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Modulation of virion incorporation of Ebolavirus glycoprotein: Effects on attachment, cellular entry and neutralization

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Abstract

The filoviruses Ebolavirus (EBOV) and Marburgvirus (MARV) cause severe hemorrhagic fever in humans and are potential agents of biological warfare. The envelope glycoprotein (GP) of filoviruses mediates viral entry into cells and is an attractive target for therapeutic intervention and vaccine design. Here, we asked if the efficiency of virion incorporation of EBOV-GP impacts attachment and entry into target cells and modulates susceptibility to neutralizing antibodies. In order to control the level of EBOV-GP expression, we generated cell lines expressing the GPs of the four known EBOV subspecies in an inducible fashion. Regulated expression of GP on the cell surface allowed production of reporter viruses harboring different amounts of GP. A pronounced reduction of virion incorporation of EBOV-GP had relatively little effect on virion infectivity, suggesting that only a few copies of GP might be sufficient for efficient engagement of cellular receptors. In contrast, optimal interactions with cellular attachment factors like the DC-SIGN protein required incorporation of high amounts of GP. Antibody-mediated neutralization of virions bearing high amounts of GP was slightly more efficient than neutralization of virions harboring low amounts of GP, suggesting that the efficiency of GP incorporation into virions might modulate susceptibility to neutralizing antibodies. Finally, regulated expression of GP in permissive 293 cells did not reduce EBOV-GP-driven infection but diminished vesicular stomatitis virus GP (VSV-G) and amphotropic murine leukemia virus (A-MLV) GP mediated entry in a dose-dependent manner. Therefore, intracellular GP does not seem to downmodulate expression of its receptor(s) but might alter expression and/or function of molecules involved in VSV-G and A-MLV-GP-dependent entry. Our results suggest that the efficiency of virion incorporation of GP could impact EBOV attachment to target cells and might modulate control of viral spread by the humoral immune response.

Keywords: Ebolavirus; Glycoprotein; Receptor; Attachment factor; Expression level; DC-SIGN

Introduction

The filoviruses Ebolavirus (EBOV) and Marburgvirus (MARV) are negative stranded RNA viruses that cause severe hemorrhagic fever in humans (Feldmann et al., 2003; Geisbert and Hensley, 2004). The genus EBOV contains four subspecies, *Zaire ebolavirus* (ZEBO), *Sudan ebolavirus* (SEBOV), *Ivory coast ebolavirus* (ICEBOV) and *Reston ebolavirus* (REBOV), while Lake Victoria MARV is the only member of the MARV genus. The EBOV subtypes exhibit differential pathogenicity in humans. ZEBOV infection is lethal in up to 90% of the cases, while REBOV is considered nonpathogenic (Feldmann et al., 2003; Geisbert and Hensley, 2004). However, the underlying pathogenicity determinants are incompletely understood. The first step of the viral life cycle, entry into target cells, is mediated by the envelope glycoprotein (GP) (Kawaoka, 2005). EBOV- and MARV-GP is encoded by the fourth gene of the viral genome (Sanchez et al., 1993; Volchkov et al., 1992; Will et al., 1993). The EBOV- but not the MARV-GP gene contains two open reading frames. The major product synthesized from the EBOV-GP gene is a secreted form of GP, termed small GP (sGP) (Sanchez et al., 1996; Volchkov et al., 1995), which is not produced in MARV infected cells. The role of sGP in EBOV infection is incompletely defined (Kindzelskii et al., 2000; Maruyama et al., 98 A.D.; Yang et al., 1998). About 20% of the EBOV-GP message is transcriptionally edited by the viral

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polymerase to encode a membrane bound from of GP (Volchkov et al., 1995). The membrane bound EBOV- and MARV-GP is incorporated into budding virions and mediates entry into target cells (Kawaoka, 2005).

An N-terminal signal peptide targets the nascent GP into the endoplasmatic reticulum, where the polypeptide chain is modified with high mannose carbohydrates (Feldmann et al., 1991, 1994; Will et al., 1993). Subsequently, posttranslational modification of GP continues in the Golgi apparatus, and upon transport into the trans-Golgi, GP is cleaved by the cellular endoprotease furin into the subunits GP_1 and GP_2 (Volchkov et al., 1998). GP_1 and GP_2 remain covalently associated via disulfide bonds (Volchkov et al., 1998) and form trimeric heterodimers in the cellular membrane (Malashkevich et al., 1999), where they are incorporated into budding virions (Jasenosky and Kawaoka, 2004). Expression of GP of the highly pathogenic ZEBOV subspecies downmodulates cellular surface proteins, triggers detachment of adherent cells in culture and disrupts the integrity of the vascular endothelium (Chan et al., 2000; Simmons et al., 2002; Takada et al., 2000; Yang et al., 2000). Its has therefore been proposed that GP is an important determinant of viral pathogenicity and might be responsible for development of hemorrhages (Yang et al., 2000). While the latter concept has been challenged (Geisbert et al., 2003), the cytopathic effect of GP is undisputed. In fact, a major purpose of sGP synthesis might be to limit expression of GP in infected cells (Volchkov et al., 2001).

The functional organization of EBOV-GP is similar to that of various other viral glycoproteins, termed class I fusion proteins (Kawaoka, 2005). Thus, the GP_1 subunit is believed to interact with cellular receptors, while the GP_2 subunit, which is oriented perpendicular to the viral membrane, drives fusion of the viral and a cellular membrane, thereby allowing delivery of the viral genome into the cellular cytoplasm (Kawaoka, 2005). The fusion machinery in GP_2 has been defined on a functional and structural basis (Ito et al., 1999; Malashkevich et al., 1999; Watanabe et al., 2000; Weissenhorn et al., 1998a, 1998b). However, the elements in GP_1 required for recognition of cellular receptors are less clear. Recent mutagenic analysis suggested that the N-terminal 150 amino acids might be involved in receptor binding (Manicassamy et al., 2005). In contrast, a C-terminal mucin-like domain, which exhibits high variability between the EBOV subspecies and constitutes a determinant of cytopathicity (Simmons et al., 2002; Yang et al., 2000), is dispensable for GP-driven infection (Yang et al., 2000). Engagement of cellular receptors by GP_1 is believed to introduce EBOV into endosomal vesicles, where cathepsin mediated proteolysis of GP_1 triggers the membrane fusion activity of GP_2 (Chandran et al., 2005; Kawaoka, 2005; Schornberg et al., 2006). While the cellular receptor(s) for filoviruses remain elusive, binding of GP to several cellular lectins was shown to profoundly augment filovirus infection (Alvarez et al., 2002; Becker et al., 1995; Gramberg et al., 2005; Lin et al., 2003; Simmons et al., 2003; Takada et al., 2004).

