Ebola virus glycoprotein GP is not cytotoxic when expressed constitutively at a moderate level

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Transient expression of Ebola virus (EBOV) glycoprotein GP causes downregulation of surface proteins, cell rounding and detachment, a phenomenon believed to play a central role in the pathogenicity of the virus. In this study, evidence that moderate expression of GP does not result in such morphological changes was provided. It was shown that GP continuously produced in 293T cells from the Kunjin virus replicon was correctly processed and transported to the plasma membrane without affecting the surface expression of β1 and α5 integrins and major histocompatibility complex I molecules. The level of GP expression in Kunjin replicon GP-expressing cells was similar to that observed in cells infected with EBOV early in infection and lower than that produced in cells transfected with plasmid DNA, phCMV-GP, expressing GP from a strong promoter. Importantly, transient transfection of Kunjin replicon GP-expressing cells with GP-coding plasmid DNA resulted in overexpression of GP, which lead to the downregulation of surface molecules and massive rounding and detachment of transfected cells. Here, it was also demonstrated that cell rounding and downregulation of the surface markers are the late events in EBOV infection, whereas synthesis and massive release of virus particles occur at early steps and do not cause significant cytotoxic effects. These findings indicate that the synthesis of EBOV GP in virus-infected cells is controlled well by several mechanisms that do not allow GP overexpression and hence the early appearance of its cytotoxic properties.

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INTRODUCTION

Ebola virus (EBOV) is the aetiological agent of a severe haemorrhagic fever with mortality rates up to 90 % (Peters *et al.*, 1996; Pourrut *et al.*, 2005). EBOV and Marburg virus (MARV) constitute the family *Filoviridae* in the order Mononegavirales, a group of enveloped, non-segmented, negative-stranded RNA viruses. Glycoprotein GP is responsible for cell targeting and virus entry by mediating receptor binding and membrane fusion (Chan *et al.*, 2000b; Ito *et al.*, 1999; Yang *et al.*, 1998). GP consists of disulfide-linked GP1 and GP2 subunits generated by proteolytic cleavage of the GP precursor by the cellular subtilisin-like protease furin (Jeffers *et al.*, 2002; Volchkov *et al.*, 1998). Surface GP is a trimer that forms spikes at the surface of infected cells and virions. Significant amounts of surface GP are shed from the surface of infected cells due to cleavage by the cellular metalloprotease tumour necrosis factor alpha-converting enzyme (TACE; Dolnik *et al.*, 2004).

Unlike the GP of the closely related MARV, that of EBOV is encoded by two overlapping reading frames. Expression of EBOV GP requires insertion of a non-templated adenosine residue at the so-called editing site during GP mRNA synthesis (Sanchez *et al.*, 1996; Volchkov *et al.*, 1995). During virus replication, expression of surface GP is therefore limited since most GP gene-specific mRNAs (80 %) direct synthesis of the secreted non-structural glycoprotein, sGP (Sanchez *et al.*, 1998; Volchkova *et al.*, 1998).

Expression of EBOV GP from recombinant adenoviruses or transfected plasmids causes massive rounding and cell detachment in several cell lines including human umbilical vein endothelial cells (HUVEC). These so-called cytotoxic properties of EBOV GP are believed to contribute to the disruption of blood vessel integrity and thus play a major role in the haemorrhagic symptoms developed during the disease (Ray *et al.*, 2004; Yang *et al.*, 2000). Morphological changes observed upon transient expression of GP in cells correlate with downregulation of surface expression of several proteins including certain integrins and immune molecules (Chan *et al.*, 2000b; Simmons *et al.*, 2002; Takada *et al.*, 2000; Yang *et al.*, 2000). Recently, Sullivan *et al.* (2005) demonstrated that GP exerts its toxic effects by altering the

transport of a specific subset of cellular proteins, depending on the cell type, through a process dependent on dynamin, a cellular GTPase involved in protein trafficking. The importance of GP cytotoxicity in viral pathogenesis is however controversial, since direct damage to the microvascular endothelia by virus replication is very limited and only documented in animal models at terminal stages of the disease (Geisbert *et al.*, 2003c; Ryabchikova *et al.*, 1999).

In this study, we demonstrate that massive release of virus particles from EBOV-infected 293T cells precedes the appearance of cytotoxic effects and/or downregulation of cell surface markers. Moreover, using the Kunjin flavivirus self-replicon system (Khromykh & Westaway, 1997), we show that a continuous and moderate expression of EBOV GP does not cause cell rounding or detachment. Cytotoxic effects of GP were however observed in the Kunjin replicon GP-expressing cells when the GP protein was overexpressed from a transiently transfected plasmid DNA encoding the GP gene under the control of a strong promoter, CMV (cytomegalovirus). Taken together, our results suggest that moderate levels of GP expression, as seen in the Kunjin replicon system or at early steps of EBOV infection, may have a limited impact on the functioning of cells.

METHODS

Cell lines and viruses. Human embryonic kidney 293T and Vero E6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS; Eurobio) 100 U penicillin ml^{-1} and 0·1 mg streptomycin ml^{-1}. 293T cells expressing Kunjin replicons were grown in the presence of puromycin (1 μg ml^{-1}).

Recombinant EBOV subtype Zaire strain Mayinga (recEBOe+) has been described previously (Volchkov *et al.*, 2001). Stock virus was propagated in Vero E6 cells and stored at −80 °C. For virus titration, Vero E6 cells were inoculated with different dilutions of culture medium containing EBOV and incubated for 1 h at 37 °C. Fresh medium containing 5 % FCS was then added; cells were cultivated for 7–9 days and subsequently fixed and stained with 10 % formaldehyde containing 0·1 % crystal violet. Cytopathic effect was evaluated under a light microscope and the $TCID_{50}$ was calculated using Karber's formula. 293T cells were infected at an m.o.i. of 4. After incubation with virus for 1 h at 37 °C, fresh medium containing 10 % FCS was added to get 5 % FCS final concentration. Aliquots of medium were collected at different intervals up to 8 days post-infection. Proteins from cells and culture supernatant were collected and analysed separately. Mock-infected cells were used as a negative control.

Recombinant plasmids. The GP gene of Zaire EBOV was extracted from pGEM-mGP8A (Volchkov *et al.*, 1995) using *Bam*HI and *Hin*dIII endonucleases and cloned into the phCMV plasmid linearized with *Bam*HI, a vector designed to express high amounts of the protein of interest (Yee *et al.*, 1994). The restriction sites in the vector and insert were filled-in with Klenow enzyme before ligation. The resulting plasmid was designated phCMV-GP. The GP gene was also cloned into the pIRES2-eGFP plasmid (Clontech), a vector designed for simultaneous expression of the protein of interest and enhanced green fluorescent protein (eGFP). In this case, the GP gene was extracted from phCMV-GP using *Eco*RI endonuclease and introduced into the unique *Eco*RI restriction site of the plasmid vector. The resulting plasmid was designated pIRES2-eGFP-GP.

