CONSERVED RECEPTOR-BINDING DOMAINS OF LAKE VICTORIA MARBURGVIRUS AND ZAIRE EBOLAVIRUS BIND A COMMON RECEPTOR

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Running Title: Filoviruses use a common receptor

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The GP1,2 envelope glycoproteins of filoviruses (marburg- and ebolaviruses) mediate cell-surface attachment, membrane fusion, and entry into permissive cells. Here we show that a 151-amino acid fragment of the Lake Victoria marburgvirus GP1 subunit bound filovirus-permissive cell lines more efficiently than full-length GP1. A homologous 148-amino acid fragment of the Zaire ebolavirus GP1 subunit similarly bound the same cell lines more efficiently than a series of longer GP1-truncation variants. Neither the marburgvirus GP1 fragment, nor that of ebolavirus, bound a non-permissive lymphocyte cell line. Both fragments specifically inhibited replication of infectious Zaire ebolavirus, as well as entry of retroviruses pseudotyped with either Lake Victoria marburgvirus or Zaire ebolavirus GP1,2. These studies identify the receptor-binding domains of both viruses, indicate that these viruses utilize a common receptor, and suggest that a single small molecule or vaccine can be developed to inhibit infection of all filoviruses.

Filoviruses cause severe hemorrhagic fevers in human and nonhuman primates, with case fatality rates that reach 88%. The family Filoviridae contains two genera, Marburgvirus (species Lake Victoria marburgvirus) and Ebolavirus (species Côte d'Ivoire ebolavirus, Reston ebolavirus, Sudan ebolavirus, and Zaire ebolavirus) (1). Like all mononegaviruses, filoviruses are enveloped and contain nonsegmented single-stranded RNA genomes of negative polarity (2).

Filoviral envelope glycoproteins (GP1,2), are type 1 transmembrane and class I viral fusion proteins that mediate cell association, fusion of

1 The abbreviations used are: CoV, coronavirus; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GP, glycoprotein; HIV-1, human immunodeficiency virus, type 1; MARV-Ang, Lake Victoria marburgvirus strain Angola; MARV-Mus, Lake Victoria marburgvirus strain Musoke; MLV, Moloney murine leukemia virus; PBS, phosphate-buffered saline; RBD, receptor-binding domain; SARS, severe acute respiratory syndrome; VSV, vesicular stomatitis Indiana virus; ZEBOV-May, Zaire ebolavirus strain Mayinga.
viral and cellular membranes, and entry of the viral core into the cytosol (3-5). The GP_{1,2} precursor assembles as a trimer, and is modified by N-glycosylation in the endoplasmic reticulum. Trafficking of the trimeric GP_{1,2} precursor to the Golgi apparatus leads to refinement of N-glycosylation and addition of O-glycans (6-9). Furin-like proteases cleave the polypeptide into the ectodomain GP_1, and the transmembrane GP_2 subunits, both of which remain connected through an intramolecular disulfide bond (GP_{1,2}). Mature GP_{1,2} trimers are then incorporated into virions during budding (6,7,10).

The filoviral GP_1 subunit mediates cell-surface receptor binding (8,11). Approximately half of the molecular weight of GP_1 is due to N- and O-glycans, many of which are located at the subunit’s C-terminus, in a region described as the mucin-like domain (12,13). This domain contributes to cytopathicity observed in GP_{1,2}-expressing cell lines, and has been suggested to play a critical role in pathogenesis of filoviral disease (14-16). However, its deletion enhances rather than decreases the efficiency of GP_{1,2}-mediated infection (13,16-18). Receptor binding is followed by endocytosis of the virions (19), acidification of the endocytic vesicle (4,5,20), and proteolytic processing of GP_1 by endosomal cathepsins (18,21). Conformational changes in the filoviral GP_2 subunit facilitate lipid mixing and fusion of the viral and cellular membranes, in a sequence of steps thought similar to those mediated by orthomyxo- and retroviral transmembrane proteins (22-25).