GP is a target for neutralizing antibodies, and such antibodies can provide protection against EBOV infection in small animal models (Wilson et al., 2000). However, the generation of neutralizing antibodies is inefficient in infected individuals (Peters and LeDuc, 1999). Nevertheless, GP-specific neutralizing antibodies have been detected in convalescent donors (Maruyama et al., 1999). Because of its indispensable function in the viral life cycle and its susceptibility to antibody inhibition GP is a key target for therapeutic intervention and vaccine development. Thus, immunization with GP encoding adenoviruses (Sullivan et al., 2000, 2003) or vesicular stomatitis viruses (Jones et al., 2005) protected macaques from filovirus infection, and these vaccines are attractive candidates for evaluation in humans.

It is appreciated that expression levels of GP in infected cells impact cellular viability and virus production (Simmons et al., 2002; Volchkov et al., 2001). However, it is unclear if the efficiency of GP incorporation into budding virions affects the interaction with cellular factors and the recognition by the humoral immune response. For simian immunodeficiency virus (SIV), it has been reported that the efficiency of envelope protein (Env) incorporation into virions can profoundly modulate infectivity and neutralization sensitivity (Yuste et al., 2004, 2005), and a correlation between Env content in virions and virion infectivity has also been observed for human immunodeficiency virus type-1 (HIV-1) (Bachrach et al., 2005). By employing cell lines that express EBOV-GP of the four subspecies upon induction, we investigated how the level of EBOV-GP incorporation into virions affects the interaction with cellular factors and the susceptibility to antibody neutralization. We provide evidence that relative few copies of GP are sufficient to allow robust infection, while only high amounts of GP allow optimal engagement of cellular lectins. We also show that the density of GP on virions can impact neutralization efficiency and that the expression of high levels of GP by target cells does not interfere with EBOV-GP mediated infection.

Results

Inducible expression of EBOV-GPs by 293 T-REx cells

We sought to investigate if the efficiency of virion incorporation of EBOV-GP affects the interaction of virions with cellular factors and their recognition by neutralizing antibodies. In order to generate virions harboring different copy numbers of GP, we employed a tetracycline inducible system to express GP. Thus, expression constructs for the GPs of the four EBOV subspecies were stably introduced into 293 T-REx cells, which express transgenes upon induction with tetracycline. The 293 T-REx cell line, a derivative of the 293 cell line, was chosen because these cells are readily transfectable and should allow efficient production of reporter viruses bearing EBOV-GPs. Western blot analysis of 293 T-REx cell lines stably transfected with EBOV-GP expression constructs revealed that all cell lines expressed the EBOV-GPs in a doxycycline (a tetracycline derivative)-dependent fashion, while no GP signal was detected in parental 293 control cells (Fig. 1A). The rabbit serum used for detection of EBOV-GP expression was raised against ZEBOV-GP and preferentially recognizes this protein (data not shown); therefore, differences in the signal intensities between

