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Enhancement of Ebola Virus Infection via Ficolin-1 Interaction with the Mucin Domain of GP Glycoprotein

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ABSTRACT

Ebola virus infection requires the surface viral glycoprotein to initiate entry into the target cells. The trimeric glycoprotein is a highly glycosylated viral protein which has been shown to interact with host C-type lectin receptors and the soluble complement recognition protein mannose-binding lectin, thereby enhancing viral infection. Similarly to mannose-binding lectin, ficolins are soluble effectors of the innate immune system that recognize particular glycans at the pathogen surface. In this study, we demonstrate that ficolin-1 interacts with the Zaire Ebola virus (EBOV) glycoprotein, and we characterized this interaction by surface plasmon resonance spectroscopy. Ficolin-1 was shown to bind to the viral glycoprotein with a high affinity. This interaction was mediated by the fibrinogen-like recognition domain of ficolin-1 and the mucin-like domain of the viral glycoprotein. Using a ficolin-1 control mutant devoid of sialic acid-binding capacity, we identified sialylated moieties of the mucin domain to be potential ligands on the glycoprotein. In cell culture, using both pseudotyped viruses and EBOV, ficolin-1 was shown to enhance EBOV infection independently of the serum complement. We also observed that ficolin-1 enhanced EBOV infection on human monocyte-derived macrophages, described to be major viral target cells,. Competition experiments suggested that although ficolin-1 and mannose-binding lectin recognized different carbohydrate moieties on the EBOV glycoprotein, the observed enhancement of the infection likely depended on a common cellular receptor/partner. In conclusion, ficolin-1 could provide an alternative receptor-mediated mechanism for enhancing EBOV infection, thereby contributing to viral subversion of the host innate immune system.

IMPORTANCE

A specific interaction involving ficolin-1 (M-ficolin), a soluble effector of the innate immune response, and the glycoprotein (GP) of EBOV was identified. Ficolin-1 enhanced virus infection instead of tipping the balance toward its elimination. An interaction between the fibrinogen-like recognition domain of ficolin-1 and the mucin-like domain of Ebola virus GP occurred. In this model, the enhancement of infection was shown to be independent of the serum complement. The facilitation of EBOV entry into target host cells by the interaction with ficolin-1 and other host lectins shunts virus elimination, which likely facilitates the survival of the virus in infected host cells and contributes to the virus strategy to subvert the innate immune response.

Ebola virus (EBOV), a member of the *Filoviridae* family, can cause a severe, often fatal, hemorrhagic fever (HF) in humans and nonhuman primates (1). The first *Ebolavirus* species was discovered in 1976 in the Democratic Republic of Congo (previously called Zaire), near the Ebola River (2). Subsequently, 28 outbreaks appeared sporadically until March 2014, when the most devastating outbreak occurred in western Africa, including the countries of Guinea, Liberia, and Sierra Leone (3–6). Most outbreaks are caused by the *Zaire Ebola virus* subspecies (7), which is the most pathogenic (case fatality rate, up to 90%). EBOV is classified in the category A agents of the Centers for Disease Control and Prevention (CDC) because it represents a substantial threat to public health, and consequently, its handling requires a biosafety level 4 (BSL-4) laboratory. The development of effective therapies against EBOV became an urgent priority during the spread of the most recent epidemics throughout Africa, which, in addition to causing the loss of thousands of human lives, caused economic and social instability (8).

The trimeric transmembrane glycoprotein (GP) of EBOV plays a crucial role in EBOV infection by mediating its cellular attachment and entry into host cells (9, 10). GP, the only viral protein at the surface of the viral particle, is a critical target for antibodies, including those leading to the antibody-dependent enhancement (ADE) of infection mediated by the complement protein C1q (11, 12). The EBOV GP is a highly glycosylated protein (the glycan contribution is half of the total GP weight, about 75 kDa) (13, 14) composed of two disulfide-linked subunits: GP1 and GP2 (15). GP1 mediates receptor binding, while the transmembrane subunit, GP2, is involved in virus-host cell membrane fusion (16). GP1 contains an N-terminal signal sequence, a receptor binding domain, a glycan cap, and a mucin-like domain

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(MLD). The majority of the N-glycosylation sites are concentrated in the MLD and glycan cap, while O-glycosylation sites are exclusively located in the MLD (17, 18).

EBOV has a wide cell tropism, and cell surface attachment occurs through GP binding to membrane lectins (DC-SIGN/L-SIGN, macrophage galactose-type lectin [MGL], lymph node sinusoidal endothelial calcium-dependent lectin [LSECtin]) and other receptors expressed by macrophages, dendritic cells, and endothelial cells (19–23). EBOV entry may also occur through the direct interaction of phosphatidylserine residues on the viral envelope with the cell surface receptor T cell immunoglobulin mucin domain 1 (TIM-1), resulting in virus infection enhancement (24). Previous work indicated a role for endogenous circulating mannose-binding lectin (MBL), a member of the defense collagen family, in Ebola virus infection (25, 26). MBL plays a key role in the first line of host defense against a wide variety of viral and other pathogens by recognizing pathogen-specific surface glycans and opsonizing infectious agents either alone or in conjunction with complement (27–29). Depending on the serum conditions, MBL balances Ebola virus infection, resulting in an enhancement under conditions with low levels of complement (30). In contrast, treatment of EBOV-infected mice with high doses of recombinant MBL had a protective effect (31).

Similarly to MBL, ficolins are members of the defense collagen family of proteins, which recognize a variety of pathogens, trigger activation of the lectin complement pathway, and mediate opsonophagocytosis (32, 33). Ficolins are oligomeric proteins assembled from trimeric subunits composed of collagen-like and fibrinogen (FBG)-related recognition domains with binding specificity for N-acetylated groups (34–36). Three ficolins have been identified in human serum and have been quantified to be present at levels of about 5 μg/ml (ficolin-2 or L-ficolin), 18 μg/ml (ficolin-3 or H-ficolin), and 1 μg/ml (ficolin-1 or M-ficolin), with the last value being close to that of circulating MBL (1.2 μg/ml) (37–40). There are few reports concerning virus recognition by ficolins, apart from the interaction of L-ficolin with hepatitis C and influenza A virus surface glycoproteins (41–44).

The aim of the present study was to investigate the interaction of EBOV GP with human ficolins. The observed interaction was shown to be ficolin-1 specific and was found to be mediated by binding of the FBG domain to the EBOV GP MLD. The presence of ficolin-1 resulted in the enhancement of EBOV infection in cell culture in either the absence or the presence of complement proteins. Ficolin-1 enhanced virus entry and likely competed with MBL for a common receptor at the cell surface. Our data suggest that, in addition to MBL, ficolin-1 could provide an alternative receptor-mediated mechanism for enhancing EBOV infection and could participate in EBOV subversion of the host innate immune system.

MATERIALS AND METHODS

Cells. Both Vero E6 cells (clone E6 of African green monkey kidney cells; ATCC CRL-1586) and human embryonic kidney (HEK) 293T cells (ATCC CRL-1573) were grown in Dulbecco's modified Eagle medium (DMEM) containing 0.11 g/liter pyruvate and 4.5 g/liter glucose (Gibco). For cell culture, the media were supplemented with 10% inactivated fetal calf serum (FCS; Gibco) and 1% antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin; Gibco). Cells were cultured at 37°C in a 5% CO_2 atmosphere. Baby hamster kidney 21 (BHK-21) cells (ATCC CCL-10) stably expressing T7 were maintained in Glasgow medium (Gibco) supplemented with 10% FCS and 10% tryptose phosphate broth.

Generation of human MDMs. Monocyte-derived macrophages (MDMs) were prepared from thrombocyte-depleted blood from human donors (EFS-Lyon, Lyon, France). Peripheral blood mononuclear cells were separated using Ficoll density gradient centrifugation (Ficoll-Paque Plus; GE Healthcare). Subsequently, monocytes were isolated onto magnetic beads coated with anti-human CD14 antibodies using an autoMACS Pro separator device (Miltenyi Biotec). Human blood monocytes, seeded at 1×10^6 cells per well in 24-well multiwell (MW) plates, were differentiated into macrophages by exposure to 10 ng/ml of human macrophage colony-stimulating factor (catalog number 130-096-491; Miltenyi Biotec) and cultivated in RPMI 1640, GlutaMAX, and HEPES (catalog number 72400-054; Invitrogen) with 10% human autologous fibrin-depleted plasma in a humidified atmosphere at 37°C with 5% carbon dioxide for 7 days.

Viruses. The recombinant vesicular stomatitis virus (VSV) expressing the glycoprotein of Zaire EBOV (Mayinga strain) (rVSV-GP) was generated by reverse genetics using a clone of the VSV Indiana serotype containing the EBOV GP open reading frame cloned instead of VSV G protein (45). BHK-21 cells were seeded in 75-cm^2 flasks 1 day prior to infection. Plasmids carrying the N, P, and L proteins and the full-length sequence of the recombinant VSV expressing the GP of Zaire EBOV Mayinga (rVSV-GP) were transfected using the FuGENE 6 reagent (Promega) following the manufacturer's recommendation. Plasmid amounts were 10 μg for the full-length plasmid, 3 μg for pBS-N, 5 μg for pBS-P, 2 μg for pBS-L, and 1 μg for pCMV-T7. After 48 h, a passage was done on Vero E6 cells, and virus was harvested 48 h later. Wild-type VSV (wtVSV), rVSV-GP, and wild-type EBOV (Mayinga) were produced in Vero E6 cells in DMEM containing 3% FCS. Both wtVSV (Indiana serotype) and rVSV-GP were propagated under BSL-2 conditions and quantified by determination of the number of PFU. EBOV was propagated in a BSL-4 laboratory (J. Mérieux, Lyon, France) and quantified by immunohistochemistry (IHC). On the day before experimental infection, cells were seeded in multiwell plates with medium supplemented with 5% FCS. Before virus infection, cells were rinsed with glucose-free DMEM (Gibco) supplemented with 1% antibiotics. Infection was performed in glucose-free DMEM in the absence of FCS.