Here we identify fragments of the Lake Victoria marburgvirus (Musoke strain; MARV-Mus) and Zaire ebolavirus (Mayinga strain; ZEBOV-May) GP_1 subunit that efficiently bound cells permissive to filovirus infection, but not a non-permissive lymphocyte cell line. Each fragment inhibited infection of retroviruses pseudotyped with either marburgvirus or ebolavirus GP_{1,2}. Both fragments also inhibited replication of infectious Zaire ebolavirus. Our data define homologous regions of otherwise divergent filoviruses that mediate association with a common receptor. Similarities in these receptor-binding domains may provide insight into the nature of this receptor and suggest vaccine and therapeutic approaches effective against all filoviruses.

**EXPERIMENTAL PROCEDURES**

*Cells and culture conditions*—African green monkey kidney (Vero E6) cells and Jurkat lymphocytes were obtained from the American Type Culture Collection (ATCC numbers CRL-1586 and TIB-152, respectively). Human embryonic kidney 293T cells are a derivative of 293 cells (ATCC CRL1573) created by S. Haase and described originally as 293/tsA1609neo (34). Adherent cells (Vero E6 and 293T) were maintained in DMEM (GIBCO-Invitrogen), and Jurkat lymphocytes in RPMI Medium 1640 (GIBCO-Invitrogen). All media were supplemented with 10% FBS (Sigma-Aldrich), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Cellgro), and cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

*Construction of filovirus envelope glycoprotein-encoding genes and variants*—Codon-optimized Lake Victoria marburgvirus strain Musoke (MARV-Mus) open reading frames (ORFs) encoding GP_1 (amino acid residues 17-432) and GP_{1,2} (amino acid residues 17-681) lacking signal sequences were synthesized and amplified by *de novo* recursive polymerase chain reaction (PCR), using overlapping DNA oligomers based on the MARV-Mus GP_{1,2} protein sequence (GenBank accession number CAA781117). A codon-optimized Zaire ebolavirus strain Mayinga (ZEBOV-May) ORF encoding a mucin-like-domain-deleted GP_1 truncation variant (amino acid residues 33-308) (13) was synthesized based on the ZEBOV-May GP_{1,2} protein sequence (GenBank accession number NP_066246), using the same strategy. ORFs were ligated into a previously described pCDM8-derived expression vector (35), encoding the CD5 signal sequence.
upstream of the ORF insert, and the Fc region of human immunoglobulin G1 downstream (MARV-Mus GP1 17-432-Fc, ZEBOV-May GP1 33-308-Fc). Vectors encoding N- and C-terminal truncation variants were generated by inverse PCR amplification using plasmids encoding MARV-Mus GP1 17-432-Fc or ZEBOV-May GP1 33-308-Fc as templates. An ORF encoding MARV-Mus GP1,2 residues 17-681 was cloned into a variant of the pCDM8 expression vector encoding the CD5 signal sequence and a C-terminal C9 tag (amino acid sequence TETSQVAPA) derived from the rhodopsin C-terminus (MARV-Mus GP1,2). Plasmid encoding a ZEBOV-May GP1,2 variant lacking its mucin-like domain, ZEBOV-May GP1,2 \text{\(\Delta\)}309-489 (4), was generously provided by Dr. James Cunningham. Plasmids encoding MARV-Ang GP1-Fc variants were generated by altering their equivalent MARV-Mus GP1-Fc variants at codon 74 (T74A), using the QuikChange method (Stratagene).