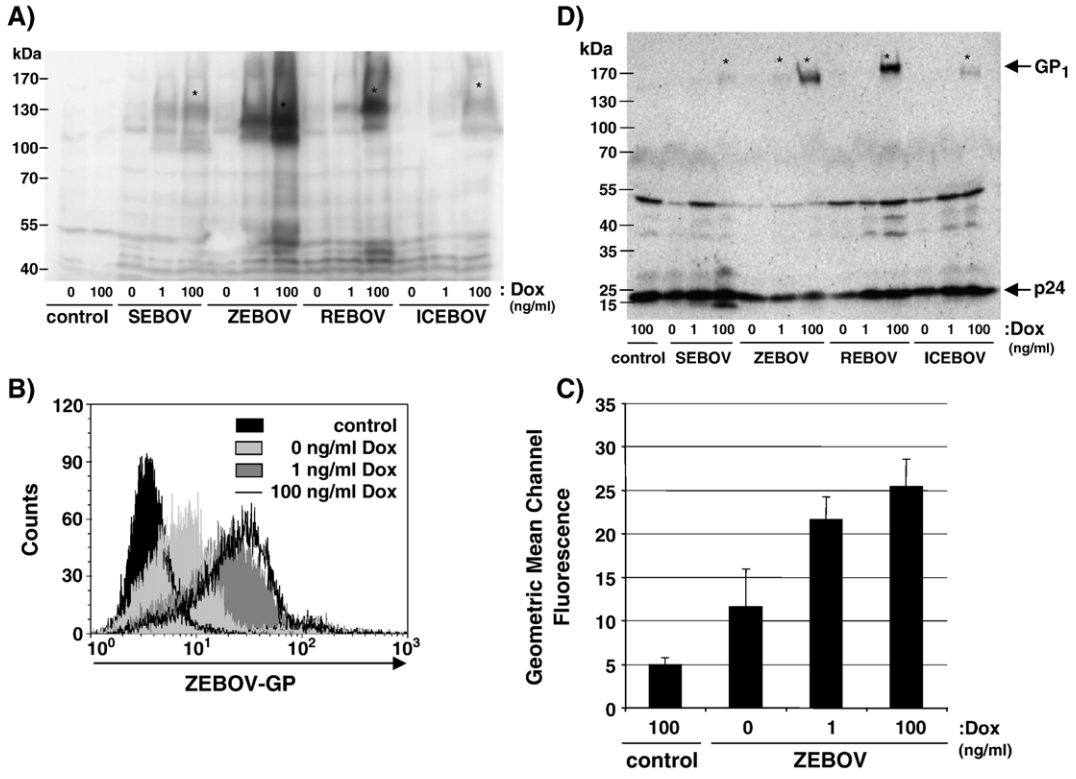

Fig. 1. Inducible expression of EBOV-GP and incorporation of different amounts of EBOV-GP into reporter viruses. (A) Inducible expression of EBOV-GP in 293 T-REx cells. The 293 cell derived cell line 293 T-REx, which encodes a tetracycline (tet)-sensitive repressor, was transiently transfected with expression constructs encoding the GPs of the four EBOV subspecies under control of a CMV promoter harboring two binding sites for the tet-repressor. After prolonged culture in the presence of selection antibiotics resistant cell populations were obtained and expanded. Expression of GP in the indicated stable cell lines was induced with the indicated amounts of doxycycline (a tetracycline derivative) and GP expression in cell lysates was analyzed by Western blot, employing a ZEBOV-GP-specific serum for detection. Similar results were obtained in two independent experiments. Asterisks mark GP detected upon induction with 100 ng/ml doxycycline. Abbreviations: Dox, doxycycline. ZEBOV, *Zaire ebolavirus*; SEBOV, *Sudan ebolavirus*; ICEBOV, *Ivory Coast ebolavirus*; REBOV, *Reston ebolavirus*; GP, glycoprotein. (B) Inducible expression of ZEBOV-GP on the surface of 293 T-REx cells. ZEBOV-GP expression in 293 T-REx cells was induced with the indicated amounts of doxycycline and analyzed by FACS employing a ZEBOV-GP-specific serum for detection. The results were confirmed in two independent experiments. The black filled histogram indicates staining of parental 293 T-REx cells with the ZEBOV-GP-specific serum. (C) Expression of ZEBOV-GP upon induction with doxycycline was assessed as described for (B) and geometric mean channel fluorescence was determined. The average of three independent experiments is shown, error bars indicate standard error of the mean (SEM). (D) Incorporation of differential amounts of EBOV-GP into lentiviral reporter viruses. The EBOV-GP transfected 293 T-REx cell lines were induced to express GP, transfected with the *env*-defective HIV-1 pNL4-3-Luc-R$^-$E$^-$ reporter virus genome, and culture supernatants harvested thereafter. Equal volumes of viral supernatants containing 2.5 ng of viral capsid antigen were pelleted and the presence of GP and capsid in viral pellets analyzed by Western blot. Similar results were obtained upon analysis of two independently generated virus stocks. Bands corresponding to GP are marked by asterisks. Densitometric analysis of ZEBOV- and REBOV-GP signals of three independent Western blot experiments yielded the following results (calculated relative to the ZEBOV-GP signal measured upon GP induction with 100 ng/ml doxycycline, which was set as 100%): 94.5 ± 15 for REBOV-GP at 100 ng/ml doxycycline; 6.9 ± 4.2 for ZEBOV-GP at 1 ng/ml doxycycline and 0.7 ± 0.4% for REBOV-GP at 0 ng/ml doxycycline. The signals measured for REBOV-GP at 1 ng/ml and for ZEBOV-GP at 0 ng/ml doxycycline were below background.

the four GPs tested do not necessarily reflect differential expression but are most likely due to differential reaction with the antiserum employed for detection. The relative gel mobility of the GPs analyzed is in agreement with documented results (Feldmann et al., 1994; Marzi et al., 2006). Several bands were observed in control and GP expressing cells (particularly in the range of 40 to 70 kDa) and most likely reflect unspecific reactivity of the rabbit serum; albeit it cannot be excluded that some signals originate from, or are enhanced by, products of GP degradation.

We next analyzed surface expression of GPs on doxycycline induced 293 T-REx cells. Relatively weak signals were observed for SEBOV- and ICEBOV-GP, while REBOV- and particularly ZEBOV-GP expression was readily detectable (Fig. 1B and data not shown), which is generally in agreement with the results obtained upon Western blot analysis of GP expression (Fig. 1A). Analysis of ZEBOV- and REBOV-GP producing cells revealed doxycycline-dependent GP expression (Figs. 1B, C and data not shown), albeit the correlation between inducer concentration and GP expression was not as strict as observed upon Western blot analysis (Fig. 1A). Notably, some ZEBOV-GP was expressed also on uninduced cells, suggesting that the negative regulation of GP expression in the absence of inductor was not entirely tight.

Having demonstrated inducible expression of GP on 293 T-REx cells, we then sought to use these cells to produce reporter viruses pseudotyped with different amounts of GP. Previous studies found that pseudotypes bearing the EBOV-GP are an adequate model to study EBOV-GP-dependent cellular entry under standard biosafety conditions (Takada et al., 1997; Wool-

Lewis and Bates, 1998; Yang et al., 1998). To this end, a proviral HIV-1 reporter genome was transiently expressed in 293 T-REx cells lines and the cells induced to produce high and low levels of GP. Western blot analysis of pelleted viral supernatants revealed that viruses produced in the presence of 100 ng/ml doxycycline had indeed incorporated readily detectable amounts of GP (Fig. 1D). In contrast, little or no GP was detected in pelleted supernatants when GP expression was induced with 100-fold less doxycycline or no doxycycline (Fig. 1D). Thus, stable introduction of EBOV-GP expression constructs into 293 T-REx allowed controlled expression of GP, which in turn enabled us to generate reporter viruses harboring different amounts of GP.

Differential induction of cell rounding upon expression of the GPs of the four EBOV subspecies

Expression of EBOV-GP alters cellular functions, a property that might contribute to EBOV pathogenesis. One consequence of GP expression is cell rounding and subsequent detachment from culture plates (Chan et al., 2000; Simmons et al., 2002; Takada et al., 2000; Yang et al., 2000). It has previously been reported that expression of ZEBOV- and SEBOV-GP triggers cell rounding with high efficiency, while this effect was less pronounced upon expression of REBOV-GP (Simmons et al., 2002). We investigated if induced expression of the GPs of the four EBOV subspecies triggered detachment of 293 T-REx cells from culture plates. In agreement with previous results (Simmons et al., 2002), we found that doxycycline induced expression of ZEBOV- and SEBOV-GP caused substantial cell detachment in a dose-dependent fashion (Fig. 2). In contrast, floating cells were only detected upon maximal induction of REBOV-GP expression, and no appreciable cell detachment was observed upon expression of ICEBOV-GP (Fig. 2). Thus, our observations generally confirm previous reports (Chan et al., 2000; Simmons et al., 2002; Takada et al., 2000; Yang et al., 2000) and indicate that the GPs expressed upon induction in 293 T-REx cell lines exhibit comparable cytopathic properties as GPs conventionally expressed upon transient transfection.

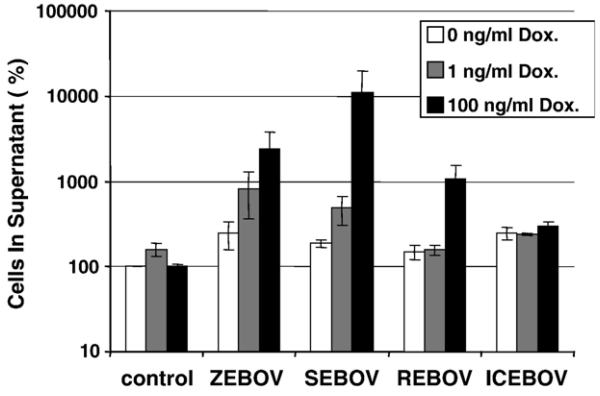

Fig. 2. The GPs of the four EBOV subspecies induce detachment of 293 T-REx cells from culture plates with differential efficiencies. EBOV-GP expression by 293 T-REx cells was induced with the indicated concentrations of doxycycline and the number of cells floating in the culture supernatants 48 h after induction was determined by FACS. Results represent the average of three independent experiments, error bars indicate SEM. In a representative experiment performed in quadruplicates, induction with 100 ng/ml doxycycline resulted in detachment of 696 ± 169 control cells, 13284 ± 5080 ZEBOV-GP T-REx cells, 20545 ± 7036 SEBOV-GP T-REx cells, 5320 ± 866 REBOV-GP T-REx cells and 2169 ± 761 ICEBOV-GP T-REx cells.