For purified EBOV production (EBOV Mayinga expressing the green fluorescent protein [GFP]), Vero E6 cells were progressively adapted to grow in serum-free medium (VPSFM; Life Technologies) for five passages. EBOV-GFP (EBPV-S-free) was inoculated at a multiplicity of infection (MOI) of 0.05, and the supernatant was harvested at 5 days postinfection. The supernatant was clarified of cell debris by low-speed centrifugation (1,500 × *g*, 10 min) and then loaded over a 20% sucrose cushion in 10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4, buffer. The virions were pelleted by ultracentrifugation for 2 h at 134,600 × *g* in an SW32 rotor (Beckman Coulter), and the pellet was suspended in 3 ml of phosphate-buffered saline (PBS) containing calcium and magnesium.

Recombinant nonreplicative mouse leukemia virus (MLV) expressing the glycoproteins of Zaire EBOV (Mayinga strain) (rMLV-GP-Z) and the Reston Ebola virus species (RESTV) (rMLV-GP-R) were generated in HEK 293T cells by cotransfections of plasmids carrying *gag* and *pol*, the GFP gene inserted between encapsidation sequences, and the gene for the filoviral glycoprotein. After 48 h, the supernatants were harvested, cleared of cell debris by centrifugation at 1,500 × *g* for 10 min, and then filtered using a 0.45-μm-pore-size filter. The particles were pelleted through a 20% sucrose cushion by ultracentrifugation at 134,600 × *g* for 2 h in an SW32 rotor (Beckman Coulter). MLV particles were titrated on Vero E6 cells by counting the number of fluorescent cells at 48 h after infection, using an Accuri C6 flow cytometer (Becton Dickinson).

On the day before the experimental infection assay, Vero E6 cells were seeded in a multiwell plate with medium supplemented with 5% FCS. Before virus incubation, the cells were rinsed with glucose-free DMEM (Gibco) supplemented with 1% antibiotics. Incubation was performed in glucose-free DMEM in the absence of FCS. Viruses were preincubated with 10 μg/ml of proteins, and infection was performed at an MOI of 1 for

1 h at 37°C. Then, the inoculum was removed, and GFP expression was measured at 2 days postinfection.

Recombinant nonreplicative VSV particles expressing the red fluorescent protein (rVSV-RFP) were pseudotyped with EBOV GP (rVSV-RFP-GP) or GP from which MLD was deleted (rVSV-RFP-GPΔmuc) as described previously (46).

Reagents. The 3327 hybridoma was generated in collaboration with Dendritics SAS (Lyon, France) using its specific techniques, as described in reference 46. Animal experimentation was performed in agreement with French regulations. The hybridoma was grown in DMEM–F-12 medium containing glutamine and 10% FCS and adapted to protein-free hybridoma medium (Invitrogen) for growth in Integra Celline high-density culture flasks. Monoclonal antibody (MAb) 3327 is IgG1(κ), as determined by the use of mouse isotyping strips (Santa Cruz). Hemagglutinin (HA) peptide, anti-HA agarose, and rabbit HA epitope tag antibody were purchased from Pierce. Both peroxidase-conjugated goat anti-rabbit and anti-mouse IgG antibodies were purchased from Jackson Immuno Research. The mouse monoclonal nucleoprotein (NP; clone ZDD4)-specific antibody of EBOV (1:500) was produced by a hybridoma in DMEM–F-12 medium (Gibco)–10% FCS containing glutamine. Human $CD14^+$ antibodies were purchased from Miltenyi Biotec (catalog number 130-050-201). Low-viscosity carboxymethyl cellulose (CMC) and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma, and True Blue peroxidase substrate and Instant Blue stain were purchased from KPL and Expedeon, respectively. Low-protein-binding (LoBind) 1.5-ml tubes were purchased from Eppendorf, and 1 M HEPES solution was from Invitrogen. Human MBL-deficient serum (<5 absorbance units, <0.016 μg/ml) was purchased from Statens Serum Institute (catalog number 36832). Human ficolin-deficient serum was prepared by incubating 3 ml of normal human serum from a healthy donor with 1 ml of acetylated BSA-Sepharose for 3 h at 4°C as described previously (47), in accordance with Etablissement Français du Sang (EFS) Rhône-Alpes agreement number 14-1940-38 regarding their use in research.

Recombinant proteins. Human recombinant wild-type ficolin-1 and its Y271F mutant were expressed in S2 insect cells and purified as described previously (48). Recombinant human ficolin-2 and ficolin-3 were produced in Chinese hamster ovary cells (47) and purified by affinity chromatography on *N*-acetyl cysteine–Sepharose for ficolin-2 (47) and on acetylated BSA-Sepharose for ficolin-3 (49). Recombinant MBL, produced and purified as described previously (50), was kindly provided by NatImmune (Copenhagen, Denmark). The trimeric FBG recognition domain of ficolin-1 (FBG) and the C-type lectin carbohydrate recognition domain of MBL (MBL-CLec) were expressed in a baculovirus/insect cell system and purified as described previously (51, 52). The molecular sizes of the proteins were estimated to be as follows: 388.1 kDa for ficolin-1, 305 kDa for MBL, 73.6 kDa for ficolin-1 FBG, and 49.1 kDa for MBL-CLec.

The recombinant GP of EBOV (Mayinga strain) was expressed in HEK 293T cells from plasmid pDISPLAY-HA-GP, kindly provided by E. Ollmann Saphire (Scripps Institute, La Jolla, CA, USA), and purified as described previously (17, 53). Two kinds of trimeric recombinant GPs were used: GP from which the transmembrane (TM) domain (residues 33 to 632) was deleted (GPΔTM) and GP from which the mucin and TM domains (the GPΔTM sequence with deletion of residues 312 to 463) were deleted (GPΔmucΔTM, abbreviated GPΔmuc). The molecular sizes of the soluble monomers were estimated from SDS-PAGE analysis to be 150 kDa for GPΔTM (53) and 50 kDa for GPΔmucΔTM (54). His-tagged recombinant EBOV GP that was devoid of the TM domain and produced in Sf9 insect cells was purchased from IBT Bioservices. The trimeric nature of the recombinant protein was assessed by native PAGE (8%) analysis under nonreducing conditions, followed by Instant Blue staining.

Interaction of ficolins with GPΔTM by overlay assay. One hundred microliters of purified ficolin solutions (0.1, 0.5, and 1 μg/spot) was dotted onto Hybond C-extra nitrocellulose membranes (Amersham). The membranes were blocked for 1 h at room temperature (RT) in Tris-buffered saline containing 0.05% Tween 20, 10 mM $CaCl_2$, and 5% skim milk. The membranes were then incubated overnight at RT in the same buffer containing 2 μg/ml of purified GPΔTM, washed three times for 20 min each time, and incubated for 1 h at RT with rabbit anti-HA antibody (1/200). After three 20-min washes, the membranes were incubated for 1 h with an anti-rabbit immunoglobulin horseradish peroxidase conjugate (1/10,000). After three 20-min washes, the interaction was detected by enhanced chemiluminescence (ECL). The experiment was performed twice.

Virus infection assay in the presence of defense collagens. Vero E6 cells were seeded to obtain plates of confluent cells after 24 h of culture in 24-well MW plates. Replicative viruses (wtVSV, rVSV-GP, and EBOV) and nonreplicative GP-pseudotyped particles (rMLV-GFP-GP, rVSV-RFP-GP, rVSV-RFP-GPΔmuc) were incubated with 10 μg/ml of defense collagens (MBL, ficolin-1, ficolin-2, or ficolin-3) for 1 h at 37°C in 10 mM HEPES, 5 mM $CaCl_2$ buffer (buffer A) in low-protein-binding (LoBind) 1.5-ml Eppendorf tubes. During this time, cells were rinsed with fresh glucose-free DMEM containing 1% antibiotics. Then, the virus-protein mixtures were incubated with the cell monolayer at 37°C in a 5% CO_2 atmosphere for 1 h at the following MOIs: 5×10^{-4} for wtVSV-GP, 1×10^{-4} for rVSV-GP, 2×10^{-4} for EBOV, 2×10^{-5} for purified serum-free EBOV, 3 for rVSV-RFP-GP and rVSV-RFP-GPΔmuc, and 1 for rMLV-GFP-GP in 24-well culture plates and 2×10^{-3} for EBOV in a 12-well MW plate (55). For each condition, a low MOI was selected to obtain the number of PFU without a loss of resolution after infection enhancement. Cells were rinsed with glucose-free DMEM, covered with 1.5 ml fresh medium (1:1 CMC and DMEM–5% FCS), and cultured for an additional 48 h and 6 days for VSV and EBOV, respectively. For VSV infection, cells were fixed by adding 0.75 ml of fixing and staining solution (0.2% crystal violet, 4.5% formaldehyde, 7.5% ethanol in PBS) per well for 2 h. Wells were rinsed twice with water, and the numbers of PFU were counted. For EBOV infection, the CMC-DMEM mix was removed, and the cells were fixed by adding a 4% formaldehyde–PBS solution for 10 min and permeabilized by the use of 0.5% Triton X-100 in PBS for 4 min. Then, immunohistochemistry was performed with anti-NP antibody (1/500) followed by peroxidase-conjugated goat anti-mouse immunoglobulin antibody (1/1,000). Plaques were visualized by adding 250 μl of True Blue substrate. For the recombinant viruses expressing a reporter gene, MLV-GFP and rVSV-RFP, cells were covered with fresh medium and cultured for an additional 48 h and 8 h, respectively. The experiments were performed three times.