Expression of filovirus envelope glycoprotein variants—For protein purification, 293T cells were transfected with plasmids encoding MARV-Mus GP1 17-432-Fc or ZEBOV-May GP1 33-308-Fc, their truncation variants, or control proteins (severe acute respiratory syndrome coronavirus strain Tor2 S(318-510)-Fc (SARS-CoV RBD-Fc) and human immunodeficiency virus 1 (HIV-1) strain ADA gp120-Fc (36,37)), using the calcium-phosphate method. Cells were washed in Dulbeccos’s phosphate-buffered saline (GIBCO-Invitrogen) 6 h post transfection, and grown at 37°C in 293 SFM II medium (GIBCO-Invitrogen) supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin, 100 µM MEM non-essential amino acids solution (GIBCO-Invitrogen), 2 mM sodium butyrate (Sigma-Aldrich), and 4 mM L-glutamine (Sigma-Aldrich). Medium was harvested after 48 h, and cell debris was removed by centrifugation and filtration through a 0.22 µm-pore size filter (Corning). Proteins were precipitated with protein A-Sepharose fast flow beads (Amersham) at 4°C for 16 h in the presence of Complete protease inhibitor (Roche, Mannheim). Beads were washed once with 30 bed volumes of 0.5 M sodium chloride/phosphate-buffered saline pH 7.4 (NaCl: Fisher Scientific, Fair Lawn, NJ; PBS: GIBCO-Invitrogen) and once with 10 bed volumes of PBS. Proteins were eluted with 50 mM sodium citrate/50 mM glycine pH 2 (sodium citrate: Fisher Scientific; glycine: BIO-RAD, Hercules, CA), neutralized with sodium hydroxide (Fisher Scientific), dialyzed in PBS, and concentrated with Centricon centrifugal filter units (Millipore, Billerica, MA). Purified proteins were assayed for size and concentration by comparison to bovine serum albumin standards (SIGMA-Aldrich) by SDS-PAGE followed by Bio-Safe Coomassie (BIO-RAD) staining, and by using the Micro BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer’s instructions.

Cell-binding assays—293T cells and Vero E6 cells were detached with PBS/5mM EDTA (GIBCO-Invitrogen) 48 h after plating, resuspended in an equal volume of PBS/5mM MgCl2 (SIGMA-Aldrich), and washed twice in PBS/2% goat serum (SIGMA-Aldrich). Jurkat lymphocytes were harvested and washed twice in PBS/2% goat serum. GP1-Fc constructs, truncation variants thereof, and control proteins were added to 5x10^5 cells to a final concentration of 100 nM, and incubated on ice for 1.5 h. Cells were washed twice in PBS/2% goat serum, and incubated for 45 min. on ice with a 1:40 dilution of goat Fc-specific fluorescein isothiocyanate (FITC) conjugated anti-human IgG antibody (SIGMA-Aldrich) in PBS/2% goat serum. Cells were washed three times with PBS/2% goat serum, once in PBS, and fixed with PBS/2% formaldehyde (SIGMA-Aldrich). Cell-surface binding of constructs was detected by flow cytometry with 10,000 events counted per sample. Baseline fluorescence was determined by measuring cells treated only with goat Fc-specific FITC-conjugate anti-human IgG antibody, which was then subtracted from binding values of the tested constructs and control proteins.

Infection assay with filovirus envelope glycoprotein-pseudotyped retroviruses—To generate retroviral pseudotypes, 293T cells were transfected by the calcium phosphate method with plasmid encoding MARV-Mus GP1,2, ZEBOV-May GP1,2 \text{\(\Delta\)}309-489, or vesicular stomatitis Indiana virus (VSV) G protein, together with the pQCXIX vector (BD Biosciences) expressing green fluorescent protein (GFP), and plasmid encoding the Moloney murine leukemia virus (MLV) gag and pol genes (38) using equal concentrations of each plasmid. Cell supernatants were harvested 48 h post transfection, cleared of cellular debris by centrifugation and filtration through a 0.45 µm-
pore size filter (Corning) and stored at 4°C. Supernatants containing pseudotyped viruses were added to 293T or Vero E6 cells in the presence or absence of the indicated concentrations of filovirus Fc truncation variants, or control proteins. After 5 h, cells were washed once in PBS, and replenished with fresh media. After 48 h, cells were imaged by fluorescent microscopy, and detached with trypsin for analysis by flow cytometry.

**Infection assay with recombinant green-fluorescent-protein-expressing Zaire ebolavirus**—All experiments with infectious filovirus were performed under biosafety level 4 conditions. Vero E6 cells were infected with a GFP-expressing ZEBOV-May created by reverse genetics (39). Virus was incubated with cells at a multiplicity of infection equal to 1 for 1 h in the presence or absence of 800 nM of filovirus truncation variants or control protein. Virus was removed, cells were washed in PBS, and media and protein were replenished. After 48 h, cells were fixed in 10% neutral-buffered formalin. After 3 days of fixation, cells were removed from the biosafety level 4 suite in and the percent of GFP-expressing cells was measured with a Discovery-1 automated microscope (Molecular Devices Corp., Sunnyvale, CA) by measuring 9 individual spots per well.