Relatively low levels of virion incorporation suffice for efficient EBOV-GP-driven infection

The level of virion incorporation of GP can determine the efficiency of HIV-1 and SIV entry into susceptible cells (Bachrach et al., 2005; Yuste et al., 2004, 2005). We employed reporter viruses harboring different amounts of EBOV-GP to assess if the efficiency of EBOV-GP-driven entry also depends on the amount of GP present in the viral envelope. Reporter viruses encoding the luciferase gene and bearing different levels of EBOV-GP were produced as described above. As a control, reporter viruses bearing vesicular stomatitis virus glycoprotein (VSV-G) were produced upon cotransfection of constant amounts of plasmids encoding VSV-G and the reporter virus genome (both expressed constitutively) into parental 293 T-REx cells, which were subsequently incubated with the indicated doxycycline concentrations (Fig. 3). All virus stocks were normalized for equal content of p24-capsid antigen and used for infection of 293T and Huh-7 cells, which are both susceptible to EBOV-GP and VSV-G-driven infection. Cells infected with these reporter viruses express luciferase under control of the viral promoter and only upon successful integration of the proviral genome into the cellular genome. Therefore, luciferase activities in lysates of reporter virus inoculated cells are an adequate parameter for infection efficiency. All EBOV-GP bearing viruses generated in the absence of doxycycline and thus in the absence of appreciable GP expression were noninfectious (Fig. 3). Thus, the low levels of GP present on uninduced ZEBOV-GP 293 T-REx cells (Fig. 1B) and possibly the other GP transfected cells were not sufficient to support infectious cellular entry of viruses produced in these cells, because either no GP or insufficient GP copies were incorporated into budding virions. In contrast, reporter viruses produced by cells in which GP expression was induced by 1 and 100 ng/ml doxycycline were highly infectious for both 293T and Huh-7 cells (Fig. 3). In the case of ZEBOV- and SEBOV-GP, no substantial difference in infectivity was observed between viruses produced by cells induced with 1 or 100 ng/ml doxycycline. In contrast, ICEBOV-GP and particularly REBOV-GP bearing viruses were reproducibly up to 10-fold more infectious when GP expression in virus producing cells was induced by 100 instead of 1 ng/ml doxycycline (Fig. 3). This doxycycline-dependent increase in GP expression, virion incorporation (Fig. 1) and infectivity suggests that a relatively high number of virion associated REBOV- and ICEBOV-GP copies might be necessary for full infectivity. However, the infectivity of reporter viruses bearing VSV-G also increased slightly upon addition of rising amounts of doxycycline to virus producing cells (Fig. 3). Thus, albeit EBOV-GP and VSV-G bearing viruses were generated under differential conditions (EBOV-GP expression was induced by doxycycline while VSV-

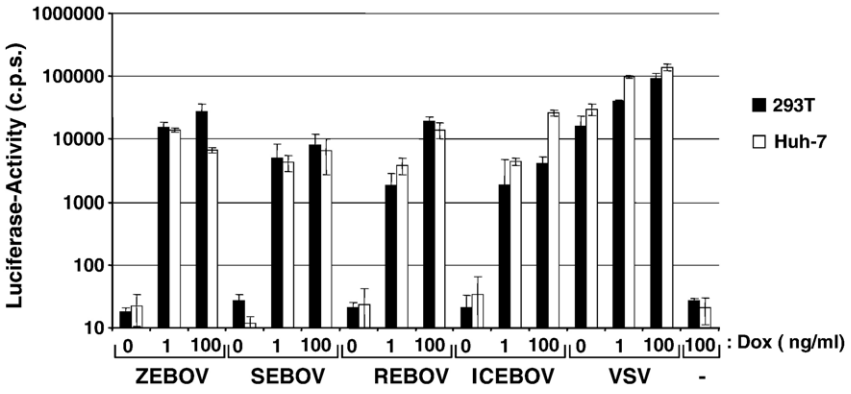

Fig. 3. Impact of the efficiency of GP incorporation into virions on viral infectivity. Reporter viruses bearing different amounts of the GPs of the four EBOV subspecies were produced as described in the legend to Fig. 1. VSV-G harboring control viruses were generated upon cotransfection of parental 293 T-REx cells with plasmids encoding VSV-G and the reporter virus genome (both designed for constitutive expression). Equal volumes of viral supernatants containing 0.1 ng of p24-capsid antigen were added to 293T and Huh-7 cells and the luciferase activities in cellular lysates determined 3 days after infection. The results of a representative experiment performed in triplicates are shown, error bars indicate standard deviation (SD). Comparable results were obtained in two separate experiments performed with independent virus stocks.

G expression was doxycycline-independent) and can therefore not be directly compared, these results indicate that the increased infectivity of viruses bearing high amounts of REBOV- and ICEBOV-GP could at least in part be due to unspecific effects. We finally analyzed if induction of GP expression by doxycycline concentrations of less than 1 ng/ml resulted in reporter viruses with intermediate infectivity, but found that viruses produced under these conditions were barely or non infectious (data not shown). In summary, we conclude that a substantial range of virion associated GP copy numbers allows for constant and robust infectivity of EBOV-GP bearing pseudotypes.