MDM infection with EBOV in the presence of defense collagens. MDMs were seeded in a 24-well plate (1×10^6 per well) and were infected with EBOV at an MOI of 0.1 for 48 h. The virus was preincubated with 10 μg/ml of defense collagens (MBL, ficolin-1, and the ficolin-1 Y271F mutant) for 1 h at 37°C in RPMI 1640–GlutaMAX–HEPES in low-protein-binding (LoBind) 1.5-ml Eppendorf tubes. Virus infection was quantified at 48 h postinfection by fluorescence-activated cell sorting analysis using the GFP reporter gene.

Flow cytometry. (i) Vero E6 cell experiments. For Vero E6 cell experiments, 2×10^5 noninfected and infected Vero E6 cells were rinsed and detached by the use of trypsin solution (Gibco) for 5 min at 37°C. The cells were rinsed in 1% BSA–PBS solution and pooled by centrifugation at $400 \times g$ for 1 min. The supernatant was removed, the cells were fixed in 4% paraformaldehyde–PBS solution for 20 min at RT, and the pellet was rinsed with 1% BSA–PBS. At 2 days postinfection, binding was measured on a MACSQuant VYB analyzer (Milenyi Biotec) using the GFP reporter gene and the 488-nm (excitation) and 525/50-nm (emission) channel, and the data were analyzed with MACSQuantify software. At 8 h postincubation, binding was measured on the MACSQuant analyzer using the RFP reporter gene and the 561-nm (excitation) and 586/15-nm (emission) channel. Both GFP- and RFP-positive populations were estimated after forward scatter (FSC) and side scatter (SSC) gating on the cells. For each condition, 20,000 events were analyzed, and the experiment was performed three times.

(ii) Macrophage experiments. For macrophage experiments, cells were analyzed as described above for Vero E6 cells using a Beckman Coulter Gallios flow cytometer.

Virus-cell binding assay. rVSV-GP was incubated with 10 μg/ml of ficolin-1 for 1 h at 37°C in 100 μl buffer A. During this time, the cells were rinsed with cold glucose-free DMEM and incubated with a cold solution of 3.4 μg/ml MBL in 250 μl buffer A per well of a 24-well plate or with buffer A for 2 h and then rinsed. After 1 h, 1 ml of glucose-free DMEM was added to the rVSV-GP-defense collagen solutions, the mixture was added to cell monolayers (MOI, 0.002 on a 24-well plate) at 4°C, and the cells were incubated for 2 h to allow virus binding. The wells were rinsed twice with 1 ml cold glucose-free DMEM. Virus was titrated by a plaque-forming assay. The experiment was performed twice.

Similarly, rVSV-RFP-GP was incubated with 10 μg/ml of ficolin-1 and MBL for 1 h at 37°C. Competition was performed under cold conditions with 4 μg/ml of MBL and ficolin-1. Particles were incubated at 4°C for 2 h to allow binding (MOI, 3 on a 24-well plate). RFP reporter gene expression was quantified at 8 h postbinding.

SPR analyses with immobilized GP proteins and data evaluation. Surface plasmon resonance (SPR) analyses were performed using a Biacore 3000 instrument (GE Healthcare). Both recombinant GPΔTM and GPΔmucΔTM were diluted to 20 μg/ml in 10 mM Na acetate, pH 4.0, and immobilized on the surface of a CM5 sensor chip (GE Healthcare) using amine coupling chemistry until coupling levels of 3,700 relative units (RU) (GPΔTM) and 3,000 RU (GPΔmucΔTM) were reached. The reference surface was obtained by performing the immobilization step without the addition of protein. The running buffer for immobilization was 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, pH 7.4, containing 0.005% surfactant P20 (GE Healthcare). Forty microliters of soluble defense collagens in 10 mM HEPES, 150 mM NaCl, pH 7.4, containing 5 mM $CaCl_2$ and 0.005% surfactant P20 was injected at a flow rate of 20 μl/min over the GPΔTM, GPΔmucΔTM, and reference surfaces. The surfaces were regenerated with 10 μl of 1 M NaCl, 10 mM EDTA, pH 7.4. The binding curves shown were obtained after automatic subtraction of the signal recorded on the reference surface. The experiments were performed twice.

Kinetic data were analyzed by global fitting to a 1:1 Langmuir binding model of both the association and dissociation phases for at least six concentrations of ficolin-1 (0.25 to 4 nM) or MBL (0.5 to 8 nM), using BIAevaluation (version 3.2) software (GE Healthcare). The apparent equilibrium dissociation constants (K_Ds) were calculated from the ratio of the dissociation constant (K_d) and association rate constant (k_a) (K_d/k_a). The values provided below are the means ± standard deviations (SDs) from two independent experiments. Although the interaction of oligomeric ficolin-1 and MBL with the trimeric GPΔTM is inherently more complex than a simple 1:1 binding model, this model was used for data fitting for comparison purposes and yielded satisfactory chi-square values (<3.85).

Statistical analyses. For the *in vitro* studies, the two-tailed unpaired Student's *t* test was performed. Values of P of <0.05 were considered significant.

RESULTS

GP interaction with ficolins. The interaction of the GP of EBOV (Mayinga) with ficolin-1 was first studied using a protein-protein overlay assay. Solutions of purified ficolin-1, ficolin-2, and ficolin-3 were dotted onto nitrocellulose membranes and incubated with the soluble trimeric form of GP (GPΔTM) in the presence of calcium ions. After washing of the membrane, GPΔTM bound to the membranes was detected using a specific anti-HA tag antibody and enhanced chemiluminescence. Recombinant human MBL (rhMBL), which has previously been shown to bind to EBOV GP, was used as a positive control for the interaction (25, 30). No interaction with ficolin-2 or BSA (Fig. 1A) or with ficolin-3 (data not shown) was observed, with ficolin-1 being the only protein displaying a robust signal for an interaction with GP (Fig. 1A).

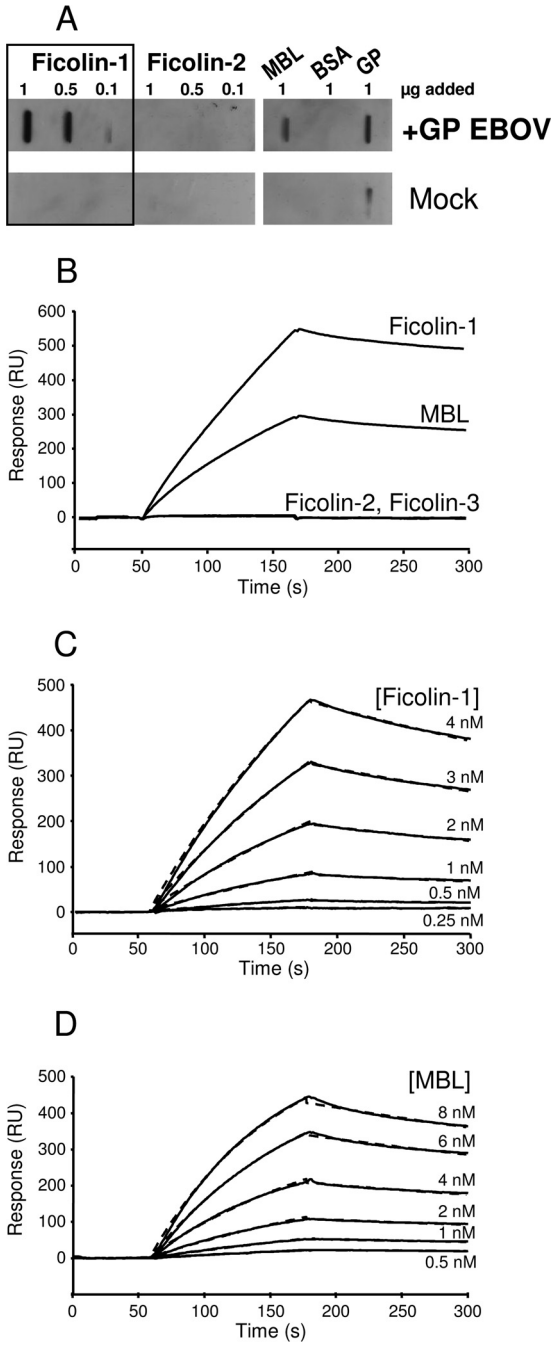

FIG 1 Interaction of ficolins and MBL with the GP of EBOV. (A) Binding detection by overlay assay. Ficolin-1 and ficolin-2 were dotted onto nitrocellulose membranes and incubated with 2 μg of purified HA-tagged GP. After three washes, bound GP was detected with a specific anti-HA tag antibody and revealed by enhanced chemiluminescence (ECL). A mock-protein incubated membrane was incubated with a specific anti-HA tag antibody. MBL and BSA were dotted as positive and negative controls of interaction, respectively. (B to D) SPR analyses of the interaction of ficolins and MBL with immobilized GPΔTM of EBOV and kinetic analyses of ficolin-1 and MBL binding. (B) Ficolin-1, -2, and -3 and MBL (5 nM) were injected over 3,700 RU of immobilized GPΔTM. (C and D) Ficolin-1 (C) and MBL (D) at the concentrations indicated in the figure were injected over immobilized GPΔTM. Fits are shown as dotted lines and were obtained by global fitting of the data using a 1:1 Langmuir binding model. The results shown are representative of those from two independent experiments.

