in infected cells as described above. We found that all the reassortants carrying the S segment of SBVp32 induced the release of IFN from infected cells, even when in combination with the M segment of SBVp32, although at lower levels compared to an NSs deletion mutant (Fig. 7B). These data indicate that for SBV, pathogenicity does not totally depend on the capability of the virus to inhibit the production of IFN but rather on its ability to induce total cellular protein shutoff. In addition, the data also suggest that virus induced global protein shutoff is not necessarily sufficient to block the production of IFN of the host.

The glycoprotein of SBVp32 facilitates early events of virus infection. The majority of the amino acid substitutions between wild type and SBV-M32 map to the Gc glycoprotein. Therefore, we reasoned that during serial passage in tissue culture the SBV glycoprotein evolved to reach a conformation to allow better entry. To test this hypothesis, we infected CPT-Tert cells with the same number of genome equivalents of SBV, SBV-M32 and SBV-SML32 (all segments from SBVp32) and the number of infected foci was quantified 8 hours post infection after immune staining of the viral nucleocapsid protein. We found a higher number of SBV positive foci in wells infected with SBVp32 and SBV-M32 than wild type SBV, indicating that the M segment of SBVp32 facilitates early events of SBV infection (Fig. 8).

SBVp32 virulence is related to increased virus replication also in the absence of an intact IFN response. Data obtained so far indicate that SBVp32 virulence is determined by its M segment and in particular by its ability to shutoff host protein synthesis and facilitate virus replication. The results obtained in NIH-Swiss mice suggest that SBVp32 virulence is therefore not linked to its ability to block the IFN response of the host. In order to support this conclusion, we compared the pathogenicity of SBV and SBVp32 in adult IFNAR$^{(-/-)}$ mice. We inoculated three groups of IFNAR$^{(-/-)}$ with SBV, SBVp32 or uninfected cell culture media as control (n = 5 per group). All the animals survived the length of the experiment and both SBV and SBVp32 infected animals developed signs of disease. However, they were more pronounced in SBVp32 infected animals. In addition, SBVp32 infected animals displayed a statistically significant ($p<0.05$) more pronounced loss in weight compared to mock-infected controls (Fig. 9A).

Next, we analysed virus distribution in a variety of organs by *in situ* hybridization (ISH) in three groups of IFNAR$^{(-/-)}$ mice (n=2 per group) infected with either SBV, SBVp32 or uninfected cell culture media. Mice were euthanized 3 days post-infection and organs were then collected for histological examination and ISH (Fig. 9B). The liver and spleen of SBV infected animals were the only organs displaying histopathological changes consisting with mild to moderate inflammation. SBV mRNA was detected by *in situ* hybridization in both macrophages and hepatocytes in the liver and in

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307 macrophages in the spleen. SBV mRNA was also detected in the mandibular and mesenteric lymph
308 nodes. On the other hand, moderate to severe necrosuppurative hepatitis, suppurative splenitis and
309 lymphadenitis were found in samples derived from SBVp32 infected animals. These lesions were
310 associated with the presence of moderate to high amounts of SBV mRNA in hepatocytes and
311 macrophages. One animal showed non-suppurative interstitial nephritis that was associated with the
312 presence of mild to moderate SBV mRNA. Degenerative and inflammatory lesions were identified in
313 the heart while mild pathomorphological changes were present in the small intestine and the spinal
314 cord but no SBV mRNA was detected in the latter. Table 2 displays the organs in which the presence
315 of SBV mRNA was evaluated.

Discussion

In this study we showed that a cell culture adapted strain of SBV (SBVp32) has a more virulent phenotype than its wild type parental strain due to amino acid substitutions in the Gc glycoprotein. Importantly, we show that SBVp32 virulence is increased by the capacity of the Gc to facilitate host protein shutoff and early events of virus replication, at least in the experimental models employed in this study. Surprisingly, SBVp32 is more virulent than wild type SBV but carries a defective S segment that is unable to inhibit the production of IFN and fails to induce total cellular protein shutoff in infected cells. This defect is however compensated in SBVp32 by Gc. Altogether our data indicate that the induction of total cellular protein shutoff in SBV is determined by multiple genes while the ability to inhibit the production of IFN maps mainly to the NSs protein.