Optimal usage of cellular lectins for augmentation of infectivity requires high levels of EBOV-GP incorporation into virions

Several cellular lectins recognize carbohydrates in EBOV-GP and augment infectious viral entry into already permissive cells (Alvarez et al., 2002; Becker et al., 1995; Gramberg et al., 2005; Lin et al., 2003; Simmons et al., 2003; Takada et al., 2004). In some cases, EBOV-GP engagement of lectins might even be sufficient to allow infection of otherwise nonpermissive cells (Alvarez et al., 2002), albeit this concept is currently under debate. In any event, filovirus binding to cellular lectins might promote viral spread in infected patients and the parameters controlling this interaction merit investigation. We asked if the efficiency of EBOV-GP interactions with lectins depends on the level of GP incorporation into virions. In order to address this question, we infected permissive 293 T-REx cells expressing lectins known to augment filovirus infection with reporter viruses bearing different amounts of EBOV-GP. VSV-G bearing viruses were produced as described above and employed as controls. All input viruses were normalized for comparable infection of parental 293 T-REx cells, thereby allowing direct comparison of lectin mediated augmentation of infection. As expected from previous reports (Alvarez et al., 2002; Becker et al., 1995; Lin et al., 2003; Simmons et al., 2003; Takada et al., 2004), all lectins examined enhanced infection driven by the GPs of the four EBOV subspecies, albeit with differential efficiency, while lectin expression did not appreciably modulate VSV-G-dependent infection (Fig. 4). Lectin engagement by ZEBOV- and REBOV-GP bearing reporter viruses was moderately but consistently enhanced upon incorporation of high amounts of GP into virions (Fig. 4), suggesting that the number of ZEBOV- and REBOV-GP trimers incorporated in the viral envelope can limit the interaction with lectins. In contrast, a less pronounced effect was observed with SEBOV-GP bearing viruses and virion incorporation of ICEBOV-GP, which generally interacted with lectins with low efficiency, did not seem to modulate lectin usage (Fig. 4), suggesting that other constrains might limit the lectin engagement of these GPs. Thus, in some cases, the number of EBOV-GP copies in the viral envelope can modulate virus capture by cellular lectins and might impact viral spread in lectin expressing cells.

Virion incorporation of high amounts of GP increases neutralization sensitivity

The filovirus GP is the major target for neutralizing antibodies. For SIV it has been observed that the number of GP copies incorporated into the viral envelope can modulate the efficiency of antibody mediated neutralization (Yuste et al., 2005). We compared neutralization of reporter viruses bearing low and high amounts of ZEBOV-GP by a rabbit serum raised against ZEBOV-GP. VSV-G bearing viruses produced in the presence of doxycycline were used as controls, and all viruses were normalized for comparable infection of 293 T-REx cells in the absence of antiserum. As expected, VSV-G-driven infection could not be blocked by the antiserum (Fig. 5), and several control sera had no appreciable effect on ZEBOV-GP-dependent infection (data not shown). In contrast, incubation of ZEBOV-GP bearing viruses with antiserum before their addition to target cells diminished infection efficiency, albeit with moderate efficiency. Notably, at a serum dilution of 1:25, but not at higher dilutions, the infection by viruses bearing relatively high amounts of ZEBOV-GP was more efficiently

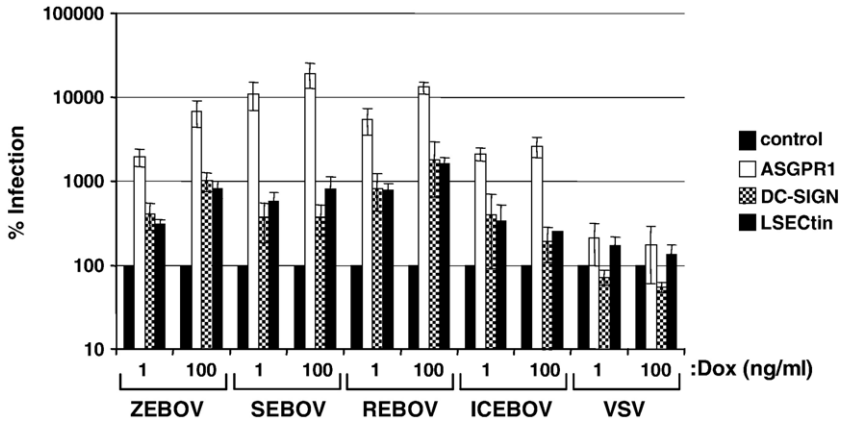

Fig. 4. Virion incorporation of high amounts of ZEBOV- and REBOV-GP is required for optimal engagement of attachment factors. Reporter viruses bearing different amounts of the GPs of the four EBOV subspecies were produced as described in the legend to Fig. 1. VSV-G harboring control viruses were generated upon cotransfection of parental 293 T-REx cells with plasmids encoding VSV-G and the reporter virus genome. Viruses were normalized for comparable infection of parental 293 T-REx cells and employed to infect 293 T-REx cell lines expressing the indicated lectins. Luciferase activities in cellular lysates were determined three days after inoculation, and values obtained upon infection of parental 293 T-REx cells were set as 100%. The results represent the average of two to five independent experiments carried out in triplicates. Error bars indicate SEM.

reduced than infection with viruses bearing relatively low amounts of ZEBOV-GP (Fig. 5, 66% reduction compared to 36% reduction, average of three experiments with independent virus stocks), suggesting that the GP density in the viral envelope might impact EBOV susceptibility to neutralizing antibodies.

Expression of ZEBOV-GP in target cells does not interfere with ZEBOV-GP-dependent entry but blocks VSV-G-driven infection in a dose-dependent manner

The expression of viral GPs can interfere with surface expression of the respective viral receptors. For example, the HIV-1 Env traps the CD4 receptor in the endoplasmatic reticulum, thereby leading to reduced expression of CD4 on the cell surface (Hoxie et al., 1986; Martin and Nayak, 1996a, 1996b). Therefore, induction of EBOV-GP expression in permissive 293 T-REx cells might downmodulate the so far unidentified EBOV receptor(s) and might reduce EBOV-GP-driven entry. We investigated this possibility by infecting ZEBOV-GP expressing and control 293 T-REx cells with pseudotypes bearing ZEBOV-GP, VSV-G or the GP of amphotropic murine leukemia virus (A-MLV). Treatment of control 293 T-REx cells with different doses of doxycycline had no appreciable impact on infection by all pseudotyped viruses tested (Fig. 6, left panel). Similarly, doxycycline mediated induction of ZEBOV-GP expression in 293 T-REx cells did not alter susceptibility to ZEBOV-GP-dependent infection (Fig. 6, right panel). However, expression of ZEBOV-GP reduced VSV-G and A-MLV-GP-driven entry in a dose-dependent fashion (Fig. 6, right panel), suggesting that ZEBOV-GP production

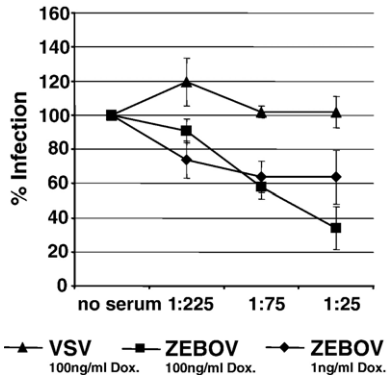

Fig. 5. Increasing the amount of GP on the virion surface increase neutralization sensitivity. Reporter viruses bearing different amounts of ZEBOV-GP were produced as described in the legend to Fig. 1. VSV-G harboring control viruses were generated upon cotransfection of parental 293 T-REx cells (induced with 100 ng/ml doxycycline) with plasmids encoding VSV-G and the reporter virus genome. Viruses were normalized for comparable infection of 293T cells. Equal volumes of virus stocks of comparable infectivity were incubated with ZEBOV-GP-specific rabbit serum for 30 min at 37 °C and subsequently added to 293T cells. Luciferase activities in cellular lysates were measured 3 days after infection. The average of three separate experiments performed in triplicates with independent virus stocks are shown. Error bars indicate SEM.