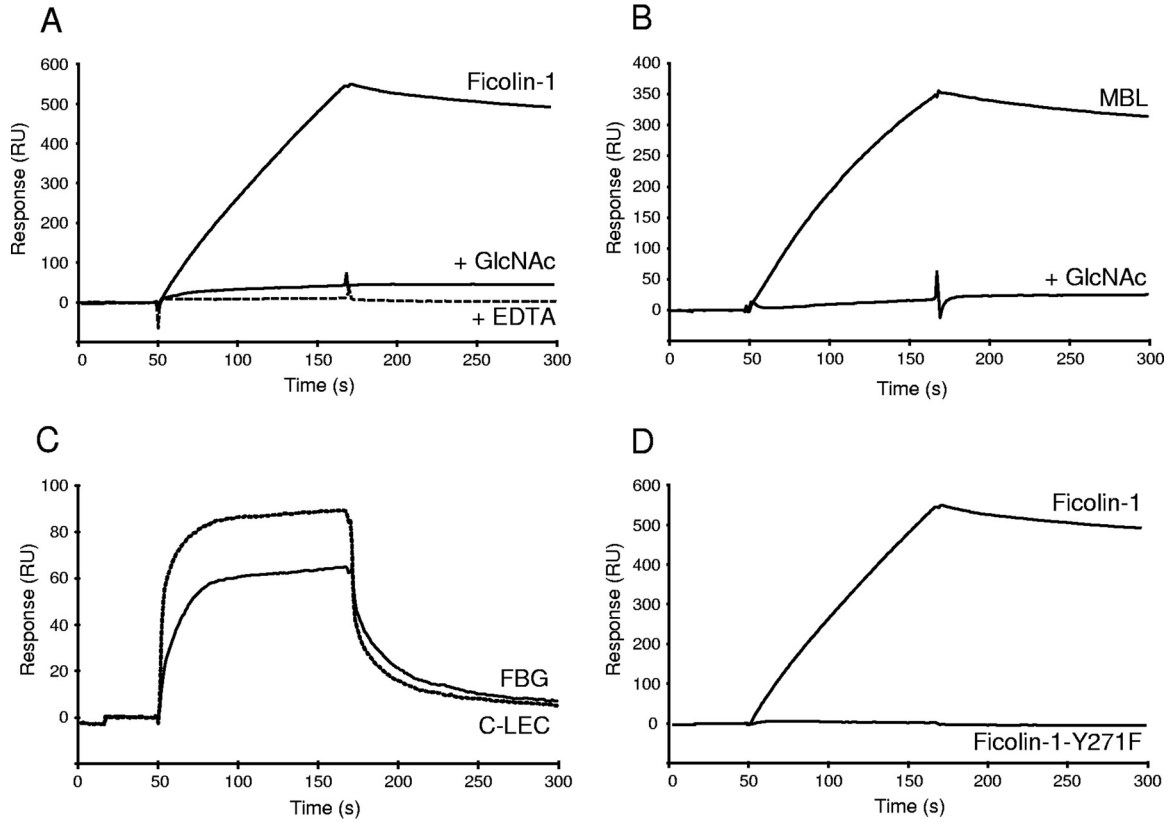

FIG 2 Characterization of the interaction of ficolin-1 with GPΔTM glycans by SPR. (A) Ficolin-1 (5 nM) was injected over 3,700 RU of immobilized GPΔTM in the presence or absence of 10 mM GlcNAc (a competitor of the ficolin-1–ligand interaction) and 5 mM EDTA (a calcium chelator). (B) MBL (5 nM) was injected over immobilized GPΔTM in the presence or absence of 10 mM GlcNAc, a competitor of the MBL-ligand interaction. (C) The FBG domain of ficolin-1 (113 nM) and the lectin domain of MBL (C-LEC; 170 nM) were injected over immobilized GPΔTM. (D) Wild-type ficolin-1 and its Y271F mutant (5 nM) were injected over immobilized GPΔTM. The results shown are representative of those from two independent experiments.

Analysis of ficolin-1 binding to GP by SPR spectroscopy. SPR spectroscopy was used to further investigate the interaction of the defense collagens and the trimeric recombinant GPΔTM. Both ficolin-1 and MBL readily bound to immobilized GPΔTM in the presence of calcium ions, whereas no interaction was observed when ficolin-2 and ficolin-3 were injected (Fig. 1B), in accordance with the data obtained by the overlay assay. Ficolin-1 and MBL bound to GPΔTM in a dose-dependent manner (Fig. 1C and D). Kinetic analyses of the ficolin-1 binding data yielded association and dissociation rate constants of $1.38 \pm 0.67 \times 10^6\ M^{-1}\ s^{-1}$ and $1.08 \pm 0.63 \times 10^{-3}\ s^{-1}$, respectively, using a global fitting to a 1:1 Langmuir interaction model. The deduced apparent equilibrium dissociation constant (K_D) was 1.19 ± 1.35 nM, indicative of high affinity. Similarly, the MBL data analysis yielded k_a, K_d, and K_D values of $1.31 \pm 0.26 \times 10^6\ M^{-1}\ s^{-1}$, $8.06 \pm 8.69 \times 10^{-4}\ s^{-1}$, and 0.72 ± 0.76 nM, respectively, which are in the same range as those obtained for ficolin-1.

Ficolin-1 binding to GPΔTM was calcium dependent, as shown by the lack of an interaction in the presence of EDTA (Fig. 2A). Considering that binding of ficolin-1 to its acetylated ligands is Ca^{2+} dependent, we next examined the effect of the acetylated carbohydrate *N*-acetyl-D-glucosamine (GlcNAc) on the interaction between ficolin-1 and GPΔTM. The interaction was indeed inhibited in the presence of 10 mM GlcNAc (Fig. 2A). In the same way, GlcNAc, known to be an MBL ligand, inhibited binding of this lectin to GPΔTM (Fig. 2B). These results strongly suggest that binding of ficolin-1 and MBL to GPΔTM is mediated by the FBG and C-lectin recognition domains of these proteins, respectively.

We have previously solved the crystal structure of the trimeric FBG domain of ficolin-1 alone and in complex with acetylated carbohydrates (51). This domain was found to bind to immobilized GPΔTM (Fig. 2C), but the dissociation was faster than that observed with the full-length oligomeric protein, which formed a stable complex because of its binding multivalency (Fig. 2A). Comparable results were obtained with the C-type lectin domain of MBL (Fig. 2C), as observed previously for the binding of MBL oligomers and the MBL lectin domain to mannose-conjugated BSA (52). These data suggest that the efficient binding of ficolin-1 (and of MBL) to GP requires oligomerization of the recognition protein, which promotes avidity through a multivalent interaction.

We have also generated a mutant of ficolin-1 (in which Tyr271 of the FBG domain is replaced by Phe) which lacks the ability to bind to acetylated ligands (48). The ability of this mutant to interact with immobilized GP was compared with that of wild-type ficolin-1. As shown in Fig. 2D, no detectable interaction was observed when the Y271F mutant was used, providing further evidence that ficolin-1 recognizes acetylated glycans of GP through its FBG domain.

Ficolin-1 binds to the mucin domain of EBOV GP. It was

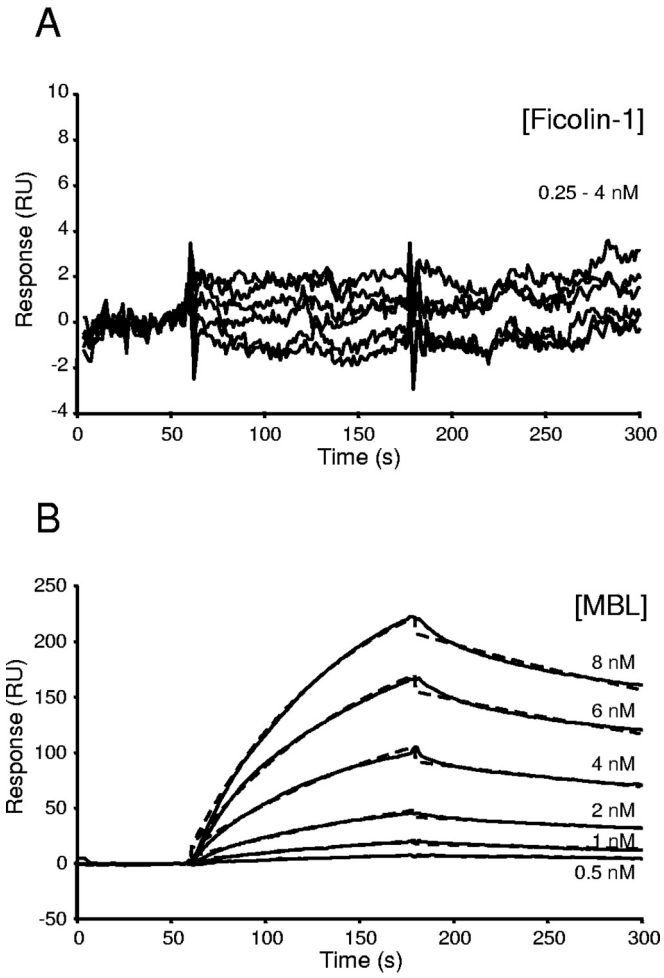

FIG 3 Results of SPR analysis of the interaction of ficolin-1 with GPΔmucΔTM of EBOV. (A) Ficolin-1 at concentrations ranging from 0.25 nM to 4 nM was injected over 3,000 RU of immobilized GPΔmucΔTM. (B) MBL at concentrations ranging from 0.5 nM to 8 nM was injected over immobilized GPΔmucΔTM. Fits are shown as dotted lines and were obtained by global fitting of the data using a 1:1 Langmuir binding model. The results shown are representative of those from two independent experiments.

previously shown that ficolin-1 is a sialic acid (*N*-acetylneuraminic acid)-binding protein able to recognize various sialylated glycans (34, 48, 56, 57). EBOV GP contains a heavily glycosylated mucin-like domain which concentrates the majority of sialylated O-linked glycosylation sites. A soluble trimeric recombinant GP from which MLD was deleted (GPΔmucΔTM, abbreviated GPΔmuc) was used to investigate, using SPR analysis, whether the mucin domain of the viral GP is required for the interaction. Ficolin-1 or MBL at various concentrations was injected over immobilized GPΔmuc. No detectable binding to GPΔmuc was observed for ficolin-1 (Fig. 3A), whereas MBL bound to GPΔmuc in a dose-dependent manner (Fig. 3B). Kinetic analysis yielded k_a, K_d, and deduced K_D values of $2.18 \pm 1.29 \times 10^6$ $M^{-1}\,s^{-1}$, $1.3 \pm 1.46 \times 10^{-3}\,s^{-1}$, and 1.09 ± 1.09 nM, respectively, using global fitting to a 1:1 Langmuir interaction model. These values are comparable to those obtained for MBL binding to GPΔTM, indicating that the mucin domain is required for the interaction of GP with ficolin-1 but is dispensable for MBL binding.