The S segment of SBV encodes the nucleocapsid and the non-structural protein NSs in overlapping reading frames. We have previously shown that the SBV NSs protein is a virulence factor that inhibits the synthesis of IFN of infected cells and controls the innate immune system of the host by blocking cellular protein production (11, 12). Thus, it is not surprising that the S segment of SBVp32 lost the ability to repress the production of IFN given that it was serially passaged in CPT-Tert cells (sheep cells that don't to produce IFN upon viral infection). The NSs protein of SBVp32 also showed a decreased ability to induce total cellular protein shutoff. These observations suggest that IFN antagonism and the capacity to induce total cellular protein shutoff by the NSs protein are linked and require an arginine residue at position 49. Residue 49 is part of a nucleolar localization signal. Thus the change from arginine to glycine in SBVp32 could likely be associated with a change in the cellular localization of NSs leading to its loss of function and evidenced as an attenuated phenotype in suckling mice. However, pathogenicity is restored and even enhanced in those reassortants that contain also the Gc glycoprotein of SBVp32. The SBVp32 Gc protein acquired the ability to induce total cellular protein shutoff during serial passage but cannot compensate the IFN antagonism of NSs. Altogether, these data suggest that the capacity to inhibit IFN production by SBV maps mainly to the NSs protein. On the other hand, SBV virulence is associated with the ability of the virus to

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induce total cellular protein shutoff rather than IFN antagonism. This is further supported by the fact that SBVp32 induces more severe clinical signs in adult IFNAR$^{(-/-)}$compared to wild type SBV. These data also indicates that the ability to block IFN and induce cellular protein shutoff by the NSs protein come with a "cost" for the virus that is easily disposed once no longer required.

SBVp32 cannot fully inhibit the synthesis of IFN and its replication is impaired in IFN competent cells, however it is still more pathogenic than wild type SBV in suckling mice. A possible explanation for these contrasting observations is that in infections with viruses expressing the SBVp32 Gc protein, cellular shutoff precedes the induction of IFN thus quickly overcoming the upregulation of ISGs and leading to pathogenicity. We need to take into consideration that we quantified the amount of IFN released into the supernatant of infected cells early in infection (16 h post infection). It is possible that a different effect on IFN synthesis is seen at later time points during infection and more studies will be required to address this point. In addition, the IFN bioassays were performed in primary cell cultures derived from adult animals. We have previously shown, like for other viral infections (23, 24), that there is an age-dependent resistance to SBV infection in suckling mice (11) that could be attributed to the development of anatomic barriers, the reticularendothelial system, the IFN and immune responses and thus the increased pathogenicity of SBVp32 could be due to many factors. It still remains to be determined if SBVp32 is more pathogenic also in ruminants and how this virus controls the innate immune system in its natural host.

Deletion of the NSs protein in wild type SBV does not lead to a full recovery of protein expression in infected cells (Fig. 4B) indicating that other viral proteins are involved in inducing cellular protein shutoff. In addition, only eight serogroups encode NSs proteins among the viruses of the 15 orthobunyavirus serogroups for which genomic data are available highlighting the fact that orthobunyaviruses use alternative mechanisms to cope with the innate immune system of the host (25, 26). Here we observed that the L segment of SBV contributes to the induction of cellular protein shutoff given that a reassortant comprising M and S segments of wild type SBV and the L segment of SBVp32 (SBV-L32) was less capable of shutting down protein expression than wild type

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SBV (Fig. 5A). Although we did not map the particular residues associated with this, we can speculate that mutation at position 130 could be involved since it sits within the endonuclease motif of the viral polymerase. The endonuclease activity of the bunyavirus polymerase allows a "cap-snatching mechanism" by which the viral polymerase cleaves the 5' cap of cellular mRNAs to use as primers for viral mRNA transcription therefore reducing the cellular pools of mRNA for translation (27). We also found that the SBV NSm protein contributes to cellular protein shutoff (Fig. 4D). Interestingly, in another study complete deletion of the NSm coding region resulted in a virus that, within the context of a wild type M and L segments (SBV-ΔNSm) was unable to induce cellular protein shutoff but had no effect on the ability to control the production of IFN of infected cells (Elliott and Shi personal communication). This is in agreement with studies carried out on Bunyamwera virus (BUNV) where deletions of internal domains of NSm resulted in a similar phenotype, although a complete NSm deletion mutant could not be rescued by reverse genetics (28) suggesting that NSm might play different roles in different orthobunyaviruses. Although BUNV NSm has been involved in playing a role in assembly and morphogenesis, its role in controlling the innate immunity of the host requires further investigation.

The molecular mechanism of SBV Gc-induced protein shutoff remains to be determined. However given that the Gc glycoprotein of other orthobunyaviruses including SBV transit from the endoplasmic reticulum (ER) to the Golgi apparatus (29) (not shown) it can be expected that they trigger the ER stress response. Several viruses use this strategy to favour translation of viral proteins (30). Viral infection can quickly lead to excess protein and the accumulation of misfolded products in the ER which ultimately results in the phosphorylation of eIF2. eIF2 phosphorylation results in cap-dependent protein synthesis shutoff reliving the burden of the accumulation of proteins in the ER and evidenced as a reduction in total cellular protein expression (31).