**RESULTS**

*MARV-Mus GP1 truncation variant 38-188-Fc efficiently binds to filovirus-permissive cells*—The envelope glycoproteins of a number of viruses include discrete, independently folded domains that bind cellular receptors as efficiently as their entire ectodomain regions. We sought to identify similar receptor-binding domains (RBDs) of MARV-Mus and ZEBOV-May. To determine the location of the MARV-Mus GP1 RBD, we synthesized a codon-optimized gene encoding the full-length mature MARV-Mus GP1 protein fused to the Fc region of human immunoglobulin G1 at the C-terminus (17-432-Fc). Four sets of seven truncation variants were created, starting at N-terminal residues 17, 38, 61, or 87, and ending at C-terminal residues 432, 308, 265, 230, 188, 167, or 134 (Fig. 1A). All 28 constructs expressed efficiently in 293T cells as Fc-fusion proteins (Fig. 1B). Equivalent concentrations of each variant were incubated with MARV-Mus-permissive African green monkey kidney Vero E6 and human embryonic kidney 293T cells, and with non-permissive Jurkat lymphocytes (5), and cell-surface association was determined by flow cytometry (Fig. 2A-C). The RBD of the severe acute respiratory syndrome coronavirus (SARS-CoV) S protein (residues 318-510) and HIV-1 gp120, expressed as Fc-fusion proteins (SARS-CoV RBD-Fc, gp120-Fc), were used as controls (36,37). As previously reported, SARS-CoV RBD-Fc efficiently bound SARS-CoV-permissive Vero E6 cells but not 293T cells or Jurkat lymphocytes (40). Also expectedly, gp120-Fc bound CD4-expressing Jurkat lymphocytes, but not Vero E6 or 293T cells. All 28 MARV-Mus proteins bound to Vero E6 and 293T cells, with varying efficiencies, whereas little or no association was observed with Jurkat lymphocytes in most cases. Successive truncation of the C-termini of MARV-Mus GP1 variants initiated with residues 17, 38, 61, or 87 led to successively increased cell-surface binding to Vero E6 cells, up through the C-terminal truncation at residue 188 (Fig. 2A). Further truncation beyond residue 188 decreased cell association. A single exception to this trend was observed with the 87-432-Fc variant, which bound Vero E6 cells with higher affinity than 87-308-Fc and 87-265-Fc. Variants initiated with residues 38, 61, and 87 bound more efficiently than those initiated with residues 17, with MARV-Mus 38-188-Fc consistently binding most efficiently to Vero E6 and 293T cells (Fig. 2B). These data identify a cell-binding region of MARV-Mus, located between GP1 residues 38 and 188.

*ZEBOV-May GP1 truncation variant 54-201-Fc efficiently binds to filovirus-permissive cells*—Deletion of the mucin-like domain has been demonstrated to markedly increase efficiency of ZEBOV GP1,2-mediated infection (13,16-18). To determine the location of the ZEBOV-May GP1 RBD, we synthesized a codon-optimized gene encoding the mature ZEBOV GP1 protein, lacking its mucin-like domain, and fused to the IgG1 Fc region (33-308-Fc). Three sets of four truncation variants were created, starting at N-terminal residues 33, 54, or 76, and ending at C-terminal residues 308, 201, 172, or 156 (Fig. 1C). With the exception of variant 76-172-Fc, all variants expressed efficiently (Fig. 1D). As with the MARV-Mus variants, equivalent concentrations of each variant were incubated with ZEBOV-May-permissive Vero E6 and 293T cells, and with non-
permissive Jurkat lymphocytes, and cell association was again assayed by flow cytometry. All 11 ZEBOV-May GP1 variants bound to Vero E6 and 293T cells, whereas binding to Jurkat lymphocytes was negligible in all cases (Fig. 2D-F). ZEBOV-May GP1 truncation variants showed a pattern of association to Vero E6 and 293T cells similar to that observed with MARV-Mus variants. In particular, 54-201-Fc and 76-201-Fc bound more efficiently than all other ZEBOV-May GP1 variants assayed, with 54-201-Fc binding slightly but consistently better than 76-201-Fc to Vero E6 cells (Fig. 2D-E). These data identify a cell-binding region of ZEBOV-May, located between GP1 residues 54 and 201, which corresponds to the cell-binding region of MARV-Mus (see Fig. 7 for alignment).