Considering the lack of interaction of the ficolin-1 Y271F mutant, devoid of a sialic acid-binding capacity, and the requirement of the GP mucin-like domain for ficolin-1 binding, our data strongly suggest that ficolin-1 recognizes sialylated glycans of the mucin domain of GP. In addition, we did not detect any interaction between wild-type ficolin-1 and immobilized purified recombinant EBOV expressing GP lacking the TM domain (rEBOVGPΔTM) expressed in Sf9 insect cells (data not shown), which is consistent with the absence of glycan sialylation of recombinant glycoproteins expressed in Sf9 cells (58).

Ficolin-1 enhances pseudovirus GP infectivity. In order to determine the role of the ficolin-1–GP interaction in virus infection, a plaque assay was performed in Vero E6 cells, using a recombinant vesicular stomatitis virus expressing the EBOV GP spike protein (rVSV-GP) at the surface of the viral particle (Fig. 4A and B).

We first verified that preincubation of the wild-type virus (expressing the G spike protein) with human ficolin-1, ficolin-2, and MBL did not result in enhancement of virus infection (data not shown). Then, rVSV-GP was preincubated with MBL, the three recombinant human ficolins, or specific anti-GP MAb 3327. Preincubation of the virus with MBL and ficolin-1 resulted in an increase in the level of virus infection (152% and 182%, respectively) compared to that obtained with nonpreincubated virus ($P = 0.022$ and $P < 0.0001$, respectively, two-tailed unpaired Student's *t* test) (Fig. 4A). Preincubation of rVSV-GP with ficolin-2 or ficolin-3 had no influence on infection ($P > 0.05$, two-tailed unpaired Student's *t* test), whereas the viral infection was completely inhibited by preincubation with the neutralizing MAb 3327 ($P < 0.0001$, two-tailed unpaired Student's *t* test), as expected for this antibody, which targets the fusion peptide of GP (46).

To further characterize the ficolin-1–rVSV-GP interaction, the virus was preincubated with increasing concentrations of ficolin-1 (2.5, 5, and 10 μg/ml) (Fig. 4B). Ficolin-1 at the three concentrations induced a statistically significant dose-dependent enhancement of rVSV-GP infection (213, 236, and 300%, respectively) compared with that achieved with nonpreincubated virus. These data indicate that the activity of ficolin-1 on rVSV-GP was specific and could be characterized as a dose-dependent enhancement of infection.

The involvement of the mucin domain of the viral GP in infection was next investigated using recombinant VSV particles expressing RFP (rVSV-RFP) from which G protein was deleted and that were pseudotyped with either GP or GPΔmuc. Because these constructs were unable to replicate, infection of Vero E6 cells was detected by evaluation of the expression of the RFP reporter gene using flow cytometry. Preincubation of rVSV-RFP-GP with 10 μg/ml of ficolin-1 resulted in an enhancement of the infection in comparison with the level of infection achieved with the nonpreincubated particles (Fig. 4C). In contrast, no ficolin-1-enhancing effect was observed when the rVSV-RFP-GPΔmuc particles were used, thereby confirming the involvement of the mucin domain in the GP–ficolin-1 interaction. The rVSV-RFP-GPΔmuc particles yielded a higher infection rate than the particles with an intact GP, suggesting that removal of the mucin domain may facilitate virus binding, as reported previously (59).

The role of the ficolin-1–GP interaction in EBOV infection was also investigated using a recombinant murine leukemia virus expressing either the EBOV-GP (strain Zaire) or RESTV-GP (strain

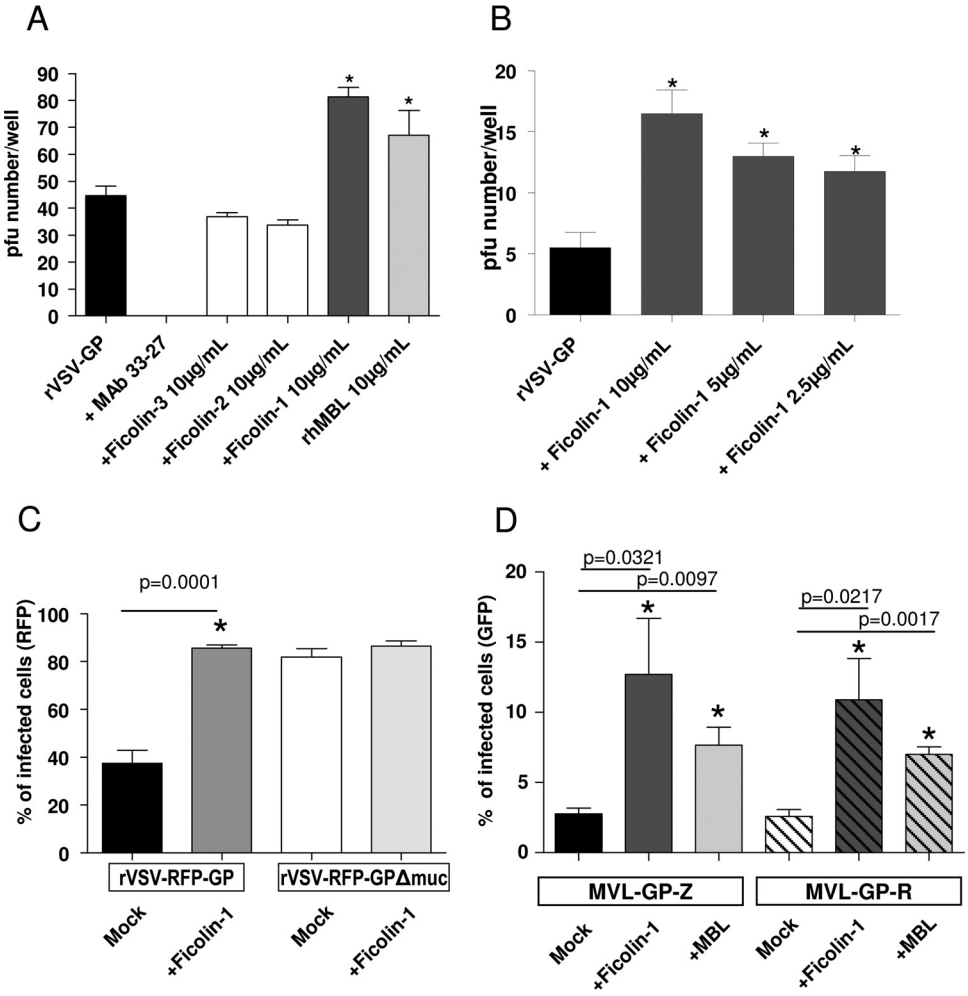

FIG 4 Results of recombinant pseudovirus-GP infection assays. rVSV-GP, MLV-GPs, and rVSV-RFP-GPs were preincubated with the defense collagens for 1 h at 37°C before infection of Vero E6 cells for 1 h at 37°C in the absence of serum at an MOI of 1×10^{-4} (rVSV-GP), 1 (MLV-GP), and 3 (rVSV-RFP-GP). At 2 days postinfection, rVSV-GP replication was measured by an assay for determination of the number of PFU, while MLV-GP infection was analyzed by flow cytometry. (A and B) Cells were infected with rVSV-GP preincubated with neutralizing MAb 3327 (1:50 dilution), ficolin-3, ficolin-2, ficolin-1, and MBL (10 μg/ml) (A) or with increasing concentrations of ficolin-1 (2.5, 5, and 10 μg/ml) (B), as indicated beneath the bars. The results for the preincubated groups were compared to those for the nonpreincubated group. *, a statistically significant difference ($P < 0.05$, two-tailed unpaired Student's *t* test). (C) Cells were infected with rVSV-RFP-GP and rVSV-RFP-GPΔmuc that had been preincubated with 10 μg/ml of ficolin-1, as indicated beneath the bars. At 8 h postinfection, RFP from infected cells (the percentage of cells positive above the RFP intensity threshold) was detected by flow cytometry. The results are the means from three independent experiments. (D) Cells were infected with MLV-GP-Z (EBOV) or MLV-GP-R (RESTV) that had been preincubated with 10 μg/ml of ficolin-1 or MBL, as indicated beneath the bars. At 2 days postinfection, GFP from infected cells (the percentage of cells positive above the GFP intensity threshold) was detected by flow cytometry. The results shown are the means from three independent experiments.