To generate pKUNrep4-GP, the GP gene was amplified by PCR using primers supplemented with *Mlu*I restriction site (underlined): GP-MluI-F 5′-AAAACGCGTTGCAGTTACCTCGTGATCGATTC-3′ and GP-MluI-R 5′-TTTACGCGTCTAAAAGACAAATTTGCATATACAG -3′. The resulting PCR fragment was cloned into the unique *Mlu*I restriction site of the pKUNrep4 vector (Varnavski *et al.*, 2000). Sequences of all recombinant plasmids were confirmed by sequence analysis.

Establishment of cell lines expressing Kunjin virus replicons. 293T cells were transfected with pKUNrep4 or pKUNrep4-GP using Exgen 500 transfection reagent (Euromedex) following the manufacturer's instructions. Briefly, cells grown in 21 cm^2 Petri dishes (~50 % confluence) were transfected with 2 μg plasmid. After 6 h of incubation, the transfection mixture was removed and replaced by culture medium containing 5 % FCS. At 24 h post-transfection, puromycin (1 μg ml^{-1}) was added to the culture medium. After 5 days of cultivation, clones of cells were isolated and screened by Western blot for the expression of Kunjin NS5 protein alone or Kunjin NS5 plus EBOV GP. Clones expressing Kunrep4 and Kunrep4-GP replicons were designated Kun and KunGP, respectively, and were then cultured in the presence of puromycin (1 μg ml^{-1}). Kun14 and KunGP12 cell clones were assayed after 5–10 passages and in total were passaged more than 30 times.

Immunoblotting analysis. 293T cells were lysed in 350 μl (50 μl per cm^2 of cells) of 1× Laemmli sample buffer [2 % SDS, 5 % 2-mercaptoethanol, 13 % (w/v) glycerol and 0·2 % bromophenol blue] and heated for 15 min at 95 °C. Cell lysates were subjected to digestion with EndoH or PNGase F performed following the manufacturer's instructions (New England Biolabs) or used untreated. Proteins were separated on 10 % SDS-PAGE and blotted onto PVDF membrane (Millipore). Primary antidodies used were horse anti-EBOV (1 : 5000), rabbit anti-GP2 of EBOV (1 : 2000), rabbit anti-VP24 of EBOV (1 : 4000), mouse anti-NS5 of Kunjin virus (1 : 50) and rabbit anti-actin antibodies (1 : 100; Sigma). Horseradish peroxidase-conjugated secondary antibodies used were rabbit anti-horse (1 : 50 000; Sigma) and goat anti-rabbit antibodies (1 : 20 000; Dako) and anti-mouse antibodies (1 : 20 000; Dako). Proteins were visualized using SuperSignal West Dura Extended Duration substrate following the manufacturer's instructions (Perbio Science).

RT-PCR. Total RNA was extracted from KunGP12 and KunGP8 cell clones using RNeasy Mini kit (Qiagen). cDNA of the GP gene was generated using the following oligonucleotides: pKUN-F 5′-CAACCTCCCCTTCTACGAGCGGCTC-3′ and pKUN-R 5′-CAAACGCACACCGGCCTTATTCCAAG-3′, and OneStep RT-PCR kit (Qiagen). PCR products were separated by electrophoresis on a 1 % agarose gel, the GP-specific band was extracted from the gel using QIAquick Gel Extraction kit (Qiagen) and then used for sequencing.

Pulse–chase labelling and immunoprecipitation. Pulse–chase analysis was performed as described previously (Volchkov *et al.*, 1998) on KunGP12 cells grown until confluent (approx. 72 h), on 293T cells 16 h post-transfection with phCMV-GP vector as a positive control and with a GFP-expressing plasmid as a negative mock-control. Briefly, cells were starved for 1 h with methionine- and cysteine-free DMEM medium, and were then labelled with 100 μCi (3·7 MBq) ml^{-1} [^{35}S]Promix (Cys–Met) (Amersham Biosciences) for 45 min in the case of the KunGP12 cells or 30 min in the case of transient transfection. After the pulse-labelling, cells were chased for different time intervals. Finally, labelled cells were lysed at 4 °C in Co-IP buffer (1 % Nonidet P-40, 0·4 % sodium deoxycholate, 0·5 % BSA, 5 mM EDTA, 100 mM NaCl, 20 mM Tris/HCl, pH 7·6, 25 mM iodacetamide, 1 mM PMSF). Immunoprecipitation was performed using horse anti-EBOV and rabbit anti-horse antibodies (Sigma) coupled to protein A–Sepharose (Sigma). Samples were separated on 10 % SDS-PAGE and analysed by autoradiography.

Immunofluorescence analysis. Cells grown on glass coverslides were washed twice with PBS, fixed for 5 min with 3 % paraformaldehyde at room temperature, rinsed twice with PBS, incubated with 0·1 M glycine for 10 min at room temperature and then washed with PBS. To avoid non-specific binding, slides were first incubated with blocking buffer [PBS, 2 % BSA, 5 % (w/v) glycerol, 0·2 % Tween 20] for 5 min and then with anti-GP KZ52 antibodies diluted 1 : 250 in blocking buffer for 1 h incubation at room temperature. For intracellular labelling of Kunjin NS5 cytoplasmic protein, cells were permeabilized using PBS containing 0·2 % Triton X-100 for 5 min at room temperature, washed twice and incubated for 1 h with Kunjin anti-NS5 rabbit antibodies. The slides were then treated for 1 h with a mixture of secondary antibodies: fluorescein isothiocyanate (FITC)-coupled rabbit anti-human antibodies (1 : 200) and tetramethylrhodamine isothiocyanate (TRITC)-coupled rabbit anti-mouse antibodies (1 : 300; Dako). Finally, the coverslides were washed and mounted with fluorescent mounting medium (Dako). Microscopic analysis was performed using an Axiovert 200M fluorescence microscope (Zeiss).

Flow cytometry. 293T cells were transfected with phCMV or phCMV-GP and analysed after 24 h of growth. EBOV-infected cells or the mock control were analysed 24 and 48 h post-infection. Kun14 and KunGP12 cells were grown until confluent prior to flow cytometry analysis. Cells were collected, pooled with floating cells and were washed in cold PBS supplemented with 2 % FCS (PBS/FCS). Cells were incubated for 1 h with primary antibodies, washed three times with PBS/FCS and then incubated for 30 min with secondary antibodies. β1 integrin and MHC I molecules were detected using phycoerythrin (PE)-coupled antibodies – CD29 (1 : 10; Becton Dickinson) and HLA-ABC (1 : 12·5; BD Biosciences). α5 integrin was labelled using biotin-conjugated mouse anti-CD49 (1 : 5; BD Biosciences) and followed by incubation with PE-coupled streptavidin (Catlag). EBOV GP was detected by using anti-GP human KZ52 antibodies (1 : 150) followed by incubation with swine anti-human FITC-coupled antibodies (1 : 50; Dako). Cells were analysed using Becton Dickinson or Beckmann Coulter flow cytometers and CellQuestPro software (BD Biosciences).