MARV strains Angola and Musoke GP1 truncation variants bind to filovirus-permissive cells with comparable efficiency—The largest and most severe marburgvirus disease outbreak to date occurred in Angola in early 2005 (41,42). The envelope glycoprotein amino acid sequence of the strain responsible for this outbreak, MARV Angola (MARV-Ang), is homologous to that of the MARV-Mus strain (43). In particular, a comparison between MARV-Mus GP1 amino acid residues 38-188 with the corresponding region of MARV-Ang yielded only one amino acid change, threonine 74 to alanine (T74A). This alteration was introduced into four MARV-Mus GP1 truncation variants (MARV-Ang GP1 38-188-Fc, 38-167-Fc, 61-188-Fc, and 61-167-Fc; Fig. 3A). Cell association of each of these variants was compared with those of MARV-Mus. Each MARV-Ang variant bound Vero E6 cells slightly less efficiently than its MARV-Mus counterpart (Fig. 3B). These data largely exclude the possibility that more efficient cellular association of the MARV-Ang cell-binding region contributes to increased severity of disease.

Both MARV-Mus and ZEBOV-May GP1 cell-binding regions inhibit entry of retroviruses pseudotyped with the GP1,2 of either filovirus—To determine if the identified GP1 cell-binding regions associated with factors necessary for infection, we assayed the ability of MARV-Mus 38-188-Fc and ZEBOV-May 54-201-Fc to inhibit entry of pseudotyped retroviruses. A Moloney murine leukemia virus vector expressing green fluorescent protein (GFP) was pseudotyped with the GP1,2 of MARV-Mus (MARV/MLV), a mucin-like-domain-deleted GP1,2 of ZEBOV-May (ZEBOV/MLV), or with the G protein of the vesicular stomatitis Indiana virus (VSV/MLV). Vero E6 cells were incubated with these pseudotyped retroviruses and varying concentrations of MARV-Mus 38-188-Fc, ZEBOV-May 54-201-Fc, or SARS-CoV RBD-Fc (Fig. 4A-C). No Fc fusion protein inhibited VSV/MLV, but both MARV-Mus 38-188-Fc and ZEBOV-May 54-201-Fc efficiently inhibited both MARV/MLV and ZEBOV/MLV. SARS-CoV RBD-Fc did not inhibit infection of either pseudotyped virus. MARV-Mus 38-188 was the more potent of the two cellular binding domains, inhibiting MARV/MLV and ZEBOV/MLV with an apparent IC50 of 50-100 nM in this assay (Fig. 4A). These data indicate that MARV-Mus 38-188-Fc and ZEBOV-May 54-201-Fc bind specifically to a common cell-surface factor critical to filovirus entry. Accordingly, and by analogy with other viral entry proteins, we hereafter refer to these cell-binding regions of MARV-Mus and ZEBOV-May GP1 as receptor-binding domains (RBDs).