Reston) spike protein (MLV-GP-Z or MLV-GP-R, respectively) at the surface of the viral particle (Fig. 4D). Infection of Vero E6 cells by recombinant MLV-GP-Z or MLV-GP-R was detected by evaluation of the expression of the GFP reporter gene using flow cytometry analysis. Preincubation of MLV-GP-Z or MLV-GP-R with 10 μg/ml of ficolin-1 or MBL resulted in an enhanced infection compared to the level of infection achieved with nonpreincubated pseudoviruses (Fig. 4D). Both ficolin-1 and MBL enhanced MLV-GP-Z infection (358% [$P = 0.0321$, two-tailed unpaired Student's *t* test] and 176% [$P = 0.0097$, two-tailed unpaired Student's *t* test], respectively) and MLV-GP-R infection (323% [$P = 0.0217$, two-tailed unpaired Student's *t* test] and 171% [$P = 0.0017$, two-tailed unpaired Student's *t* test], respectively). A comparable enhancement of infection was observed when EBOV was compared to RESTV ($P > 0.05$, two-tailed unpaired Student's *t* test). Similar results were obtained when infections were performed at 4°C, which allows virus attachment but not cell entry (data not shown). In addition to confirming the ficolin-1-mediated enhancement of EBOV infection, these results show that this effect is common for several Ebola virus strains.

Ficolin-1 enhances EBOV infection independently of complement proteins. We next investigated whether the ficolin-1-mediated enhancement of infection observed with VSV-GP was similar in an EBOV infection model. As shown in Fig. 5A, ficolin-1 and MBL enhanced EBOV infection in the absence of serum. The enhancement of infection was restricted to ficolin-1 and MBL, since no effect was observed using ficolin-2 or ficolin-3 ($P > 0.05$, two-tailed unpaired Student's *t* test). As observed previously with

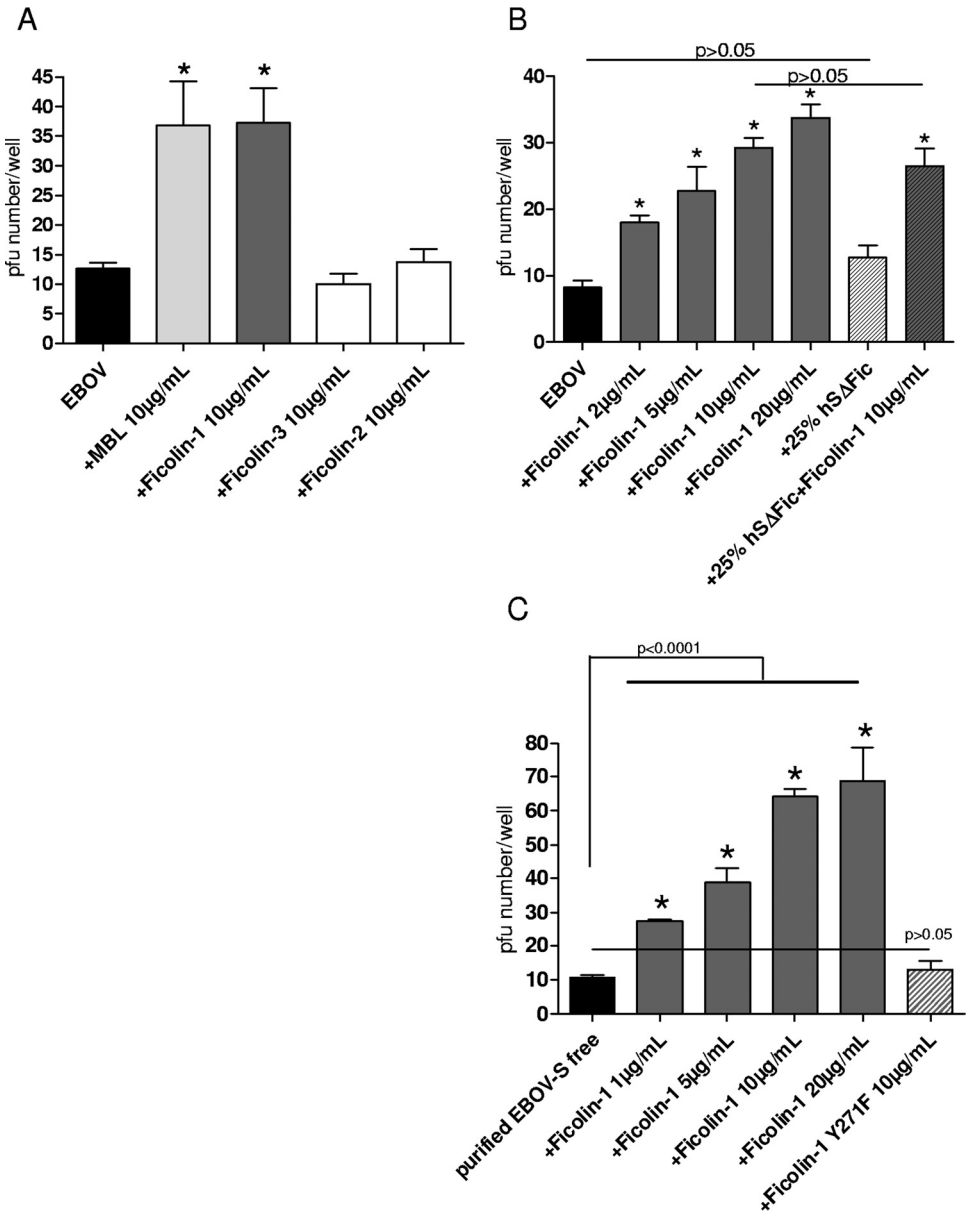

FIG 5 Results of EBOV infection enhancement assays. EBOV was preincubated with the defense collagens for 1 h at 37°C. Vero E6 cells were infected at an MOI of 2×10^{-4} (A and B) or 2×10^{-5} (C) in 24-well MW culture plates. Virus replication was measured at 6 days postinfection by an IHC assay (using EBOV NP antibody). EBOV was preincubated with 10 μg/ml of MBL, ficolin-1, ficolin-2, or ficolin-3 (A) or increasing ficolin-1 concentrations (2 to 20 μg/ml) and 25% ficolin-depleted human serum (hSΔFic) or 25% hSΔFic reconstituted with 10 μg/ml ficolin-1 (B), as indicated beneath the bars. (C) Purified EBOV produced in Vero E6 cells adapted to serum-free medium (EBOV-S free) was preincubated with increasing doses of ficolin-1 (1 to 20 μg/ml) or 10 μg/ml of the ficolin-1 Y271F mutant, as indicated beneath the bars. The result for the pretreated groups were compared to those for the nonpreincubated EBOV group. *, a statistically significant difference ($P < 0.05$, two-tailed unpaired *t* test).

VSV-GP, ficolin-1 enhanced EBOV infection in a dose-dependent manner (2 to 20 μg/ml) (Fig. 5B).

The MBL protein was previously shown to inhibit EBOV infection in the presence of active complement in serum (26, 30, 31). In order to confirm this observation, EBOV was preincubated with 25% either MBL-deficient human serum (hSΔMBL) or hSΔMBL supplemented with 10 μg/ml recombinant MBL (data not shown). No statistically significant difference was observed in the infection assay when the virus was preincubated or not preincubated with MBL-deficient serum ($P > 0.05$, two-tailed unpaired Student's *t* test). MBL addition to MBL-deficient serum induced a statistically significant inhibition in the level of EBOV infection (19%) compared to that achieved with nonpreincubated virus ($P = 0.0182$, two-tailed unpaired Student's *t* test).

We next investigated whether a similar effect of serum proteins could be observed in the case of ficolin-1 and EBOV infection (Fig. 5B). The preincubation of EBOV with 25% ficolin-depleted human serum (hSΔFic) had no significant effect on virus infection ($P > 0.05$, two-tailed unpaired Student's *t* test). The addition of ficolin-1 (10 μg/ml) to ficolin-depleted serum resulted in an en-

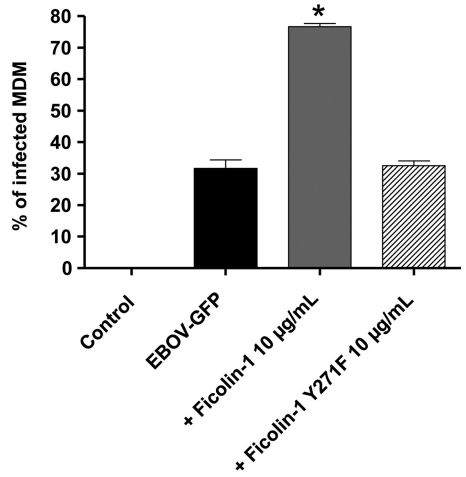

FIG 6 EBOV infection enhancement on human macrophages. EBOV was preincubated with 10 μg/ml ficolin-1 (the wild type or its 271F mutant) for 1 h at 37°C. MDMs were infected at an MOI of 0.1 in 24-well culture plates. EBOV replication was quantified at 2 days postinfection by detection of GFP from infected cells (the percentage of cells positive above the GFP intensity threshold) by flow cytometry. Control, noninfected cells.