RESULTS

EBOV infection of 293T cells

To understand better the importance of GP-induced cytotoxicity, we studied EBOV infection in 293T cells, which are known to be especially sensitive to the expression of this viral glycoprotein (Chan *et al.*, 2000a; Simmons *et al.*, 2002; Yang *et al.*, 2000). 293T cells were infected with EBOV at an m.o.i. of 4 and monitored during the following 8 days. At 24 h post-infection, cells did not show any remarkable morphological differences when compared to mock-infected cells (Fig. 1a). A low percentage of floating cells (approx. 10–15 % of total cells) was observed after 48 h post-infection and the number of detached cells did not exceed 25 % even after 6–8 days post-infection. However, most infected cells became rounded between 24 and 48 h post-infection (60–90 %). Indeed, cell rounding was also seen with the mock control although to a lesser extent and later post-infection coinciding with culture medium acidification. Release of virus particles was at maximum during the first 24–48 h of infection and no further increase in virus titres was observed thereafter (Fig. 1b, right panel). Analysis of culture supernatants by Western blot using anti-VP24 and anti-GP2 antibodies also showed that during first 48 h of infection maximal amounts of the viral proteins were released from the cells (Fig. 1b, left panel).

Cytotoxicity caused by transient expression of EBOV GP from plasmids or recombinant adenovirus vectors is known to coincide with alterations in the surface expression of certain cellular molecules, including β1 and α5 integrins but also MHC I proteins in 293T cells (Simmons *et al.*, 2002; Takada *et al.*, 2000). However, only limited data are available concerning cell surface marker expression during EBOV infection (Harcourt *et al.*, 1999). We thus infected 293T cells with EBOV at an m.o.i. of 4 and analysed surface expression of β1 and α5 integrins and MHC I molecules by flow cytometry. At 24 h post-infection the levels of cell surface markers were not yet affected, whereas over 50 % of cells were clearly positive for the surface expression of GP (Fig. 1c). Alteration of the expression of surface markers became detectable only in about 20–30 % of the cells after more than 48 h post-infection when the majority of cells ($>$90 %) were GP-positive. Taken together, these results indicate that significant amounts of GP are produced in virus-infected cells without causing cytotoxic effects and also that cytotoxicity caused by EBOV replication is a relatively late event when compared with systems transiently expressing GP.

Expression of EBOV GP from Kunjin virus replicons

We felt that there was a large difference in the kinetics of protein expression between EBOV infection and cells transfected with the GP-expressing plasmids. In the transient expression system, the synthesis of GP occurs rapidly and reaches its highest level soon after transfection. In contrast, in EBOV-infected cells, synthesis of this protein occurs gradually and the level of expression depends on the virus replication rate. We therefore generated an expression system where the level of protein synthesis could be regulated, but more importantly, was dependent on cell viability. The flavivirus Kunjin (KUN) replicon-based system was previously used to develop stable cell lines continuously expressing different heterologous genes (Khromykh & Westaway, 1997; Varnavski *et al.*, 2000). In this system, KUN replicon RNA is initially synthesized from transfected plasmid DNA containing the replicon coding sequence placed under the control of a CMV promoter (Fig. 2a). Subsequent synthesis of the replicon RNA molecules is directed by the Kunjin non-structural (NS) proteins encoded by the replicon RNA. In the KUN replicon vector pKUNrep4 (Varnavski *et al.*, 2000), genes encoding structural proteins of the Kunjin virus are deleted and replaced by a sequence encoding a polyprotein consisting of fused puromycin acetyl transferase (PAC), Foot-and-mouth disease virus (FMDV)-2A autoprotease and the gene encoding the protein of interest (Fig. 2a). Kunjin virus NS proteins required for replication of the replicon RNA are synthesized independently from the encephalomyelocarditis virus internal ribosome entry site (IRES). To express EBOV GP from