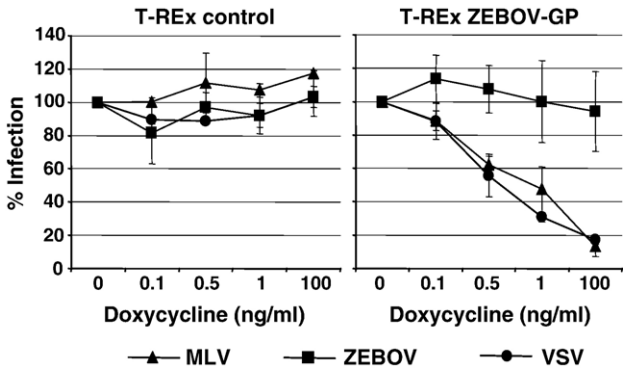

Fig. 6. Expression of ZEBOV-GP blocks VSV-G and A-MLV-GP but not ZEBOV-GP-driven entry. Parental 293 T-REx cells (left panel) and ZEBOV-GP transfected 293 T-REx cells (right panel) were induced with the indicated amounts of doxycycline and the cells inoculated with reporter viruses bearing VSV-G, A-MLV-GP or ZEBOV-GP. Luciferase activities in cellular lysates were determined 3 days after infection. The average of three independent experiments is presented, error bars indicate SEM.

might interfere with cellular factors required for VSV-G and A-MLV-GP-dependent infection.

Discussion

EBOV-GP mediates the first indispensable step in the viral life cycle, virus entry into cells, and is the major target of neutralizing antibodies. However, it is unclear if the level of EBOV-GP incorporation into virions impacts the interaction with cellular factors and the susceptibility to neutralizing antibodies. By employing reporter viruses bearing low and high amounts of EBOV-GP, we provide evidence that increased GP incorporation into virions can augment engagement of cellular attachment factors and increases neutralization by antibodies. Moreover, we show that EBOV-GP expression does not reduce susceptibility to EBOV-GP-dependent infection, suggesting that GP does not interfere with expression of its cognate cellular receptor(s). However, EBOV-GP expression diminished VSV-G and A-MLV-GP-driven infectious entry and might therefore modulate cellular functions exploited by VSV, A-MLV and possibly other viruses for infectious entry.

We employed a doxycycline inducible system in order to achieve regulated production of EBOV-GP. Upon transfection and subsequent selection with antibiotics, 293 cell lines were obtained that express the GPs of the four EBOV subspecies in a doxycycline-dependent manner (Figs. 1A–C). Comparable induction of GP expression upon doxycycline induction was observed over several passages, and the cells could be employed to generate reporter viruses bearing different amounts of GP (Fig. 1D). Thus, the generated cell lines constitute valuable tools to assess GP function. Despite the clear induction of GP expression upon addition of doxycycline to cell lines, we only managed to induce relatively low and relatively high amounts of GP but failed to consistently induce a range of intermediate levels of expression. This limitation might be in part inherent to the particular inducible expression system used, which was mainly designed to guarantee either complete shut down or full expression of the gene of interest. More recently generated tetracycline inducible systems allow efficient induction of intermediate expression of the gene under study (Berens and Hillen, 2004) and might be more suitable for fine regulation of GP expression. Finally, we note that reduction of doxycycline from 100 to 1 ng/ml resulted in clearly diminished GP expression as judged by Western blot, while FACS analysis revealed only a moderated decrease of GP expression on the cell surface (compare Figs. 1A and B, C). Once a certain level of GP expression is reached, the protein might thus accumulate preferentially in intracellular compartments and to a lesser degree at the cell surface. Remarkably, the efficiency of GP incorporation into lentiviral particles seemed to correlate roughly with the amount of GP present in cell lysates and not with surface expressed GP, albeit we cannot exclude that the apparent reduction of virion incorporation of GP was partly exacerbated by a nonlinear decrease of reactivity of the antiserum upon analysis of very low amounts of GP. In any event, these observations raise the possibility that a substantial fraction of virion incorporation of GP occurred intracellularly. In fact, Sandrin and colleagues observed that incorporation of certain heterologous GPs into retro- and lentiviral particles can occur in late endosomes (Sandrin et al., 2004; Sandrin and Cosset, 2006).

Expression of ZEBOV- and SEBOV-GP was previously shown to cause cell rounding and detachment of cells from culture plates, while the effects of REBOV-GP on cellular attachment to culture plates were less profound (Simmons et al., 2002). These observations were made with cell lines transiently expressing GP or cells infected with adenovirus encoding GP. Similarly, in 293 T-REx cells, which express GP upon induction with doxycycline, profound cellular detachment was observed upon induction of ZEBOV- and SEBOV-GP expression, whereas REBOV-GP and ICEBOV-GP were less active in this system (Fig. 2). It should be noted, however, that in the T-REx cell system, SEBOV-GP was more active compared to ZEBOV-GP, at least under conditions of maximum induction of GP expression, while the reverse finding was made with transiently transfected cells (Simmons et al., 2002). Differences in GP expression levels might account for this discrepancy. Thus, GP produced in 293 T-REx cells generally resembled GP generated in widely used transient systems in its ability to diminish cellular attachment to culture plates, indicating that during selection of stable cells no GP variants with reduced interference with cellular adhesion functions were selected.

Cellular entry of enveloped viruses is brought about by a series of complex interactions of the viral envelope GP with cellular factors (Dimitrov, 2004; Smith and Helenius, 2004). GP engagement of cellular receptors activates GP-driven fusion of the viral and a cellular membrane, which ultimately results in the introduction of the viral genome into the cellular cytoplasm. Albeit several candidate molecules have been proposed (Chan et al., 2001; Takada et al., 2000), the cellular receptor for EBOV remains elusive and the processes activating GP-driven membrane fusion are therefore incompletely understood. Our results indicate that a range of GP copies presented on the surface of virions is sufficient to allow entry into cells with comparably high efficiency (Fig. 3). Therefore, even EBOV particles bearing relatively few copies of GP might be highly infectious, which would have implications for strategies aiming at the inhibition of GP function (as discussed below).

