Differential induction of cellular detachment by envelope glycoproteins of Marburg and Ebola (Zaire) viruses

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Marburg (MBG) and Ebola (EBO) viruses are human pathogens that have caused fatal epidemics of haemorrhagic fever characterized by massive virus replication, widespread infection of numerous tissues and fatal tissue necrosis (Peters et al., 1996). The molecular mechanism(s) underlying these disease manifestations are poorly understood. MBG and EBO each express a unique but related type I transmembrane glycoprotein (GP) (Sanchez et al., 1993; Will et al., 1993) that probably mediates binding and fusion with target cells. However, the complete functions of all GP products remain unknown. Here, we investigated the possibility that filovirus GP expression may directly disrupt target cell function and therefore contribute to pathogenesis independent of virus replication.

EBO GP produced in an infected cell is cleaved intracellularly to produce membrane-bound GP2, a transmembrane protein that mediates membrane fusion, and associated GP1, an extracellular protein that presumably mediates virus attachment to a receptor at the cell surface (Volchkov et al., 1998a). Free GP1 is also shed in a nonvirion-bound form and has been hypothesized to be pathogenic (Volchkov et al., 1998b). MBG GP is processed similarly (Volchkov et al., 2000). In addition, pre-translational processing of EBO GP results in the production of two EBO glycoprotein products, a shorter secreted product (sGP) derived from unedited mRNA transcripts and a polypeptide precursor to the longer membrane-bound form (GP1/GP2) encoded by edited transcripts (Sanchez et al., 1996; Volchkov et al., 1995). While GP1/GP2 mediates infection in a variety of cells, EBO sGP has been reported to bind to uninfected cells such as neutrophils (Yang et al., 1998); the interpretation of these findings has been challenged (Maruyama et al., 1998), and the precise functional relevance of EBO sGP in pathogenesis is unknown. In contrast, MBG GP transcripts apparently do not undergo such editing (Bukreyev et al., 1995; Will et al., 1993), and express exclusively the GP1/GP2 polypeptide which is cleaved and assembled into the full-length membrane-bound GP1/GP2 complex. These distinctions underscore the possibility that GP products from different filoviruses may induce different dysregulatory phenotypes in host cells.

In initial studies, genes encoding MBG GP and the Zaire (Z) subtype of EBO GP (provided by A. Sanchez, Centers for Disease Control and Prevention, Atlanta, GA, USA) were cloned into the mammalian expression vector pCMV4neo (Goldsmith et al., 1994) and separately co-transfected with pNL-Luc-E+ (Connor et al., 1995), the HIV-1 NL4-3 provirus carrying a luciferase reporter gene driven by the 5′ LTR (provided by N. Landau, Salk Institute, La Jolla, CA, USA, via the AIDS Research and Reference Reagent Program) into 293T cells in order to produce pseudotype virus stocks as previously described (Chan et al., 2000). To assess and compare production...
Fig. 1. Induction of cellular detachment by EBO-Z GP, but not MBG GP. (A) Production of similar levels of functional MBG and EBO-Z GP by 293T cells as assessed by infection of Vero cells by HIV-1 pseudotype viruses packaged by VSV-G, MBG or EBO-Z GP and produced from transfected 293T cells. Displayed values are typical of three separate infections. (B) Assessment of detachment after transient transfection of EBO-Z or MBG GP into 293T cells. Detachment was quantified as described in the text, and was assessed in parallel with determination of expression levels in (C). Values are typical of three separate transfections. (C) Consistent expression of full-length EBO-Z GP (left) and MBG GP (right) in 293T cells after transient transfection as assessed by SDS–PAGE of cellular lysates under reducing conditions and Western blot using chemiluminescent detection with the ECL system (Amersham) using separate polyclonal guinea pig antisera recognizing EBO-Z GP and MBG GP products, respectively. Although these polyclonal antisera recognized a number of variable background bands, the labelled GP products were reproducibly detectable. Consistent expression of sGP was not observed as expected since the EBO-Z GP cDNA used for these studies encoded only edited, full-length GP1/GP2 products and not pre-edited sGP products.

and function of MBG and EBO-Z GP in this system, Vero cells were challenged with Luc+ pseudotype viruses packaged by no GP, vesicular stomatitis virus (VSV) G protein (provided by J. Burns, University of California, San Diego, CA, USA), MBG GP or EBO-Z GP (Fig. 1A), and luciferase expression was used to quantify virus entry as previously described (Chan et al., 2000). Both MBG and EBO-Z pseudotypes infected Vero cells to comparable and significant levels, demonstrating that GP1/GP2 complexes encoded by both MBG and EBO-Z constructs were functionally competent for packaging virus and initiating target cell infections. Furthermore, MBG and EBO-Z pseudotype viruses generated from 293T transfections infected the same proportion of target cells at highest achievable titres (Chan et al., 2000), indicating similar expression of functional GP in both preparations.