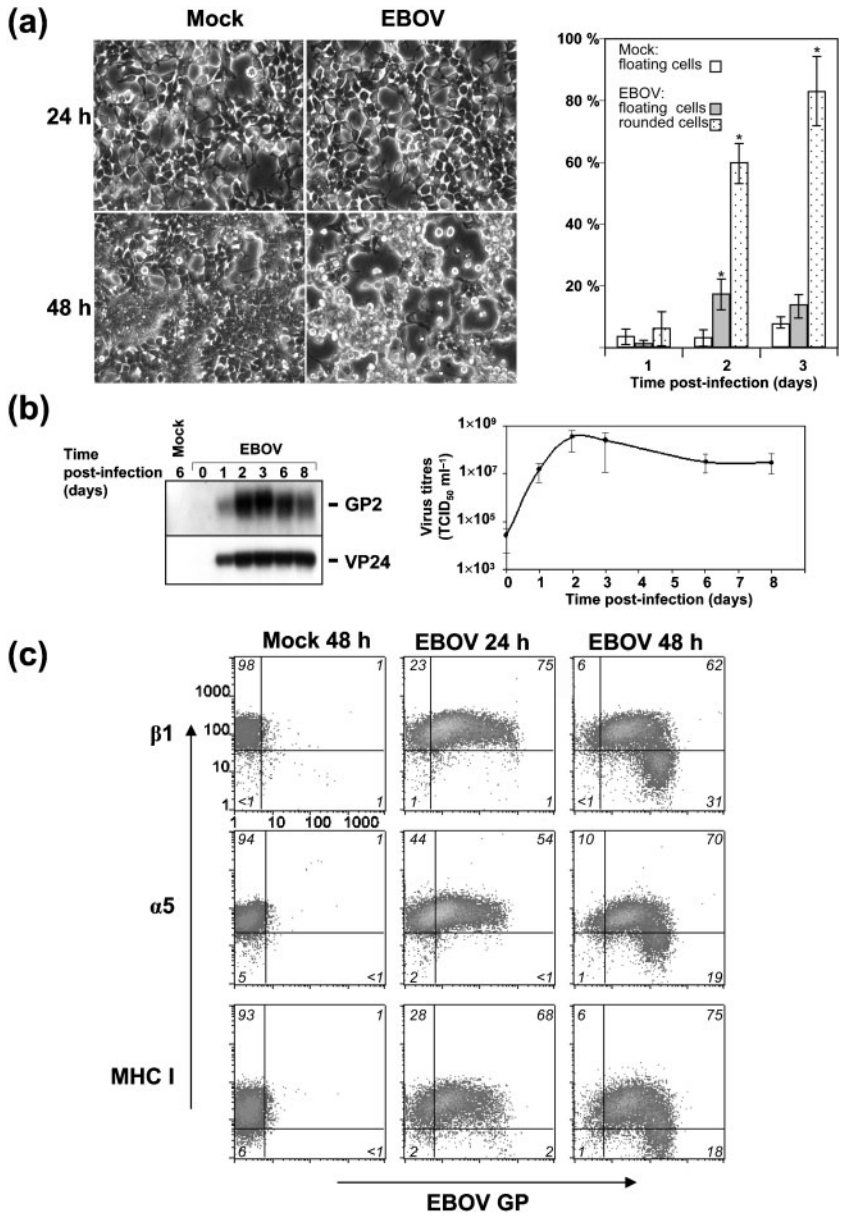

Fig. 1. EBOV infection of 293T cells. 293T cells were infected with EBOV at an m.o.i. of 4 and analysed 24, 48 h and up to 8 days post-infection. (a) Morphology of EBOV- and mock-infected cells was monitored by light microscopy 24 and 48 h post-infection (left panel). Percentage of rounded and floating cells was calculated by counting the cells on representative slides from two independent experiments (right panel). Each bar represents the mean percentage of floating or rounded cells ± SD. Asterisks indicate values that are significantly different from mock-infected cells ($P<0{\cdot}01$, Student's *t*-test). (b) Kinetics of EBOV multicycle growth. Aliquots of culture supernatants were harvested at different intervals until day 8 post-infection. Samples were analysed by Western blot using anti-GP2 and anti-VP24 antibodies (left panel) and by titration on Vero E6 cells (right panel). Data are representative of two independent experiments, mean titres are indicated ± SD. The difference between the experiments was not statistically significant ($P>0{\cdot}05$, paired Student's *t*-test). (c) Effect of EBOV infection on surface expression of selected cell surface markers. EBOV- and mock-infected cells were analysed 24 and 48 h post-infection. A mixture of anti-GP KZ52 antibodies and PE-coupled anti-β1 integrin (β1), biotin-coupled anti-α5 (α5) integrin or PE-coupled anti-MHC I (MHC I) antibodies were used followed by incubation with secondary anti-human FITC-coupled antibodies (and PE-coupled streptavidin for detection of bound biotin-coupled anti-α5 integrin antibodies). The percentage of cells in each quadrant is indicated.

this system, cDNA coding this protein was cloned into the unique *Mlu*I cloning site of the pKUNrep4 vector (Varnavski *et al.*, 2000). GP with the authentic amino acid sequence is generated following proteolytic processing of the fused polyprotein by the FMDV-2A autoprotease. The synthesis of GP with an authentic C terminus is ensured by incorporating the translation termination codon at the end of the GP sequence. Importantly, synthesis of PAC encoded by the KUNrep4 replicon (Fig. 2a) allows establishment of cell lines continuously expressing EBOV GP by selection with puromycin.

First, we analysed expression of GP in 293T cells transfected with pKUNrep4-GP in comparison to cells transfected with phCMV-GP, using the corresponding vectors lacking GP insert as controls. As shown in Fig. 2(b), phCMV-GP caused a strong cytotoxic effect 24 h post-transfection; all cells were floating in the culture medium. In contrast, transient expression of GP from pKUNrep4-GP caused only cell rounding without massive detachment of cells, which resembled EBOV-infected cells 48 h post-infection (Figs 1a and 2b). Western blot analysis demonstrated that about 20 times less GP was expressed from KUNrep4-GP replicon than from phCMV-GP (Fig. 2c). Delayed replication of KUN vectors incorporating the IRES sequence (Varnavski *et al.*, 2000) could contribute to the observed substantial difference in GP expression between pKUNrep4-GP and phCMV-GP vectors early (24 h) in transfection. Cells transfected with Kunjin vectors were then subjected to puromycin selection at a concentration of 1 μg ml^{-1}. Following treatment, several cell clones expressing Kunrep4 or Kunrep4-GP replicons were isolated and named Kun and KunGP,

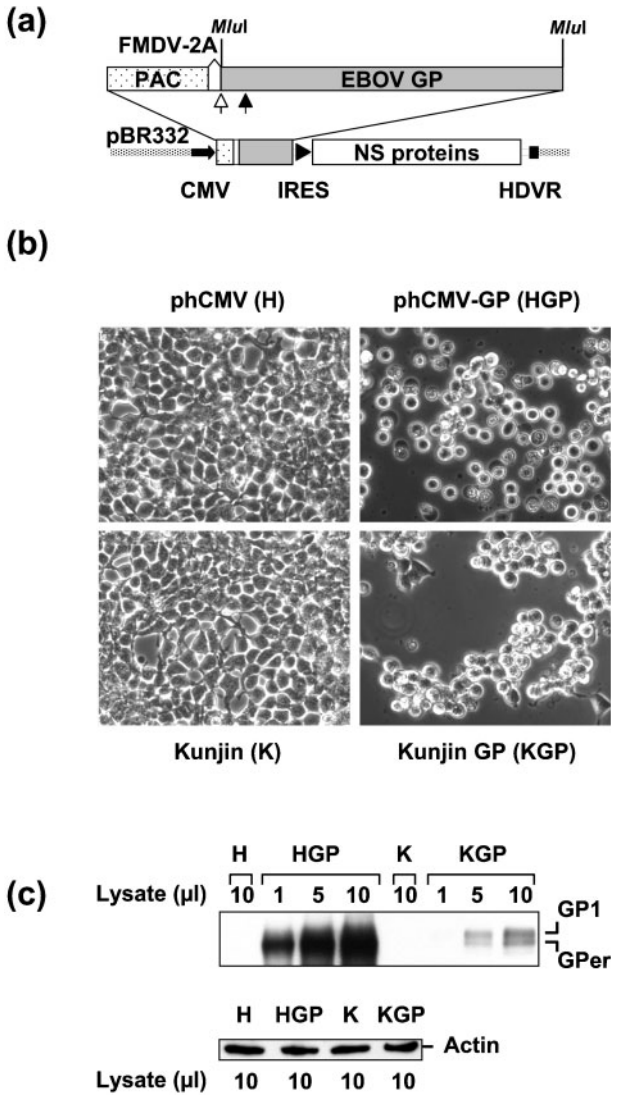

Fig. 2. Transient expression of EBOV GP using pKUNrep4-GP. (a) Schematic representation of the pKUNrep4-GP replicon vector. Boxes indicate translated regions of the KUN replicon. CMV, Eukaryotic cytomegalovirus-derived promoter directing synthesis of the RNA replicon. PAC, Puromycin acetylase gene. FMDV-2A, Foot-and-mouth disease virus-2A autoprotease that separates PAC and GP (cleavage site is indicated by white arrow). IRES (encephalomyelocarditis virus internal ribosome entry site), that directs synthesis of polyprotein encoding Kunjin non-structural (NS) proteins. HDVR, Hepatitis D virus ribozyme that generates the correct 3′ terminus of the Kunjin replicon. *Mlu*I restrictions sites used for the construct preparation are shown. The GP signal peptide cleavage site is indicated by a black arrow. 293T cells were transfected with phCMV (H), phCMV-GP (HGP), pKunrep4 (K) and pKunrep4-GP (KGP), and analysed by light microscopy 24 h post-transfection (b) and Western blot using anti-EBOV antibodies (c). Data are representative of three independent experiments. The positions of the GPer and the GP1 are indicated. To assess the amounts of cells loaded, the samples were also analysed with antibodies to actin.