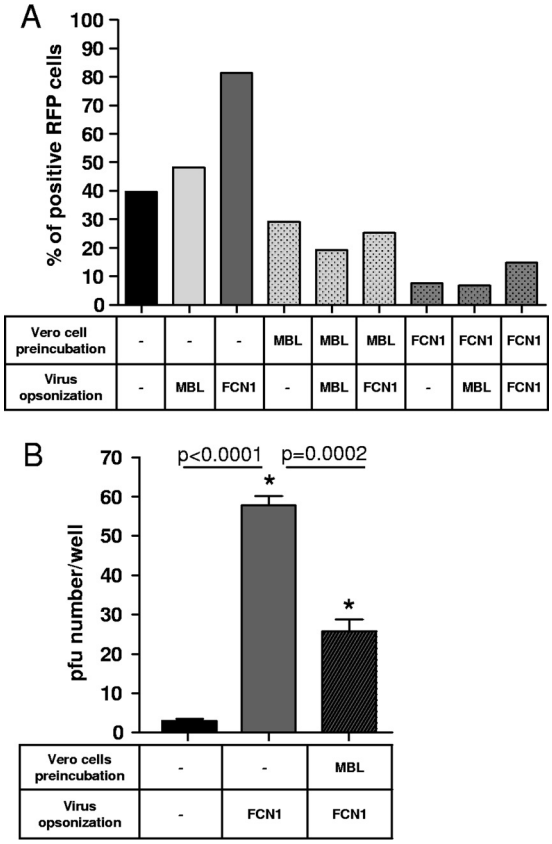

FIG 7 Competition of MBL and ficolin-1 for virus attachment. (A) Vero E6 cells, pretreated or not pretreated with a cold solution of MBL or ficolin-1 (4 μg/ml), were incubated for 2 h at 4°C with rVSV-RFP-GP particles that had been preincubated or not preincubated with ficolin-1 (FCN1) or MBL (10 μg/ml). After 8 h, virus attachment was measured by flow cytometry. (B) Vero E6 cells that had been pretreated or not pretreated with a cold solution of MBL (3.4 μg/ml) were incubated with ficolin-1-pretreated rVSV-GP or nonpretreated viral particles for 2 h at 4°C. Virus binding was measured by an assay for determination of the number of PFU, and plaques were revealed using crystal violet. *, a statistically significant difference compared to the result for the rVSV-GP control ($P < 0.05$, two-tailed unpaired Student's *t* test).

hancement of EBOV infection that was comparable to that observed with ficolin-1 in the absence of serum ($P > 0.05$, two-tailed unpaired Student's *t* test). These data indicate that ficolin-1 enhances EBOV infection independently of complement proteins.

The role of ficolin-1 was confirmed by using purified EBOV particles under serum-free conditions in order to prevent the possible adsorption of serum components to the virus particle throughout virus production. Preincubation of EBOV with increasing concentrations of ficolin-1 (range, 1 to 20 μg/ml) induced a statistically significant dose-dependent enhancement in the level of EBOV infection (219, 277, 356, and 408% at concentrations of 1, 5, 10, and 20 μg/ml, respectively) compared to that achieved with the nonpreincubated virus ($P = 0.0006$, $P = 0.0086$, $P < 0.0001$, and $P < 0.0001$, respectively, two-tailed unpaired Student's *t* test) (Fig. 5C). Moreover, to assess the involvement of the ficolin-1 sialic acid-binding capacity, the level of infection achieved with the ficolin-1 Y271F mutant was next compared to that achieved with wild-type ficolin-1 (Fig. 5C). No detectable enhancement of EBOV infection was observed using the Y271F mutant, confirming that the interaction of ficolin-1 with the sialic acid residues of GP is required to enhance infection.

Ficolin-1 enhances EBOV infection of human macrophages. We investigated whether a similar enhancement of EBOV infection by ficolin-1 could be observed on macrophages, which are major viral target cells. Human monocyte-derived macrophages (MDMs) were produced and infected with EBOV expressing the GFP reporter gene. Preincubation of EBOV-GFP with ficolin-1 resulted in a statistically significant enhancement of EBOV infection (Fig. 6) compared to the level of infection achieved with nonpreincubated virus ($P = 0.03$, Mann-Whitney test). In addition, no effect was observed when the ficolin-1 Y271F mutant was used, confirming the findings of the SPR interaction and EBOV infection enhancement experiments (Fig. 2A and 5C).

Ficolin-1 and MBL enhancement of infection depends on a common cellular receptor/partner. MBL and ficolins are innate immune proteins able to sense pathogen-associated carbohydrate motifs through their recognition domains and to enhance the phagocytosis of opsonized targets through interaction of their collagen-like regions with phagocyte receptors. Enhancement of EBOV infection of Vero E6 cells might proceed through a similar mechanism, which implies interaction of the defense collagens with a putative cell surface receptor for their collagen stalks.

To investigate the possible competition between ficolin-1 and MBL for binding to a common receptor on Vero E6 cells, a viral attachment assay was performed using rVSV-RFP-GP, and the effect of preincubation of target cells with MBL or ficolin-1 was studied. The viral particles, nonopsonized or opsonized with ficolin-1 or MBL, were incubated for 2 h at 4°C with the Vero E6 cells, which had been preincubated or not preincubated with MBL or ficolin-1 (for 2 h at 4°C) and rinsed to eliminate the unbound protein. Virus attachment to Vero E6 cells was analyzed by flow cytometry, as described above. As expected, rVSV-RFP-GP binding to Vero E6 cells was enhanced when GP-bearing particles were preincubated with either ficolin-1 or MBL compared to the level of binding of nonopsonized particles (Fig. 7A). Preincubation of Vero E6 cells with MBL slightly diminished the attachment of

nonopsonized viral particles but strongly reduced the enhanced attachment of both MBL- and ficolin-1-opsonized particles (Fig. 7A), suggesting that both proteins might recognize a common cell surface receptor. In contrast, preincubation of Vero E6 cells with ficolin-1 virtually abolished the attachment of nonopsonized rVSV-RFP-GP particles (Fig. 7A), thus making it impossible to draw any reliable conclusions regarding the potential effect of virus opsonization under these conditions. This inhibitory effect likely arises from the strong affinity of ficolin-1 for Vero E6 cell surface sialic acids, which could further impair attachment of the virus to the cell surface.

The effect of Vero E6 cell preincubation with MBL on ficolin-1 enhancement of virus attachment to the cells was also studied under the same conditions described above, but rVSV-GP and a plaque-forming assay were used to analyze virus attachment to the Vero E6 cell monolayer. Ficolin-1 enhanced viral attachment (Fig. 7B) ($P < 0.0001$, two-tailed unpaired Student's t test), and this enhancement was reduced when the cells were preincubated with MBL (Fig. 7B) ($P = 0.00022$, two-tailed unpaired Student's t test), suggesting again that both proteins can bind to a common cell surface receptor.

DISCUSSION

Ficolin-1 has a special status in the ficolin family of innate immune recognition proteins due to its main localization at the cell surface and in secretory granules of monocytes and neutrophils and its unique binding specificity for sialylated ligands (36). Whereas the biological functions of cell surface ficolin-1 have not been elucidated, serum ficolin-1 appears to be an authentic pattern recognition molecule able to sense several bacteria of clinical interest by binding to sialylated structures of the surface proteins (reviewed in reference 60). For example, ficolin-1 binds to *Staphylococcus aureus* (a Gram-positive bacterium), to *Salmonella enterica* serovar Typhimurium LT2 (a Gram-negative bacterium) (57), and also specifically to sialic acids of the capsular polysaccharides of *Streptococcus agalactiae* (61). However, no viral ligand for ficolin-1 has been identified so far.

The current study describes the binding of ficolin-1 to EBOV GP. We demonstrated that the interaction of ficolin-1 with the viral glycoprotein arises from the known ability of ficolin-1 to bind acetylated ligands, more precisely, the sialylated moieties of the GP mucin-like domain. This assessment is based on the following observations: (i) the interaction was Ca^{2+} dependent and was inhibited by acetylated glycans, such as GlcNAc; (ii) the ficolin-1 Y271F mutant, which had an impaired sialic acid-binding capacity, did not interact with GP; (iii) removal of the mucin-like domain of GP abolished its interaction with ficolin-1; (iv) ficolin-1 did not interact with insect cells expressing GP from which sialylated glycans were deleted; and (v) sialylated moieties are present on O-linked glycans of the MLD but are largely absent from GP N-linked glycans (62). The observed ficolin-1 binding specificity for the GP mucin domain, which contains 5 N-linked glycan addition sites and 12 to 15 O-linked glycan addition sites (63), is in accordance with our previous data showing the ficolin-1 interaction with CD43, the major sialomucin exposed at the neutrophil surface (64). The unique sialic acid-binding specificity of ficolin-1, demonstrated by our previous glycan array screening analysis (48), likely accounted for the fact that human ficolin-2 and ficolin-3 were not found to interact with EBOV GP.

MBL, another member of the defense collagen family, was previously shown to interact with EBOV GP (25, 30, 31). In this study, we demonstrated that the GP binding mechanism of ficolin-1 clearly differed from that of MBL. Indeed, MBL retained the ability to bind to GP devoid of the mucin domain, in accordance with its known specificity for neutral carbohydrates, such as high-mannose N-linked carbohydrates of the GP glycan cap (25, 30). However, the possibility that MBL might also interact with certain N-linked carbohydrates of the EBOV GP MLD cannot be excluded. Interestingly, the recently described kidney collectin CL-K1, either in a recombinant form or in a native complex with the CL-L1 liver collectin (65, 66), did not interact with EBOV GP (data not shown). The difference between the two collectins and MBL might arise from their restricted carbohydrate binding specificity, although all these C-type lectins share the ability to recognize mannose residues (67), and/or the difference might arise from a lower degree of oligomerization of the CL-K1 and CL-L1/CL-K1 complexes in comparison with that of MBL. Indeed, it has been demonstrated, at least in the case of recombinant CL-K1, that it is mainly assembled from monomers and dimers of subunits composed of three polypeptide chains (67), whereas recombinant MBL exhibited higher oligomers (68).