MARV-Mus 38-188 Fc inhibits MARV/MLV entry more efficiently than other GP1 truncation variants—We investigated whether the cell-binding efficiency of MARV-Mus and MARV-Ang GP1 truncation variants correlated with their ability to inhibit entry of pseudotyped retroviruses (Fig. 5). Vero E6 cells were incubated with the indicated GP1 variants together with VSV/MLV or MARV/MLV. None of the GP1 variants inhibited VSV/MLV entry, whereas most of the MARV-Mus GP1 variants assayed inhibited that of MARV/MLV (Fig. 5). Some variation between entry inhibition and cell-binding was observed. Notably, full-length MARV-Mus GP1 (17-432-Fc) inhibited MARV/MLV entry as efficiently as the defined receptor-binding domains of MARV-Mus and MARV-Ang (38-188-Fc). Apart from this interesting exception, the MARV-Mus RBD inhibited entry more efficiently than any other GP1 variant assayed (Fig. 5). We speculate that the mucin-like domain of full-length GP1 mediates a lower affinity interaction with Vero E6 cells which may contribute to inhibition of entry, but which may be more susceptible to the wash steps of the binding assay shown in Fig. 2. Alternatively, partial misfolding of the longer truncation variants may impair cell surface association. Our data show
that variants of the MARV-Mus RBD that are slightly longer or shorter inhibit MARV/MLV less efficiently, consistent with their relatively lower affinity for filovirus-permissive cell lines.

**MARV-Mus 38-188-Fc and ZEBOV-May 54-201 inhibit replication of infectious Zaire ebolavirus.** To determine if the filovirus RBDs also inhibited infectious filovirus, Vero E6 cells were incubated with an infectious Zaire ebolavirus modified to express GFP (39), at a multiplicity of infection of 1, together with MARV-Mus 38-188-Fc, ZEBOV-May 54-201-Fc, or SARS-CoV RBD-Fc. As expected, viral replication, measured as percentage of infected cells, was specifically inhibited by both filovirus RBDs, but not by that of SARS-CoV (Fig. 6). Higher concentrations were required to inhibit infectious filovirus than those used to inhibit pseudotyped retroviruses (Fig. 4 and 6). These higher concentrations may be necessary to interfere with the greater number of GP1,2 molecules present on the filamentous filoviruses, compared to the significantly smaller retroviral pseudotypes. As observed with pseudotyped retroviruses, the MARV-Mus RBD inhibited infectious Zaire ebolavirus more efficiently than the ZEBOV-May RBD (Fig. 6). Similar inhibition of Zaire ebolavirus replication was observed in primary monocyte-derived human dendritic cells treated with ZEBOV-May or MARV-Mus RBDs (data not shown). The efficiency with which the MARV-Mus RBD inhibited ebolavirus replication is consistent with the utilization of a common entry factor by both marburg- and ebolaviruses.

**DISCUSSION**

Enveloped viruses require specific proteins on the virion surface that mediate cell attachment and fusion of the viral and cellular membranes. Viral class I fusion proteins are typically comprised of two functionally distinct domains or subunits (44,45). The N-terminal domain, GP1, in the case of filoviruses, mediates cell attachment and receptor association (8,11). Viral entry proteins attach to a number of cell-surface molecules including glycosaminoglycans and C-type lectins, and these attachments frequently make substantial contributions to the efficiency of viral entry (46-49). More critically, most enveloped viruses require one or more cellular receptors to initiate membrane fusion. Receptor-binding regions of viral fusion proteins are typically the most important antibody-neutralizing epitopes on the virion, due to the functional importance of and limited variation in this region (44,45). In some cases, such as murine and feline leukemia viruses and SARS coronavirus, the receptor-binding region is localized to a discrete, independently folded domain that can efficiently bind the cellular receptor and inhibit infection (37,50,51). These domains themselves also can be sufficient to elicit protective neutralizing antibodies (45,52).

Here we defined small domains of the GP1 proteins of two divergent filoviruses that bind filovirus-permissive cells. Several lines of evidence suggest that these domains bind a cellular receptor rather than a less specific attachment factor. First, these domains do not associate with a cell line refractory to filovirus infection. Second, they associate with filovirus-permissive cells more efficiently than larger and more heavily glycosylated GP1 variants. Indeed, ZEBOV-May 54-201-Fc includes no N-glycosylation sites that could associate with a cell-surface lectin-like molecule (MARV-Mus 38-188-Fc has two potential N-glycosylation sites). Third, each domain efficiently inhibits entry mediated by their respective GP1,2 at 50-200 nM, indicating that they associate with moderately high affinity and specifically with a factor critical to entry. Finally, they include the most highly conserved region of filovirus GP1 (17). The conservation of this region among all marburg- and ebolaviruses raises the possibility that ZEBOV-May 54-201-Fc and MARV-Mus 38-188-Fc can be used to elicit antibodies that protect against most filoviruses.