In contrast to GP interactions with receptors, which are essential for infectious entry into cells, GP binding to so-called attachment factors can strongly augment infection efficiency but is ultimately dispensable for entry (Baribaud et al., 2002). Binding of filovirus GPs to certain cellular lectins has been shown to augment infectious entry into already permissive cells (Alvarez et al., 2002; Becker et al., 1995; Lin et al., 2003; Simmons et al., 2003; Takada et al., 2004). Lectin mediated enhancement of EBOV infectivity might be due to concentration of virions on the cell surface and therefore increased entry via so far unidentified cellular receptors, albeit in some instances lectins might serve as true viral receptors (Alvarez et al., 2002). It has been shown that the number of lectin copies expressed on target cells and the presence, and most likely adequate spatial distribution, of appropriate carbohydrates on the EBOV-GP surface can determine the interaction with lectins

(Lin et al., 2003; Simmons et al., 2003). Here, we provide evidence that also the number of EBOV-GP copies inserted into the viral envelope can impact lectin engagement, with viruses bearing a higher number of GP molecules being more adept in engagement of lectins (Fig. 4), a result we confirmed for ZEBOV-GP in a transient system, as part of a separate study (Marzi et al., 2006). This correlation was particularly evident for viruses bearing ZEBOV- and REBOV-GP, while the level of ICEBOV-GP virion incorporation did not seem to alter interactions with cellular lectins (Fig. 4). Lectin binding of ICEBOV-GP bearing viruses was generally weak. Since these viruses attached robustly to control cells (data not shown) determinants in ICEBOV-GP might specifically prevent the engagement of certain lectins. Indeed, forced incorporation of high mannose carbohydrates into ICEBOV-GP allowed efficient interactions with the mannose, fucose-specific lectin DC-SIGN (data not shown), indicating that the carbohydrate modifications of ICEBOV-GP could limit lectin engagement. In summary, these observations suggest that binding of multiple GP trimers to cellular lectins might in some instances be required for optimal augmentation of EBOV infection by lectins.

Recently described vaccines that provide efficient protection against filovirus infection induce cellular as well as humoral immune responses (Jones et al., 2005; Warfield et al., 2005), and both might be required to control infection. In one study, the vaccine induced humoral immune response was clearly more prominent than the cellular response but did not efficiently neutralize the virus in in vitro systems (Jones et al., 2005). Therefore, it was suggested that a vigorous albeit nonneutralizing antibody response might be sufficient to provide protection against filovirus infection (Jones et al., 2005). Nevertheless, production of neutralizing antibodies upon vaccination is obviously desirable, and the parameters determining neutralization efficiency are of interest. We observed that the amount of GP incorporation into virions affects neutralization sensitivity, with virions bearing more GP being more susceptible to neutralization, at least when neutralization by a low dilution of antiserum was assessed (Fig. 5). Notably, the inverse correlation has been documented for a SIV variant, for which infectivity and resistance to neutralization increased with the amount of virion associated Env protein (Yuste et al., 2005). The latter observation can be explained in a straight forward fashion with more Env trimers on the virion surface requiring a higher amount of antibodies for their inactivation (Yuste et al., 2005). However, it cannot be excluded that antibodies must recognize most or all SIV Env copies on the virion surface in order to inhibit infection, while antibody binding of a few copies of virion associated EBOV-GP might be sufficient to block entry. In this case, particles harboring low amounts of GP would be less efficiently recognized by serum and therefore more resistant to neutralization, despite displaying equal infectivity as particles bearing high amounts of GP (Fig. 3).

Retroviruses (Levesque et al., 2004; Ruibal-Ares et al., 2004) and several other viruses (Breiner et al., 2001; Horga et al., 2000; Kuba et al., 2005; Naniche et al., 1993; Schneider-Schaulies et al., 1995; Yamate et al., 2005) have evolved strategies to negatively regulate the surface expression of their receptors on infected cells, most likely to avoid superinfection and to ensure that progeny particles detach efficiently from infected cells. One mechanism that causes downregulation of the HIV-1 receptor CD4 from the cell surface is Env mediated trapping of CD4 in the endoplasmatic reticulum (Hoxie et al., 1986; Martin and Nayak, 1996a, 1996b). Expression of EBOV-GP leads to downmodulation of many surface molecules (Simmons et al., 2002), including β1-integrins and α5β3 integrin (Sullivan et al., 2005; Takada et al., 2000), and members of the former group of integrins have been proposed to function as EBOV receptors (Takada et al., 2000). In our hands induced expression of ZEBOV-GP in 293 T-REx cells did not interfere with ZEBOV-GP-driven infection (Fig. 6), suggesting that in this cellular system GP does not appreciably modulate expression of its receptor. However, ZEBOV-GP expression caused a profound and dose-dependent reduction of VSV-G and A-MLV-GP-driven infection, suggesting that ZEBOV-GP interferes with the expression and/or function of cellular factors required for VSV-G and A-MLV-GP-dependent entry. It has been suggested that phosphatidylserine, an ubiquitous membrane lipid present mainly in the inner leaflet of the plasma membrane, is an entry receptor for VSV (Hall et al., 1998; Schlegel et al., 1983; Schlegel and Wade, 1983). Albeit a function of phosphatidylserine as VSV receptor has been challenged (Coil and Miller, 2004; Coil and Miller, 2005), an important role of this lipid in VSV entry, possibly after receptor engagement, is likely, and it can be speculated that EBOV-GP might alter the phosphatidylserine content of cellular membranes. Similarly, EBOV-GP might modulate surface expression of the multimembrane-spanning phosphate transporter Pit2, the entry receptor for A-MLV (Kavanaugh et al., 1994; Miller et al., 1994; van Zeijl et al., 1994). Another potential explanation for the interference of ZEBOV-GP expression with VSV-G and possibly also A-MLV-GP-driven entry is the recently documented GP interaction with the GTPase dynamin, which is involved in the release of vesicles from lipid rafts into the cellular cytoplasm (Conner and Schmid, 2003). Since VSV-G mediated infection involves virion endocytosis in a probably dynamin-dependent fashion (Daecke et al., 2005; Lee et al., 1999) and subsequent fusion with endosomal membranes, it is conceivable that GP interference with dynamin function might inhibit VSV-G-dependent cellular entry. A-MLV is believed to fuse with the plasma membrane (McClure et al., 1990), but endocytic entry of A-MLV has recently been documented (Beer et al., 2005) and dynamin might be involved in this process. However, dynamin is required for both, entry via clathrin coated pits, the route employed by VSV (Superti et al., 1987), and for cellular uptake of viruses via caveolae, the route used by EBOV-GP bearing pseudotypes (Empig and Goldsmith, 2002) and A-MLV, at least for uptake into NIH 3T3 cells (Beer et al., 2005). Therefore, one would need to argue that EBOV-GP selectively interferes with dynamin functions required for VSV-G and A-MLV-GP but not EBOV-GP-dependent entry.