The present studies were based on the fortuitous observation that EBO-Z pseudotype preparations were associated with substantial 293T cell detachment after transfection, a phenomenon that was also observed when EBO-Z GP alone was expressed in 293T cells without other viral genes. To quantify this effect, 293T cells were transfected with expression vectors carrying the EBO-Z or MBG GP as well as the parental pCMV4neo control vector as previously described (Chan et al., 1999). After a 48 h incubation, detached cells were collected, attached cells were recovered by trypsinization, and both populations were counted on a haemocytometer. Transient expression of the EBO-Z GP construct caused release of 33% of total cells (Fig. 1B). Despite using equivalent amounts of expression vectors, transfection of the MBG GP resulted in $\leq 5\%$ detachment. Of the detached cells in EBO-Z transfections, $>99\%$ were viable as assessed by Trypan blue exclusion; transmission electron microscopy of detached cells also revealed morphology consistent with cell viability (data not shown). Therefore, expression of the EBO-Z envelope glycoprotein(s) was not cytotoxic to 293T cells, but rather it interfered specifically with the mechanism of cellular attachment.

To assess directly MBG and EBO-Z GP expression levels in 293T cells, SDS–PAGE under reducing conditions and Western blotting were performed as previously described (Liu et al., 1997) on lysates [1% NP-40 lysis buffer containing 1 x stock protease inhibitor cocktail set I (CalBiochem)] obtained from transfected samples. For detection, we used polyclonal guinea pig antisera (1:1000 dilution) raised against MBG (Musoke strain) or EBO-Z (Mayinga strain) virus, respectively (provided by A. Schmaljohn, United States Army Medical Research Institute for Infectious Diseases, Fort Detrick, MD, USA), and an HRP-conjugated goat anti-guinea pig IgG (H + L) secondary antibody (1:5000 dilution; Accurate Chemical and Scientific Corp.) (Fig. 1C). Despite the presence of variable background bands, expression of both MBG and EBO-Z GP1/GP2 polyproteins was consistently and readily detected. As expected, sGP was undetectable since the cDNA used to express EBO-Z GP encoded only the edited full-length, membrane-bound GP and not the pre-edited cDNA encoding sGP (Xu et al., 1998). In view of the comparable infectious titres of both pseudotype viruses generated from 293T transfections (Fig.
secreted GP products induce detachment in cells at a 1:1 ratio, and detachment was measured at 24 h. If infected cells were re-plated together with untransfected 293T products was marked by GFP expression. After 12 h, transfected cells with pEBO-Z-IRES2-GFP, an expression vector encoding EBO-Z GP upstream of an internal ribosome entry site (IRES) site separately driving translation of an enhanced green fluorescent protein (GFP) reporter gene. In this configuration, every transfected cell that produced EBO-Z GP products was marked by GFP expression. After 12 h, transfected cells were re-plated together with untransfected 293T cells at a 1:1 ratio, and detachment was measured at 24 h. If secreted GP products induce detachment in trans, a similar ratio of GFP-positive (transfected) and GFP-negative (untransfected) cells would be expected to be evident in the detached cell population as in the attached cell population. However, if cell-associated GP causes detachment in cis, only GFP-positive transfected cells should be present in the detached population. In fact, flow cytometry revealed that 25% of cells remaining attached were GFP-positive in this experiment (Fig. 2 A), while nearly all (95%) of released cells were GFP-positive. In other experiments, the proportion of attached cells that was GFP-positive varied with the specific mixture of input cells, but the GFP-positive proportion in the detached fraction was always nearly 100%. As a specificity control, 293T cells were similarly transfected with the parental vector pIRES2-GFP and re-plated with untransfected cells. No detachment was detected in these cultures, and 28% of adherent cells expressed GFP. These results clearly demonstrate that detachment of 293T cells is caused by cell-associated EBO-Z GP rather than shed GP products.