Previous studies of Zaire ebolavirus GP1,2 are also consistent with association of these domains with a specific cellular receptor. Medina et al. have observed that a Zaire ebolavirus GP1,2 lacking residues 241-496 nonetheless retained its ability to mediate entry of a pseudotyped retrovirus (53). Manicassamy et al. have shown that short deletions and point mutations of Zaire ebolavirus GP1,2, some of them between residues 54-201, interfere with GP1,2-mediated infection (17). Finally, Chandran et al., demonstrated that digestion of Zaire ebolavirus GP1,2-pseudotyped VSV with cathepsin B or L removes all but an 18-19 kD fragment of GP1, likely localized at the N-terminus. This fragment remained attached to GP2.
through a disulfide bond and still mediated infection (18,21).

Although the genomic organization of marburg- and ebolaviruses is similar, and although they cause similar diseases of comparable severity, it has not been clear whether all filoviruses utilize a common receptor. Several observations in the literature raised the possibility that their receptors or entry mechanisms are distinct. Lake Victoria marburgvirus has been reported to be less susceptible than Zaire ebolavirus to treatment of target cells with proteases and glycosidases (5). Electron micrographs of the virus entering cells have been used to suggest that Lake Victoria marburgvirus enters cells differently than Zaire ebolavirus (54), although earlier work suggests otherwise (19). Some variation in the relative efficiencies with which Lake Victoria marburg- and Zaire ebolavirus GP1,2 mediated entry in different cell lines also raised the possibility of distinct receptors (5).

Despite these observations, our data indicate that at least one of the receptors required by each filovirus is common to both. This situation is not unprecedented. For example SARS coronavirus and human coronavirus NL63 enter cells by distinct mechanisms although angiotensin-converting enzyme 2 is an obligate receptor for both (55,56). Further study will be necessary to clarify if the down-stream entry processes of marburg- and ebolaviruses are similarly distinct.

The conservation of the filovirus receptor-binding domains and their utilization of a common receptor raise the possibility that a vaccine could elicit antibodies that neutralize both marburg- and ebolaviruses, although cross-protective antibodies have not been described to date. Our observations also indicate that small molecules could be designed to inhibit entry of all filoviruses. Such cross-protection would be useful in the rapid containment of a novel filovirus epidemic.
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FIGURE LEGENDS

FIG. 1. MARV-Mus and ZEBOV-May GP1-Fc truncation variants. A, Representation of MARV-Mus GP1 truncation variants in relation to the full-length MARV-Mus GP1,2 envelope glycoprotein (residues 1-681). sp – signal peptide; tm – transmembrane domain. Cysteine residues, predicted or experimentally confirmed disulfide bonds, and potential N-glycosylation sites are indicated (13,57). “RBD” indicates the truncation variant which most efficiently bound to cell surfaces of filovirus-permissive cells (see Fig. 2) and inhibited GP1,2-mediated infection (Fig. 4). B, MARV-Mus GP1-Fc, containing GP1 residues 17-432 fused to the Fc region of human IgG, or truncation variants of GP1-Fc containing the indicated GP1 residues, were purified from supernatants of transfected 293T cells. GP1-Fc and truncation variants were normalized for expression, as shown by Coomassie staining. C, Representation of ZEBOV-May GP1 truncation variants in relation to the full-length ZEBOV-May GP1,2 envelope glycoprotein (residues 1-676) as in (A). D, ZEBOV-May GP1-Fc, lacking its mucin-like domain fused to the Fc region of human IgG (33-308-Fc), truncation variants thereof, and control proteins (SARS-CoV RBD-Fc and HIV-1 gp120-Fc) were expressed and normalized, as in (B).

FIG. 2. Binding of MARV-Mus and ZEBOV-May GP1-Fc truncation variants to the surface of nonhuman primate and human cells. The indicated MARV-Mus (A, B, C) and ZEBOV-May GP1-Fc constructs (D, E, F) and control proteins were incubated with filovirus-permissive African green monkey kidney (Vero E6) cells (A, D), filovirus-permissive 293T cells (B, E), and filovirus-non-permissive Jurkat lymphocytes (C, F), and analyzed by flow cytometry using an Fc-specific FITC-conjugated secondary antibody. Bars indicate mean fluorescence intensity (M.F.I.) averages of two or more experiments. Error bars indicate standard deviations.