In summary, our data provide evidence that the number of EBOV-GP copies incorporated into virions might affect engagement of cellular lectins and susceptibility to antibody

mediated neutralization. Moreover, our results suggest that a relatively broad range of virion associated GP copies might mediate highly efficient cellular entry of virions. In the light of these observations, it would be interesting to investigate if the GP content of filoviruses depends on, e.g., the viral isolate, the producer cell type or the stage of infection and if differences in the amount of virion associated GP have functional consequences. These undertakings require access to P4 facilities and are beyond the scope of the present study.

Material and methods

Plasmid construction, cell culture and generation of stable EBOV-GP expressing cell lines

The ORFs of the four different Ebolavirus glycoproteins (EBOV-GPs) were generated by insertion of these genes in pcDNA4/TO (Invitrogen, CA, USA) via HindIII and XbaI as described (Gramberg et al., 2005). 293 T-REx cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL, Eggenstein, Germany) supplemented with 10% FCS, penicillin, streptomycin and 2.5 μg/ml blasticidin (Invitrogen, CA, USA). 293 T-REx cell lines stably expressing EBOV-GPs were generated by transfection with the indicated expression plasmids followed by selection for 2 weeks in DMEM containing 10% FCS, penicillin, streptomycin, 2.5 μg/ml blasticidin and 50 μg/ml zeocin (Invitrogen, CA, USA). Lectin expressing T-REx cell lines were also maintained in DMEM supplemented with FCS, penicillin, streptomycin, blasticidin and zeocin as described previously (Simmons et al., 2003). EBOV-GP and lectin expression on T-REx cells was induced with doxycycline (Sigma, Deisenhofen, Germany) at final concentrations of 0.1, 0.5, 1 or 100 ng/ml. 293T and Huh-7 cells were propagated in DMEM supplemented with 10% FCS, penicillin and streptomycin.

Western blot and FACS analysis

Western blot analysis was performed with equal amounts of 293 T-REx cells induced to express different levels of EBOV-GP for 24 h or with concentrated viral supernatants containing 2.5 ng capsid protein (p24) antigen as determined by enzyme-linked immunosorbent assay (ELISA; Murex; Abbott Diagnostics, IL, USA). Viruses were pelleted by centrifugation for 2 h at 14,000 rpm at 4 °C, lysed in SDS Laemmli buffer and boiled at 95 °C for 15 min. Cells were harvested, also lysed in SDS Laemmli buffer and boiled at 95 °C for 15 min. Proteins were then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, employing 10% PAA gels), transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and blocked o/n at 4 °C in 5% skim milk in PBS supplemented with 0.1% Tween. EBOV-GP and HIV-1 p24-antigen were detected with polyclonal rabbit sera used at a dilution of 1:1000 (the EBOV-GP-specific serum was raised against ZEBOV-GP) and appropriate secondary antibody coupled to peroxidase. Chemoluminescence detection was performed according to the manufacturer's protocol (Amersham Pharmacia Biotech Europe, Freiburg, Germany).

For FACS analysis of GP expression the indicated EBOV-GP 293 T-REx cell lines were induced to express different levels of GP over a period of 24 h, harvested and incubated with the polyclonal anti GP rabbit serum at a 1:150 dilution in a total volume of 100 μl FACS buffer (PBS with 3% FCS; 0.01% NaN_3) for 30 min at 4 °C. After a washing step, the bound antibody was detected by incubating the cells in 100 μl FACS buffer containing 0.5 μg of anti-rabbit-FITC (Dianova, Hamburg, Germany). Antibody binding was allowed to proceed for 30 min at 4 °C, then cells were washed, and EBOV-GP surface expression was quantitated by FACS analysis employing a FACSCalibur flow cytometer (Becton Dickinson, NJ, USA).

Assessment of EBOV-GP induced detachment of cells from culture flasks

2×10^5 cells per well of EBOV-GP transfected 293 T-REx cells were seeded into 12-well plates, GP expression was induced by different concentrations of doxycycline, and after 48 h, the supernatants were collected. Floating cells in the supernatants were pelleted by centrifugation, washed for two times in FACS buffer and counted by FACS analysis using a FACSCalibur flow cytometer (Becton Dickinson, NJ, USA).

Production of pseudotyped reporter viruses, infection assays and inhibition with ZEBOV-GP-specific antiserum

Pseudotypes harboring different amounts of EBOV-GP were generated by transfection of pNL4-3-Luc-R^-E^- (Connor et al., 1995) into EBOV-GP 293 T-REx cell lines, which were induced to express GP 1 h before transfection. Pseudotypes bearing constant, high amounts of GP were generated by cotransfection of 293T cells with pNL4-3-Luc-R^-E^- and ZEBOV-, A-MLV-GP or VSV-G expression plasmids. 48 h after transfection the culture supernatants were harvested, passed through 0.4 μm-pore-size filters, aliquoted and stored at −80 °C. For subsequent infection experiments, the supernatants were either normalized for comparable content of HIV-1 p24-antigen employing antigen capture ELISA (Murex; Abbott Diagnostics, IL, USA) or normalized for comparable infection of control cells, which was determined by assessing luciferase activities in the lysates of infected cells employing a commercially available kit as recommended by the manufacturer (Promega, WI, USA). Infections were carried out by seeding the indicated cell lines onto 96-well plates at a density of 1×10^4 per well followed by addition of virus normalized either for infectivity or p24-antigen content in a total volume of 100 μl per well. For serum inhibition experiments, viruses were preincubated with a polyclonal rabbit serum raised against ZEBOV-GP at the indicated dilutions for 30 min prior to infection. For analysis of interference of ZEBOV-GP expression with permissiveness to VSV-G, A-MLV-GP or ZEBOV-GP-driven infection, GP expression was induced in target cells 12 h prior to infection. For assessment of lectin mediated augmentation of EBOV-GP-dependent infection, lectin expression was induced on T-REx

target cells with 100 ng/ml doxycycline 12 h before addition of viral supernatants. Cells were incubated with virus for 12 h, and thereafter, medium was replaced and luciferase activities were determined 72 h after infection with a commercially available kit (Promega, WI, USA).

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