respectively (Fig. 3a). EBOV GP was expressed at various levels by different selected KunGP clones, which also showed differences in cell growth and attachment. Clone KunGP12 that stably expressed GP for more than 30 passages was chosen for further studies. The Kun replicon vector was also successfully used for generation of stable RK13 or HeLa cells continuously expressing EBOV GP (data not shown). We compared the amounts of GP synthesized in KunGP12 cells, EBOV-infected 293T and phCMV-GP transfected cells.

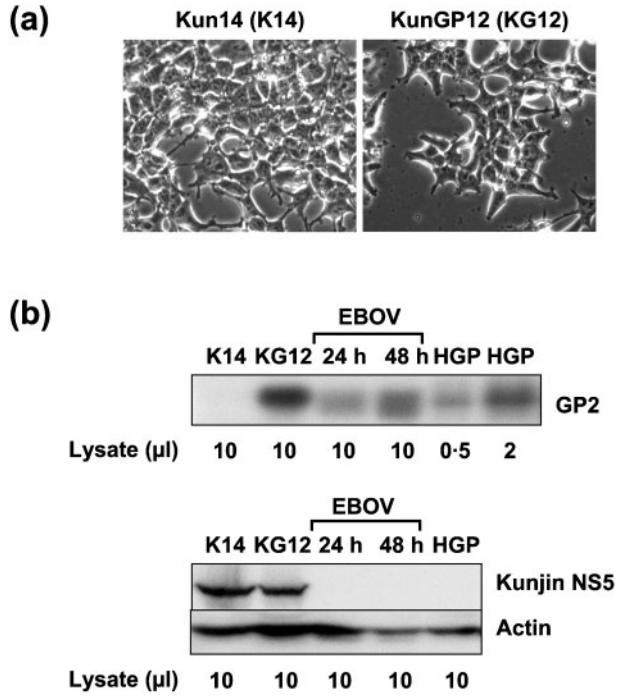

Fig. 3. Continuous expression of EBOV GP from the Kunjin replicon (KunGP12 cells). (a) Light microscopy analysis of the cells stably expressing Kunrep4 (Kun14 clone, K14) or Kunrep4-GP (KunGP12 clone, KG12) replicons. 293T cells were transfected with pKunrep4 and pKunrep4-GP, and subjected to puromycin selection. Individual clones of cells were isolated and analysed after 5–10 passages. (b) Comparison of GP expression in KunGP12 cells, EBOV-infected cells and phCMV-GP transfected cells. Kun14 and KunGP12 cells were grown until confluent; 293T cells were infected with EBOV at an m.o.i. of 4 and analysed 24 or 48 h post-infection (EBOV); 293T cells were transfected with phCMV-GP and analysed 24 h post-transfection (HGP). Cell lysates were subjected to Western blot analysis using anti-GP2 and anti-NS5 antibodies. To assess the amount of cells loaded on the gel, blots were also analysed with antibodies to actin. To obtain comparable amounts of GP2 on the Western blots, 10 (2 µl) and 40 (0·5 µl) times less phCMV-GP-transfected cells were loaded onto the gels compared with virus-infected and KunGP12 cells. Differences in the level of GP expression in KunGP12 and EBOV-infected cells (four independent experiments) were not statistically significant in comparison to that found with phCMV-GP-transfected cells ($P<0{\cdot}05$, Mann–Whitney test).

Western blot analysis using anti-GP2 antibodies showed that amounts of intracellular mature GP present in EBOV-infected and KunGP12 cells were comparable, whereas significantly more GP was found in cells transiently transfected with phCMV-GP DNA (Fig. 3b). Clone Kun14 stably expressing KUN replicon lacking GP gene insert was used as a control.

It is of interest and value to determine the molecular basis leading to the apparent absence of cytotoxic effects in cells continuously expressing EBOV GP. It is possible that the loss of GP cytotoxicity could be due to the generation of adaptive mutations in the GP gene of KUNrep4-GP replicon RNA occurring during selection and passaging of stably expressing cells. In earlier publications, the cytotoxicity of GP was attributed to a mucin domain containing clusters of potential sites of *O*-glycosidation present in the GP1 subunit (Simmons *et al.*, 2002; Yang *et al.*, 2000). Elimination of these glycosylation sites led to a gradual decrease in the level of GP cytotoxicity. In order to verify whether any mutations appeared in the GP gene of the KUNrep4-GP replicon, intracellular RNA from KunGP12 cells was isolated and analysed by sequencing following RT-PCR amplification. No mutations were detected in the GP gene of replicon RNA isolated from KunGP12 cells. The absence of cytotoxic effects in KunGP12 cells is therefore not linked to the generation of adaptive mutations in the GP coding sequence.

The absence of cytotoxic effects in KunGP12 cells could also be due to defects in GP processing and transport. We therefore first examined the processing of GP in KunGP12 cells by pulse–chase labelling followed by immunoprecipitation analysis using anti-EBOV antibodies. As shown in Fig. 4(a), the processing of GP in KunGP12 and in phCMV-GP transfected cells was not different. GP was first identified as an endoplasmic precursor GPer that was later transported into the Golgi and then cleaved into GP1 and GP2 subunits (Volchkov *et al.*, 1998). Earlier detection of the GP1 and GP2 subunits in KunGP12 cells can be explained by the longer pulse labelling necessary to enhance the sensitivity of the method. The identity of the GPer and mature GP1 bands in KunGP12 cell extracts was confirmed by EndoH treatment (Fig. 4b). As expected GPer contained high mannose sugars as indicated by its sensitivity to EndoH treatment, whereas mature GP1 contained complex sugars and was therefore EndoH resistant. Since the kinetics of GP synthesis were similar, we also compared the accumulation of different processing intermediates in both systems using Western blot analysis of cell lysates. Interestingly, the majority of intracellular GP in KunGP12 cells consisted of mature GP1–GP2 complexes, whereas a clear accumulation of GPer was found in phCMV-GP transfected cells (Fig. 4b, right panel).

Surface expression of GP by KunGP12 cells and phCMV-GP transfected cells was assayed using anti-GP human monoclonal KZ52 antibodies (Maruyama *et al.*, 1999). Immunofluorescence analysis showed that GP is efficiently transported to the plasma membrane of either cells and confirmed that normal adherence was seen with KunGP12 cells, whereas most GP-positive cells were rounded in the case of phCMV-GP (Fig. 5).