Importantly, we showed here that ficolin-1 also bound to EBOV GP exposed at the VSV and MLV surface, which resulted in enhanced infection of Vero E6 cells. The requirement for the GP mucin domain was confirmed using VSV particles with surface-exposed GP lacking this domain. Moreover, the ficolin-1-dependent enhancement of infection was observed using viral particles expressing the Zaire or Reston EBOV strain GP on their surfaces. Ficolin-1 was shown to enhance infection of Vero E6 cells when not only pseudotyped viral particles but also genuine EBOV was used. In addition, similar results were obtained with human macrophages, which are major viral targets of Ebola virus infection. Ficolin-1 enhanced EBOV infection of MDMs, while no effect was obtained with the ficolin-1 Y271F mutant, confirming the involvement of the ficolin recognition domain in virus opsonization.

Ficolin-2, ficolin-3, and the CL-K1 and CL-K1-L1 complexes displayed no ability to modulate EBOV infection, which was in accordance with their inability to interact with EBOV GP. MBL was previously shown to enhance EBOV infection in the absence of serum or in the presence of a low serum concentration (<10%) but to inhibit its infection at a higher serum concentration (30, 31), an observation that was reproduced in the present study. Interestingly, ficolin-1 was shown to enhance EBOV infection independently of serum, a result that was confirmed using a virus produced in serum-free medium. The MBL-mediated virus-neutralizing effect in the presence of serum has been shown to be dependent on complement activation (25), which suggests that the ficolin-1–GP interaction likely does not trigger activation of the lectin complement pathway. This might be related to the low complement-activating efficiency previously reported for ficolin-1 (69), but this remains to be investigated.

A further difference between ficolin-1 and MBL relies on the unique property of ficolin-1 in which it is able to bind to cell surface sialylated ligands through its FBG domain (70). Although such ligands might be present on many cell types, a preferential binding of recombinant ficolin-1 to monocytes and granulocytes rather than lymphocytes was reported (70). Recombinant ficolin-1 also bound to the surface of cells of various cell lines, including fibroblast-like COS7 cells (56), epithelial cell-like CHO cells (70),

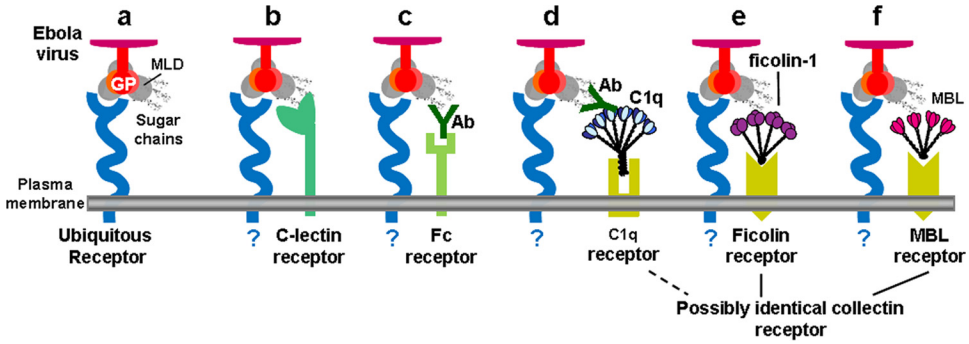

FIG 8 Putative models of EBOV GP-mediated attachment to host cells. (a) EBOV binds to the target cell surface through the interaction of GP with host cell receptors, including a putative ubiquitous receptor. (b) GP glycans, mainly provided by the MLD, are recognized by membrane-bound C-type lectin receptors. (c and d) Antibody (Ab)-dependent enhancement of infection is triggered by antibody binding to GP epitopes and subsequent interaction of its Fc region with the Fc receptor (c) or with the globular heads of soluble complement protein C1q (d); C1q then cross-links the virus to the cell surface by interacting with a cell surface receptor for its collagen-like stalks. (e) Ficolin-1 interacts through its FBG recognition domains with sialylated moieties of the MLD and enhances viral attachment through binding to a putative collectin receptor that might be shared with MBL and, possibly, C1q. (f) MBL binds to neutral GP glycans through its C-type lectin domains. Ficolin-1 enhances viral infection independently of complement, whereas the effect of MBL (infection enhancement or inhibition) depends on the complement conditions (low or high). (Adapted from *Frontiers in Microbiology* [77].)

and Vero E6 cells (data not shown). The binding of ficolin-1 and MBL to Vero E6 cells was partly mediated through their carbohydrate recognition domains, as indicated by the reduced binding of the ficolin-1 Y271F mutant and of MBL in the presence of GlcNAc and mannan (data not shown). However, preincubation of Vero E6 cells with MBL and ficolin-1 prevented viral attachment only in the latter case (Fig. 7A), which might suggest that, under certain circumstances, cell surface ficolin-1 could have a protective effect against viral infection. Cell surface expression of endogenous ficolin-1 has been shown to be restricted to monocytes and granulocytes, which seem to be refractory to specific EBOV GP-mediated entry (71, 72). Interestingly, M-ficolin expression is silenced in macrophages and dendritic cells (56, 73), which are major target cells of EBOV. It is therefore tempting to speculate that circulating ficolin-1 binds preferentially to the viral GP rather than to the surface of host target cells. However, it is not possible to verify this assumption with the current state of our knowledge, because of its dependence on the ficolin-1 concentration and relative affinities for the viral GP and host cell glycans, which are unknown at present. These considerations emphasize the dual role potentially played by human ficolin-1 when it is circulating in serum and tethered to leukocyte surfaces.

Although MBL and ficolin-1 clearly differed from each other in their mechanisms of binding to the EBOV GP, by recognizing different carbohydrate ligands located in different domains, the two soluble proteins acted as bridging molecules to facilitate virus attachment to host cell surface receptors. Both proteins have collagen-like regions able to interact with collectin receptors, including the calreticulin/CD91 complex (74), $\alpha_2ß_1$ integrin (75), and complement receptor 1 (CD35) (76), that are shared with the C1q protein, another member of the complement defense collagen family. Ficolin-1 and MBL might bind to the same cell receptor, as indicated here in the competition assay using MBL-preincubated target cells and MBL- and ficolin-1-opsonized virus (Fig. 7). Two MBL candidate receptors, the C1q-binding protein (C1qBP) and the lectin receptor dectin-2, were previously identified using RNA interference screening (30). However, C1qBP is identical to the receptor for the globular domains of C1q and has not been reported to bind to the collagen-like regions of C1q, MBL, or ficolins. In addition, this protein has no transmembrane domain and likely functions in association with other membrane receptors. Dectin-2 is a membrane C-type lectin receptor, but its specificity for the collagen-like sequences has not been described so far. Thus, the MBL and ficolin-1 receptor remains to be identified, and they may have different receptors, depending on the host cell type. It should be mentioned that this receptor might also serve as the unknown C1q receptor used to mediate antibody-dependent enhancement of viral infection (77) (Fig. 8).

Other membrane-anchored C-type lectin receptors, including the asialoglycoprotein receptor in hepatocytes, the dendritic cell-specific DC-SIGN, the liver/lymph node-specific lectins L-SIGN and LSECtin, and human macrophage lectin MGL (which are not present on the Vero E6 cells used in this study; reviewed in reference 77), have been shown to bind to the EBOV GP and to promote virus entry into various cell types. Binding of these lectins to EBOV GP was proposed to involve the mannose and/or GlcNAc residues of the N-linked carbohydrates present in the MLD or in the glycan cap. To our knowledge, ficolin-1 is the first host innate immune recognition protein described that binds exclusively to the MLD of the EBOV GP protein, in particular, to the sialylated moieties of O-linked glycans of this domain. The role of MLD is complex, as illustrated by its multiple described functions. It was hypothesized to shield the EBOV GP from neutralizing antibodies and host proteins, such as major histocompatibility complex class I and integrins (78–81). It was also involved in the antibody-dependent enhancement of viral infection via recognition by GP-specific antibodies (82). However, the removal of MLD from immunizing virus-like particles did not alter the neutralizing antibody response (54). The MLD could also interact with protective antibodies, but the epitopes identified so far have been unglycosylated peptides (63).

Although all the above-mentioned lectins have been shown to enhance virus infection through GP binding, it is likely that these molecules mediate only viral attachment (83). Indeed, following virus uptake and trafficking to late endosomes, where membrane fusion takes place, EBOV GP is cleaved by cellular proteases, thus releasing the MLD and bound proteins/receptors. Another hypothesis involving an unknown ubiquitous receptor that could

mediate both the attachment and fusion functions has also been proposed (77) (Fig. 8).

In summary, we have identified soluble ficolin-1 to be a novel actor in EBOV host cell attachment that serves as a bridging molecule between the mucin domain of GP and a host target cell receptor that remains to be identified. Our study provides further evidence that the GP glycans play a double role by shielding host cell nonlectin surface proteins and diverting the protective function of innate defense lectins. The detailed mechanisms underlying the enhancement of viral infection of target cells by ficolin-1 and the possible influence of ficolin-1 in Ebola virus pathogenesis are currently not known. However, it can be hypothesized that an increase in the local concentration of soluble ficolin-1, i.e., after secretion by stimulated monocytes or granulocytes, may allow virus capture at the host target cell surface and facilitate further interaction with its cognate receptor and subsequent membrane fusion. On the other hand, endogenous ficolin-1 exposure at the surface of certain cell types might contribute to their protection from viral entry. In any case, many facets of ficolin-1-dependent enhancement of infection remain to be deciphered, and it would be interesting to investigate among other things whether ficolin-1 modulates the viral entry driven by the GPs of other viruses, such as the Marburg virus GP.

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