Reassortants carrying the SBVp32 M segments reached higher virus titers in the brains of infected mice and were more infectious *in vitro*. We hypothesize that during serial virus passage and without pressure from neutralizing antibodies the Gc protein accumulated mutations that allow more

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efficient entry and thus faster spread. However, it remains to be determined if this virus could be viable or if it could be more pathogenic in its natural hosts given the possibility that it could be better neutralized. It is possible that faster entry or higher replication conferred by the SBVp32 Gc glycoprotein allows earlier expression of other viral proteins (such as the viral polymerase and/or NSm), leading to faster induction of cellular protein shutoff compared to wild type virus. Unfortunately, we have not been able to express the viral glycoproteins to high levels using a transient transfection system to test the role of Gc in protein shutoff independently of other viral components. These data could be an indication that expression of Gc leads to protein synthesis shutoff resulting in repression of its own plasmid.

We showed that single point mutations in Gc (1894, 2236 and 2411) rescue the inability of the S segment of SBVp32 to induce total cellular protein shutoff. There are currently less than ten SBV M segment sequences derived from field isolates, thus our understanding of the true genetic diversity of this virus and the consensus sequence circulating in affected countries is very limited. Interestingly, SBVp32 mutation at position 2236 exists in the field, indicating that it can lead to viable virus. Given that we found a consistent positive correlation between the ability of our reassortants to induce protein shutoff and be more pathogenic in our mouse model of infection we can conclude that a single amino acid change in the Gc glycoprotein can potentially rescue attenuated phenotypes based on mutations of the S segment. Importantly, we also showed that the defect in inducing host protein shutoff by a double NSs and NSm deletion mutant can be rescued by amino acid changes in the Gc glycoprotein and that at least one of the changes exists in viruses circulating in the field. Therefore live attenuated vaccines based on deletion of NSs and NSm require further careful consideration.

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Acknowledgements

We would like to dedicate this work to the memory of our friend and colleague Richard M. Elliott.

Tables

Table 1. Nucleotide differences between SBV wild type and SBVp32.

Segment	SBV nt		SBV nt	SBVp32 nt	syn/non-syn		Location
	N	NSs			N	NSs	N/NSs
S	66	41	C	T	Syn	Non-syn	N/NSs
	124	99	G	A	Non-syn	Syn	N/NSs
	167	142	A	G	Non-syn	Non-syn	N/NSs
	319	n/a	A	G	Non-syn	n/a	N
M	1016		G	A	Non-syn		NSm
	1239		T	G	Non-syn		NSm
	1502		T	C	Non-syn		Gc "hot spot"
	1894		A	G	Non-syn		Gc
	2011		C	G	Syn		Gc
	2236		A	G	Non-syn		Gc
	2411		C	T	Non-syn		Gc
	2506		T	C	Non-syn		Gc
	2575		G	A	Non-syn		Gc
L	130		T	C	Non-syn		Endonuclease region
	3044		T	C	Non-syn		
	3858		A	G	Non-syn		
	4078		C	T	Non-syn		

Table 2. Organs in which the presence of SBV mRNA was evaluated by in situ hybridization.

	sSBV		SBVp32		Controls	
	Mouse 1*	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6
Salivary gland	0	0	+	0	0	0
Mandibular lymph node	+	0	+++	+++++	0	n.d.
Pharynx	0	0	n.d.	0	0	0
Thyroid gland	0	n.d.	n.d.	0	n.d.	0
Lungs	0	0	0	0	0	0
Thymus	0	n.d.	n.d.	n.d.	n.d.	0
Heart	0	0	0	xx	0	0
Liver	+++	0	++++++	++++++	0	0
Spleen	+++++	0	+++++	+++++	0	0
Kidney	0	0	+++	0	0	0
Testis	0	0	0	0	0	0
Cerebellum	0	0	0	0	0	0
Brain stem	0	0	0	0	0	0
Cerebrum	0	0	0	0	0	0
Stomach	0	0	0	0	0	0
Small intestine	0	0	+	++	0	0
Large intestine	0	0	0	++	0	0
Pancreas	0	0	0	0	0	0
Mesenteric lymph node	++	n.d.	+++++	n.d.	0	0
Spinal cord	0	0	0	0	0	0
Bone marrow	++++	0	+++++	+++++	0	0

*Mouse 1 to 6 indicate the individual animals. 0 no positive cells; x single positive cells; xx small number of positive cells; xxx small to moderate number of positive cells; xxxx moderate number of positive cells; xxxxx moderate to large number of positive cells; xxxxxx large number of positive cells.

Figure Legends

Figure 1. SBVp32 pathogenicity maps to the M segment. A. Schematic representation of SBV reassortants generated by reverse genetics. B. Survival plots of 11-day old NIH-Swiss mice inoculated intracerebrally with 400 PFU of the indicated reassortants. Survival of mice inoculated with wild type SBV has been included in each panel to facilitate comparisons. Asterisks indicate significance levels: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$ (Log-Rank test, Mantel Cox test).