Expression of surface markers on KunGP12 cells

Flow cytometry analysis has shown that 293T cells transfected with phCMV-GP exhibit a dramatic reduction of $\alpha 5$ and $\beta 1$ integrins and MHC I proteins as early as 20–24 h post-transfection (Fig. 6a). We were therefore interested to find out if expression of GP in KunGP12 cells also influenced the expression of these markers. In accordance with the absence of cell rounding and detachment, KunGP12 cells showed no downregulation of any of these markers (Fig. 6a). Surprisingly, the levels of GP expressed at the surface of KunGP12 cells were similar to those in phCMV-GP transfected cells (Fig. 6a). This observation was in contradiction with the results of the Western blot analysis, which demonstrated that lower amounts of GP were found in KunGP12 cells compared with phCMV-GP transfected cells (Fig. 3b). Thus, it can be assumed that the relatively low efficiency of surface expression of GP in phCMV-GP transfected cells is due to inadequate traffic of GP from the endoplasmic reticulum and accumulation of this protein in this cellular compartment. It is reasonable to conclude that the expression of GP in KunGP12 cells is at the highest level that cells can tolerate without adverse effects on their survival and propagation. Higher expression of GP would result in accumulation of GP precursors in the endoplasmic reticulum and promote cytotoxic effects as seen in phCMV-GP transfected cells.

To ascertain that the presence of KUN replicons and/or expression of KUN-specific proteins did not interfere with GP-induced cytotoxic effects, KunGP12 and Kun14 cells were transfected with pIRES2-eGFP-GP, a plasmid expressing GP and eGFP from the same mRNAs (Fig. 6b). Cells were analysed 24 h post-transfection by flow cytometry for $\beta 1$ integrin and GFP expression. As shown in Fig. 6(b), expression of GP from pIRES2-eGFP-GP resulted in a reduction of the surface expression of $\beta 1$-integrin in both Kun14 and KunGP12 cells, confirming that Kun replicons do not interfere with GP-induced cytotoxicity. Moreover, these results demonstrate that the level of GP expression is a critical factor for GP-induced cytotoxicity as increasing the amount of GP synthesized in the cells caused cytotoxic effects visualized by both cell rounding and detachment and also dowregulation of surface markers.

DISCUSSION

The early appearance of the toxic effect observed during transient expression of EBOV GP from adenoviral or other conventional vectors is in contrast to the known moderate cytotoxicity and relatively low replication rate of EBOV. It appears that the replication and spread of EBOV would be hampered by the elevated cytotoxicity observed in transient

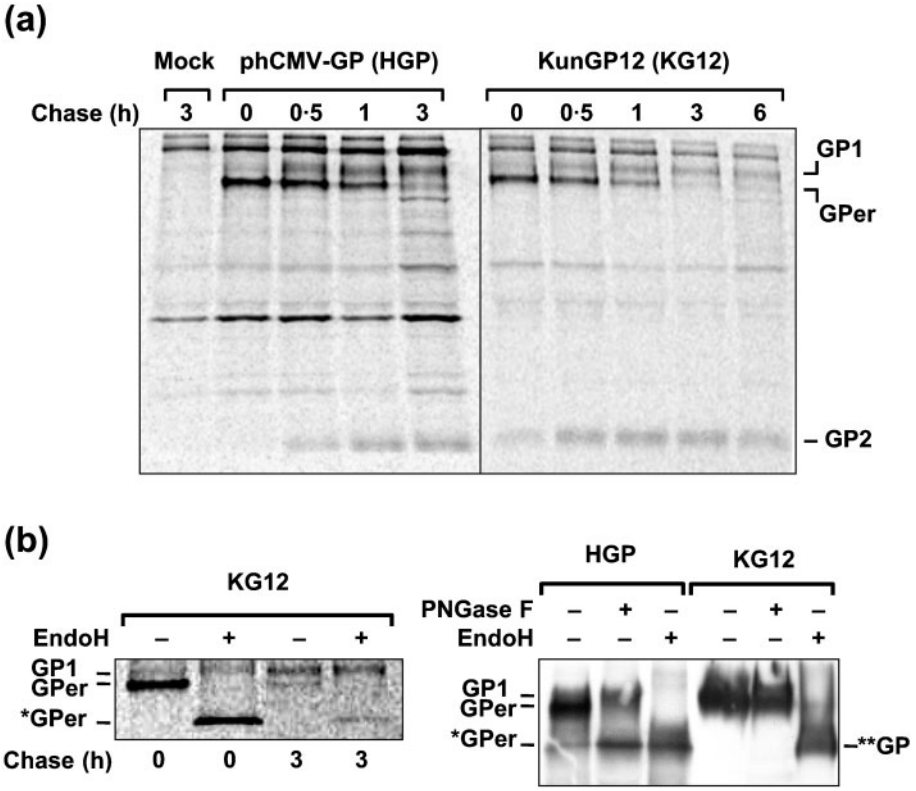

Fig. 4. Processing of EBOV GP in KunGP12 cells. (a) Pulse–chase analysis. KunGP12 and phCMV-GP-transfected cells (16 h post-transfection) were pulse-labelled with a ^{35}[S]methionine/cysteine mix for 45 min (KunGP12) or 30 min (phCMV-GP) and chased for the indicated intervals. 293T cells transfected with a GFP-expressing plasmid were used as a negative control. Proteins immunoprecipitated from cell lysates using horse anti-EBOV immunoglobulins were subjected to 10 % SDS-PAGE under reducing conditions and analysed by autoradiography. (b) EndoH digestion of GP expressed in KunGP12 cells. Left panel, samples collected immediately after pulse labelling or after 3 h of chase were treated with EndoH and subjected to 10 % SDS-PAGE. EndoH cleaves high mannose oligosaccharides present on GPer resulting in an increase in the electrophoretic mobility of this protein (*GPer). Right panel, KunGP12 cells were grown until confluent. After approximately 72 h of growth, cells were lysed and samples were treated with EndoH, PNGase F or used untreated. 293T cells transfected with phCMV-GP were collected and analysed 24 h post-transfection. Proteins were analysed by Western blot using horse anti-EBOV immunoglobulins. After PNGase F treatment GP1 and GPer co-migrated on 10 % polyacrylamide gels (**GP band).