To map the general region of EBO-Z GP necessary to induce cellular detachment, a panel of EBO-Z GP expression vectors was constructed that carry FLAG epitopes at the C-terminal end encoding: (1) full-length GP1/GP2; (2) a variant of GP1/GP2 truncated artificially at the transmembrane segment of GP2 (GP1/GP2 Trunc.) and carrying the extracellular portion of GP2; and (3) GP1 alone (expected to express a FLAG-tagged GP1 but not sGP, unless unexpected and previously unreported ‘reverse editing’ occurs). Using the monoclonal M2 antibody (International Biotechnologies Inc.) recognizing the FLAG epitope, a Western blot was performed on cellular lysates of 293T cells 48 h after transfection of these constructs. Titration of the amount of expression vector used in each transfection allowed for equivalent levels of expression from each GP construct (Fig. 2 B). In parallel, attached and released cells were counted as before. Despite comparable levels of expression by all constructs, only full-length GP1/GP2 (> 7%) and GP1/GP2 Trunc. (> 2%) forms reproducibly caused significant detachment while GP1 alone did not (Fig. 2 C). Therefore, a portion of EBO-Z GP necessary for eliciting detachment in 293T cells must be located in the extracellular domain of GP2, a region absent from either soluble GP1 or sGP products. It is important to emphasize that GP2 is normally anchored in the plasma membrane when wild-type GP1/GP2 is expressed. Therefore, in the context of our observation in Fig. 2 (A) that detachment is observed only in
cells directly expressing GP products and not in the neighbouring cells exposed to other secreted GP products, we conclude that the detachment effect is caused by the extracellular domain of GP2 anchored in the host cell membrane. Furthermore, since alterations in cellular adhesion potentially play an important role in pathogenesis, this novel process of cellular dysregulation driven only by EBO-Z GP highlights the fact that MBG and EBO-Z interact with host cells differently and may elicit disease by distinct mechanisms.

Finally, we hypothesized that expression of EBO-Z GP results in cellular detachment as a consequence of modulating a specific intracellular signalling pathway(s). To identify such a signalling cascade in target cells, we screened a panel of phosphorylation inhibitors for the ability to block 293T cell detachment and found that the Ser/Thr kinase inhibitor 2-aminopurine (2-AP) potently inhibited detachment. When 2-AP (10 mM) was added to cultures 6 h after EBO-Z GP transfection, cellular detachment at 48 h was reduced by 77% as compared to untreated transfected controls (Fig. 3A). A Western blot using the guinea pig antisera raised against EBO-Z virus on separate lysates from control transfections and transfections treated with 2-AP confirmed equivalent expression of EBO-Z GP1/GP2 in both cultures (Fig. 3B). Therefore, an as yet undefined Ser/Thr kinase activity induced by EBO-Z GP expression must be instrumental in mediating cellular detachment.

We discovered that expression of EBO-Z GP causes marked detachment of 293T adherent cells by a process that is independent of cell death and is mediated by a signalling pathway involving Ser/Thr phosphorylation. A similar effect was not evident for MBG GP, despite detection of GP expression and similar levels of envelope-mediated infectivity in pseudotype virus production as compared to EBO-Z GP. Therefore, these data reveal a functional difference between MBG GP and EBO-Z GP, perhaps not an unexpected result since they share only a 31% identity in amino acid sequence (Sanchez et al., 1998). The weak adherence of 293T cells may have made these cells especially susceptible to the detachment effect of EBO-Z GP, since more strongly adherent cells such as COS and CHO cells did not display such a response (data not shown). Nonetheless, this finding provides the first indication in a functional assay that EBO GP gene products themselves may cause cellular dysregulation, as is the case with HIV-1 gp120 and its pathogenic potential in the central nervous system (Toggas et al., 1994). It has been speculated EBO GP products shed from virions or infected cells may be important for inducing disease manifestations (Volchkov et al., 1998b; Yang et al., 1998). However, our results identify the extracellular region of the membrane-bound GP2, rather than secreted products such as sGP or shed GP1, as necessary for induction of the cellular detachment manifest in our assay system. Furthermore, the responsible GP domain maps to a region that is absent from the sGP coding sequence. Since sGP selectively binds neutrophils rather than endothelial cells (Yang et al., 1998), it thus would not be expected to have direct effects on endothelial cells. In contrast, the present findings are potentially important since dysfunction of blood vessel endothelial cells previously has been implicated in the widespread haemorrhage in visceral organs after EBO infections (Georges-Courbot et al., 1997), and these cells could be key targets of GP1/GP2-induced cellular detachment. Future experiments aimed at identifying the in vivo pathogenic potential of MBG and EBO-Z GP should clarify their impact on disease progression as well as hasten the design of more effective treatments for lethal filovirus outbreaks in the future.

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References


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