Figure 2. SBV carrying the M segment of SBVp32 reaches higher genome copy numbers in the brains of infected mice. Groups of 3 mice were infected intracranially and brains harvested at the indicated times post infection. Viral genome equivalents were quantified by qRT-PCR. Data was analysed using a 2-way ANOVA (top asterisks indicate the significance level between SBV and SBV-SML32 while bottom asterisks between SBV and SBV-M32).

Figure 3. SBV-S32 attenuation maps to nucleotide 167/142 of nucleocapsid/NSs gene. A. schematic representation displaying the position of nucleotide changes of SBVp32 compared to wild type SBV in the nucleocapsid and NSs open reading frames. Asterisks indicate non-synonymous changes. B. Survival plots of 7-day old mice inoculated intracerebrally with 400 PFU of the indicated reassortants. Asterisks indicate significance levels: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$ (Log-Rank test, Mantel Cox test). C. Replication kinetics of SBV S point mutants in CPT-Tert and primary sheep fibroblasts. The graph displays the average of two and four independent experiments respectively.

Figure 4. Mutation at nucleotide 167/142 of nucleocapsid/NSs gene of the S segment of SBVp32 impairs the ability to induce total cellular protein shutoff and to control interferon production of infected cells. A. Interferon bioassay. Primary sheep fibroblasts were infected with the indicated reassortants (MOI 1) and the amount of IFN present in supernatants was estimated by comparing the ability to induce GFP expression in A549 cells stably expressing GFP under ISRE promoter to known quantities of universal IFN. Data is expressed relative to SBV-ΔNSs, known to induce IFN

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457 synthesis. E. Western blots displaying puromycin labelled proteins 16 h after infection with the
458 indicated reassortans (MOI 1). Infection with SBV-ΔNSs was used as control given its ability to shut
459 down cellular protein synthesis. Γ-tubulin was used as a loading control and SBV N to confirm
460 infection. The numbers indicate the quantification of each lane relative to mock that was set at
461 100%.

462 **Figure 5. The M segment of SBVp32 rescues the inability of attenuated S segments to induce total**
463 **cellular protein shutoff.** Western blots displaying puromycin labelled proteins 16 h after infection
464 with the indicated reassortans (MOI 1). γ-tubulin was used as a loading control and SBV N to confirm
465 infection. The numbers indicate the quantification of each lane relative to mock that was set at
466 100%. A. Reassortants carrying the S segment of SBVp32 in combination with wild type or SBVp32 M
467 and L segments. B. Reassortants carrying a wild type SBV S segment deleted of its NSs protein in
468 combination with wild type or SBVp32 M and L segments.

469 **Figure 6. SBVp32 pathogenicity maps to the Gc glycoprotein.** A. Schematic representation of
470 SBVp32 mutations within the M segment. B. Survival plots of 8-day old NIH-Swiss mice inoculated
471 intracerebrally with 400 PFU of the indicated reassortants. Asterisks indicate significance levels ns
472 $p>0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$ (Log-Rank test, Mantel Cox test). C-D-
473 E. Western blots displaying puromycin labelled proteins 16 h after infection with the indicated
474 reassortans (MOI 1). Γ-tubulin was used as a loading control and SBV N to confirm infection. The
475 numbers indicate the quantification of each lane relative to mock that was set at 100%.

476 **Figure 7. Replication kinetics of SBVp32 reassortants in IFN competent cells.** A. Primary sheep
477 fibroblasts and were infected (MOI 0.001) with the indicated reassortants and samples collected at
478 the indicated times post infection. The graph shows the average of four independent experiments.
479 B. Interferon bioassay. Primary sheep fibroblasts were infected with the indicated reassortants (MOI
480 0.5) and the amount of IFN present in supernatants was estimated as described in Fig.4.

481 **Figure 8. SBVp32 glycoprotein is more infectious than the wild type SBV glycoprotein.** CPT-tert cells
482 were infected with 2.5 10^5 genome equivalents of the indicated viruses and the number of SBV

positive cells was counted 8 hours post infection after immune staining. 10 view fields were counted for each condition. The percentage of positive cells is presented relative to wild type SBV infection. The experiment was repeated 3 times independently. Data was analysed using ANOVA (p 0.0076).

Figure 9. **SBVp32 virulence is related to increased virus replication in IFNAR$^{(-/-)}$ knockout mice.** A. Groups of 5 mice were inoculated intraperitoneally (1,000 PFU) and the weight recorded over a 15-day period. The area under the curve was estimated for each mouse and then compared among groups (ANOVA $p<0.05$, Tukey multiple comparison test). B. Representative micrographs displaying the pathological changes observed in SBVp32-infected IFNAR$^{(-/-)}$ knockout mice (left) and the presence of viral RNA by *in situ* hybridization (ISH) (right).

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