GP expression systems, suggesting that mechanisms that prevent early cytotoxicity are likely to exist. Analysis of EBOV infection of 293T cells performed in this study demonstrate that early in EBOV infection expression of GP does not cause significant downregulation of surface molecule expression and it is not toxic for cells (Fig. 1). We have shown that a massive release of virus particles from infected cells occurred long before any significant cytotoxic effects or decrease in expression of cell surface molecules were detectable. Importantly, the level of GP synthesis in infected cells was substantially lower than in cells transiently expressing GP from plasmid DNA. As discussed previously, the increase in expression of surface GP in recombinant EBOV mutant lead to a significant increase in virus cytotoxicity (Volchkov *et al.*, 2001). The overexpression of GP resulted in the early death of infected cells and hampered virus spread. Importantly, in all expression systems used in this study, an increase in the synthesis of GP did not result in a simultaneous increase in surface expression of GP but rather in an intracellular accumulation of non-processed GPer. Significant amounts of GPer were found in cells transfected by phCMV-GP and also in cells infected by the EBOV mutant directing higher levels of GP expression (Volchkov *et al.*, 2001). No accumulation of the GP precursor however was detected in EBOV-infected cells or in the cells continuously expressing GP from Kunjin virus replicons. Recently Sullivan *et al.* (2005) demonstrated that GP exerts its cytotoxic effects by disrupting normal intracellular trafficking and that the cytotoxicity is dependent on dynamin, a cellular GTPase that mediates transport vesicle formation. The authors also postulated that both the endoplasmic precursor and the mature GP interact with

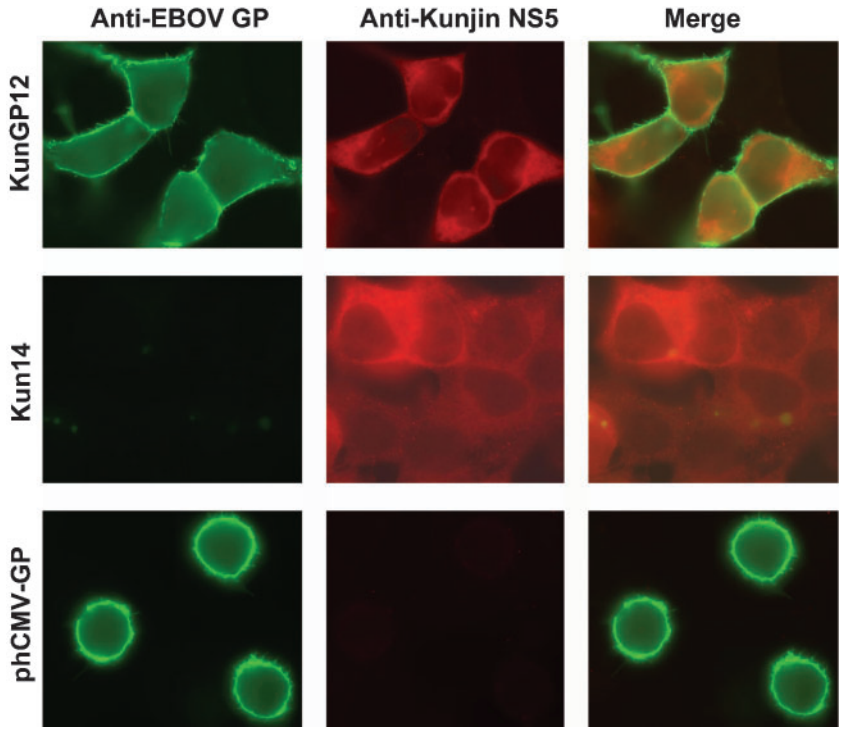

Fig. 5. Surface expression of GP in KunGP12 cells. Immunofluorescence analysis was performed on KunGP12, Kun14 cells and on 293T cells transfected with phCMV-GP (24 h post-transfection). Cells were grown on glass coverslides, fixed with paraformaldehyde, surface labelled with human anti-GP antibodies and then permeabilized using Triton X-100 and stained with anti-NS5 antibodies. Bound anti-GP antibodies were detected with FITC anti-human antibodies and anti-NS5 antibodies with TRITC anti-rabbit antibodies. Pictures shown are representative of three independent experiments.

cellular integrins thus promoting downregulation of the cell surface expression of these molecules leading to weak adhesion of the cells and their subsequent detachment. This is in agreement with our results showing that GP does not induce cytotoxic effects in cells that do not show accumulation of GP precursors and in which the GP is correctly processed. Indeed, using Kunjin replicons we demonstrated that different types of cells are capable of continuously expressing EBOV GP without showing cytotoxic effects or downregulation of cell surface markers. KunGP12, a clone of 293T cells expressing EBOV GP from the Kunjin virus replicon, was passaged as many as 30 times without any decrease in the expression of GP. The apparent absence of cytotoxic effects in these cells was shown to be due to the moderate level of GP synthesis rather than to the appearance of adaptive mutations in the GP gene. It is evident that the moderate level of GP expression observed in KunGP12 cells is not sufficient to significantly alter cellular trafficking and therefore is not toxic for the cells. It would appear that an optimal level of GP synthesis was reached during selection and passaging of cells encoding the Kunjin virus replicon-expressed GP gene that allowed cells to survive and propagate. Taken together these data suggest that previously described cytotoxic properties of GP could be largely attributed to overexpression of the protein in transient expression systems. It appears that in cells infected by EBOV several mechanisms exist that prevent cytotoxicity caused by GP expression. It has been shown previously that GP synthesis is regulated through RNA editing (Volchkov *et al.*, 1995, 2001). Moreover, the expression of GP on the surface of infected cells is downregulated through intensive shedding of this protein following cleavage by the cellular metalloprotease TACE (Dolnik *et al.*, 2004). Another mechanism of downregulation of surface GP expression involves budding of virus particles from the plasma membrane of infected cells. These events do not occur in cells transiently expressing GP gene from plasmid DNA and thus uncontrolled synthesis of GP from strong promoters results in intracellular accumulation of this protein. The mechanisms that limit the amounts of transmembrane GP on the cell surface may therefore play a major role in the pathogenicity of EBOV infection by restricting GP-induced cytotoxicity and thus allowing efficient virus production and spread in the host.

Cytotoxic effects that appear later in EBOV infection could be explained by several other factors in addition to GP overexpresssion. Indeed, electron microscopy data indicates that infected cells are overloaded with assembled viral nucleocapsids (Geisbert & Jahrling, 1995; Zaki & Goldsmith, 1999). Moreover, other viral proteins can be toxic. For example, VP40, a major structural protein of EBOV has been shown to interact with cellular tubulin and to induce budding of virus-like particles (Jasenosky *et al.*, 2001; Ruthel *et al.*, 2005; Timmins *et al.*, 2001) but also caused intensive cell rounding when overexpressed (unpublished observations).