FIG. 3. Comparison of the cell surface-binding affinities of MARV-Mus and MARV-Ang GP1-Fc truncation variants. MARV-Ang GP1-Fc truncation variants, differing from corresponding MARV-Mus GP1-Fc truncation variants at residue 74 (threonine for MARV-Mus; alanine for MARV-Ang), were characterized as in Fig. 1. A, MARV-Ang GP1-Fc truncation variants were normalized for expression and compared to the corresponding MARV-Mus truncation variants, as shown by Coomassie staining. B, The indicated MARV-Mus and MARV-Ang GP1-Fc constructs were incubated with Vero E6 cells and analyzed by flow cytometry using an Fc-specific FITC-conjugated secondary antibody. Bars indicate mean fluorescence intensity (M.F.I.) averages of two or more experiments. Error bars indicate standard deviations.

FIG. 4. MARV-Mus GP1 truncation variant 38-188-Fc and ZEBOV-May GP1 truncation variant 54-201-Fc inhibit MARV-Mus or ZEBOV-May GP1,2-mediated entry. The indicated concentrations of MARV-Mus GP1 truncation variant 38-188-Fc, ZEBOV-May GP1 truncation variant 54-201-Fc, and SARS-CoV RBD-Fc protein were incubated with Vero E6 cells together with GFP-expressing MLV pseudotyped with either MARV-Mus GP1,2 (A, D), mucin-like-domain-deleted ZEBOV-May GP1,2 (B, E), or VSV G (C, F). Entry of pseudotyped MLV was quantified by measuring green fluorescence using flow cytometry (A, B, C). Bars indicate mean fluorescence intensity (M.F.I.) averages of two or more experiments. Error bars indicate standard deviations.

FIG. 5. Comparison of the inhibitory effect of MARV-Mus and MARV-Ang GP1-Fc truncation variants on cell-entry of MLV pseudotyped with MARV-Mus GP1,2. 100 nM of the indicated MARV-Mus or MARV-Ang GP1-Fc truncation variants or SARS-CoV RBD-Fc were incubated with Vero E6 cells together with GFP-expressing MLV pseudotyped with MARV-Mus GP1,2 or VSV G. Entry of pseudotyped MLV was quantified by measuring green fluorescence using flow cytometry. Bars indicate mean fluorescence intensity (M.F.I.) averages of two or more experiments. Error bars indicate standard deviations.
FIG. 6. MARV-Mus GP₁ truncation variant 38-188-Fc and ZEBOV-May GP₁ truncation variant 54-201-Fc inhibit replication of infectious filovirus. 800 nM of MARV-Mus GP₁ truncation variant 38-188-Fc, ZEBOV-May GP₁ truncation variant 54-201-Fc, and SARS-CoV RBD-Fc were incubated with recombinant, GFP-expressing Zaire ebolavirus. Infection was quantified by measuring green fluorescence using Discovery-1 automated microscopy. Bars indicate percentage of infected cells, averaged over three experiments. Error bars indicate standard deviations.

FIG. 7. Sequence alignment of MARV-Mus and ZEBOV-May receptor-binding domains. Sequence alignment of the best cell surface-binding and GP₁,₂-mediated entry-inhibiting filovirus GP₁ truncation variants MARV-Mus 38-188 and ZEBOV-May 54-201. Highlighted residues indicate identical residues. A disulfide bond common to both receptor-binding domains is indicated with a bracket, as is a disulfide bond present only in ebolaviruses. Threonine 74 of MARV-Mus GP₁, which is an alanine in MARV-Ang GP₁, is shown in green. Arrows indicate further truncations that reduced cell-surface binding and inhibited GP₁,₂-mediated entry.
Conserved receptor-binding domains of lake Victoria marburgvirus and zaire ebolavirus bind a common receptor
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