EBOV and the closely related MARV have very similar pathogenesis (Mahanty & Bray, 2004). Surprisingly, it has been reported that the GP of MARV is not cytotoxic when transiently expressed from transfected plasmid DNA (Chan *et al.*, 2000a; Will *et al.*, 1993). However, when we expressed MARV GP from the phCMV vector, a cytotoxicity similar

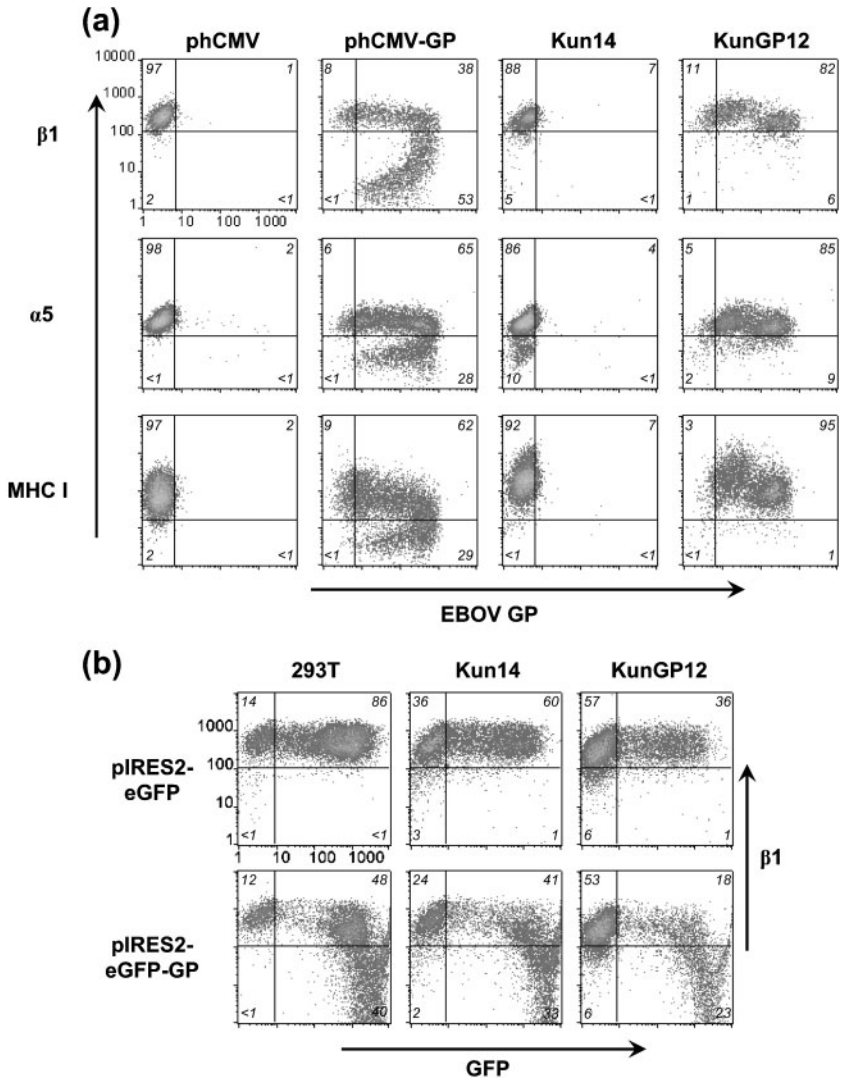

Fig. 6. Effect of GP expression on the surface expression of selected cellular markers. (a) 293T cells were transfected with phCMV or phCMV-GP and analysed 16 h post-transfection. Kun14 and KunGP12 cells were grown until confluent (approx. 72 h). Cells were harvested and analysed by flow cytometry using a mixture of human anti-GP antibodies and anti-β1 integrin antibodies conjugated with PE, anti-α5 integrin antibodies labelled with biotin or PE-coupled anti-MHC I antibodies followed by incubation with secondary anti-human FITC-labelled antibodies and PE-coupled streptavidin for detection of α5 integrin. Mean values for GP-associated fluorescence are very similar for phCMV-GP-transfected cells (mean fluorescence intensity: 244) and KunGP12 cells (mean fluorescence intensity: 195). (b) Effect of increase in GP synthesis in KunGP12 cells. KunGP12, Kun14 and 293T cells were transfected with either pIRES2-eGFP-GP or pIRES2-eGFP lacking the GP insert. Cells were collected 24 h post-transfection and analysed by flow cytometry for β1 integrin and eGFP expression. The transfection efficiency with Exgen 500 transfection reagent was lower in Kun14 and KunGP12 cells compared with 293T cells. The percentage of cells in each quadrant is indicated. Data represent findings from multiple experiments.

to that caused by EBOV GP was also observed, although it occurred later in transfection (48 h) and required higher amounts of plasmid DNA (unpublished data). Interestingly, these two viruses differ with respect to their GP expression strategy. The GP gene of MARV encodes only GP synthesized from a single open reading frame (Will *et al.*, 1993). The difference in the organization of the GP genes suggests that more GP should be expressed in cells infected with MARV than in EBOV-infected cells. In this respect, the lower cytotoxicity of MARV GP (in comparison with the GP of EBOV) means that the requirement to control GP-induced cytotoxicity was evolutionarily linked only to EBOV and resulted in very special strategy for GP expression (transcriptional RNA-editing). However, when overexpressed the surface glycoproteins of both filoviruses are similar in inducing strong cytotoxic effects.

Cytotoxicity caused by EBOV GP expression is believed to play an important role in the haemorrhagic manifestations widely observed in patients and laboratory-infected monkeys (Simmons *et al.*, 2002; Sullivan *et al.*, 2005; Yang *et al.*, 2000). However, direct damage of endothelial cells by virus replication is unlikely to be the first determinant of vascular injury. Involvement of the endothelial cells in virus replication was not confirmed in several detailed analyses of virus infection in monkeys (Geisbert & Jahrling, 2003; Geisbert *et al.*, 2003a, b, c; Ryabchikova *et al.*, 1999). It appears that damage to the endothelial barrier leading to loss of vascular integrity and haemorrhage syndrome, more likely results from the effect of the aberrant cytokine profiles of cells of the mononuclear phagocytic system, which are the primary target cells of the virus (Bray & Geisbert, 2005; Geisbert & Jahrling, 2003; Gibb *et al.*, 2002; Gupta *et al.*, 2001; Ryabchikova *et al.*, 1999). Here, downregulation of the cell surface markers, a phenomenon observed in virus-infected cells at later stages of infection could be very important. It has been reported that infection of these cells leads to their incomplete activation and triggers an inappropriate immune response contributing to the high pathogenicity of the disease (Bray & Geisbert, 2005; Gibb

et al., 2002; Gupta *et al.*, 2001; Stroher *et al.*, 2001). The immune response is known to be subtly regulated by cell-to-cell contacts and interactions between cell surface proteins. Thus, the effects caused by GP expression even at a moderate level could contribute to the inhibition of the host's ability to mount an effective immune defence. Molecular mechanisms that allow cells, particularly dendritic cells, to escape the cytotoxic effects caused by virus replication and GP expression for an extended period have not as yet been determined. Understanding these mechanisms will make an important contribution in the fight against filovirus infections.

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