A Novel L-ficolin/Mannose-binding Lectin Chimeric Molecule with Enhanced Activity against Ebola Virus*‡§

Received for publication, January 23, 2010, and in revised form, May 28, 2010 Published, JBC Papers in Press, June 1, 2010, DOI 10.1074/jbc.M110.106260

Ian C. Michelow†¶, Mingdong Dong§, Bruce A. Mungall†, L. Michael Yantosca‡, Calli Lear†, Xin Ji**, Marshall Karpel‡, Christina L. Rootes§, Matthew Brudner‡, Gunnar Houen¶¶, Damon P. Eisen**†, T. Bernard Kinane§, Kazue Takahashi‡, Gregory L. Stahl‡¶, Gene G. Olinger§, Gregory T. Spear**, R. Alan B. Ezekowitz‡, and Emmett V. Schmidt‡

From the †Program of Developmental Immunology, Department of Pediatrics, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114, ‡Interdisciplinary Nanoscience Center (iNANO), University of Aarhus, DK-8000 Aarhus, Denmark, §Australian Animal Health Laboratory, Commonwealth Scientific and Industrial Research Organisation (CSIRO) Livestock Industries, Geelong, Victoria 3220, Australia, ¶¶Department of Clinical Biochemistry and Immunology, Statens Serum Institut, DK-2300 Copenhagen, Denmark, **Department of Immunology/Microbiology, Rush University Medical Center, Chicago, Illinois 60612, ††Department of Clinical Biochemistry and Immunology, Statens Serum Institut, DK-2300 Copenhagen, Denmark, §§Victorian Infectious Diseases Service, Royal Melbourne Hospital, Parkville 3050, Australia, and ‡‡Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115.

Ebola viruses constitute a newly emerging public threat because they cause rapidly fatal hemorrhagic fevers for which no treatment exists, and they can be manipulated as bioweapons. We targeted conserved N-glycosylated carbohydrate ligands on viral envelope surfaces using novel immune therapies. Mannose-binding lectin (MBL) and L-ficolin (L-FCN) were selected because they function as opsonins and activate complement. Given that MBL has a complex quaternary structure unsuitable for large scale cost-effective production, we sought to develop a less complex chimeric fusion protein with similar ligand recognition and enhanced effector functions. We tested recombinant human MBL and three L-FCN/MBL variants that contained the MBL carbohydrate recognition domain and varying lengths of the L-FCN collagenous domain. Non-reduced chimeric proteins formed predominantly nona- and dodecameric oligomers, whereas recombinant human MBL formed octadecameric and larger oligomers. Surface plasmon resonance revealed that L-FCN/MBL76 had the highest binding affinities for N-acetylgalcosamine-bovine serum albumin and mannan. The same chimeric protein displayed superior complement C4 cleavage and binding to calreticulin (cC1qR), a putative receptor for MBL. L-FCN/MBL76 reduced infection by wild type Ebola virus Zaire significantly greater than the other molecules. Tapping mode atomic force microscopy revealed that L-FCN/MBL76 was significantly less tall than the other molecules despite similar polypeptide lengths. We propose that alterations in the quaternary structure of L-FCN/MBL76 resulted in greater flexibility in the collagenous or neck region. Similarly, a more flexible in the collagenous or neck region. Similarly, a more flexible

Development of effective therapies against antimicrobial-resistant and emerging infectious diseases is an urgent priority (1). Ebola and Marburg viruses, members of the Filovirus family, are Centers for Disease Control and Prevention Category A agents that represent a substantial threat to public health because they cause rapidly fatal hemorrhagic fevers. As such, they also pose a potential danger if manipulated as bioweapons. Therefore, a key unmet need is the development of effective therapies against this class of viral pathogens. To that end, we set about developing large molecule therapies that are aimed at viral elimination.

Our thinking was influenced by previous work from our and other laboratories that indicates a role for endogenous circulating mannose-binding lectin (MBL)3 as a first line host defense against a wide variety of viral and other pathogens (2). Mannose-binding lectin may be considered an ante-antibody because it serves as a broad spectrum recognition molecule and acts as an opsonin either alone or in conjunction with complement in the first minutes or hours after exposure to an infectious agent (3). Mannose-binding lectin, which is a C-type lectin, recognizes specific configurations of N-acetylgalactosamine but not galactose and sialic acid, the penultimate and ultimate sugars of most mammalian glycoproteins, respectively. Therefore, MBL recognizes the conserved sugars that decorate the

*This work was supported, in whole or in part, by National Institutes of Health Grants U01 AI070330 (to E. V. S.) and R01 HL52886, R01 HL56086, R21 HL092469, and P50 DE016191 (to G. L. S.). This work was also supported by United States Department of Defense, Defense Threat Reduction Agency Biological Therapeutics Program Grants 4.10006 and 4.10007 (to G. G. O.).

†The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure.

‡To whom correspondence should be addressed: Dept. of Pediatrics, Massachusetts General Hospital, Harvard Medical School, 55 Fruit St., GRJ-1402, Boston, MA 02114. Fax: 617-724-3248; E-mail: imichelow@partners.org.

§Present address: Merck Research Laboratories, Rahway, NJ 07065.

**The abbreviations used are: MBL, mannose-binding lectin; AFM, atomic force microscopy; BSA, bovine serum albumin; CRD, carbohydrate recognition domain; HeV, Hendra virus; HIV, human immunodeficiency virus; L-FCN, L-ficolin; MASP, MBL-associated serine protease; NIV, Nipah virus; rhMBL, recombinant human MBL; VBS, Veronal-buffered saline; hMBL, human MBL; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; C4, complement component 4; GP, glycoprotein; env neg, envelope-negative; EMEM, Eagle’s minimum essential medium; eGFP, enhanced green fluorescent protein.
Novel Chimeric Molecule Active against Ebola Virus

cell walls of many Gram-positive and Gram-negative bacteria as well as the surface glycoproteins of viruses like influenza virus, HIV, severe acute respiratory syndrome coronavirus, and Ebola virus (2, 4). The potential clinical applications of MBL as a therapeutic agent are illustrated by animal studies in which MBL effectively protected mice against Staphylococcus aureus (5) and Pseudomonas aeruginosa (6) and by in vitro studies showing activity against influenza virus (7).

Mannose-binding lectin has a C-terminal carbohydrate recognition domain (CRD) followed by a neck region that forms an α-helical coiled coil that favors the formation of trimers that are stabilized by an N-terminal collagen helix (8, 9). Mannose-binding lectin is a hexamer of trimers and resembles the first complement component, C1q. The complex quaternary structure of MBL makes it difficult to produce recombinant material with a cost-effective process. We therefore sought to develop an idealized chimeric fusion protein that maintained the binding repertoire of MBL and the ability to activate complement but represented a less complex quaternary structure. The design of the novel chimeric proteins was derived from a novel paradigm described by Hartshorn and co-workers (10–13) who showed that chimeric molecules consisting of fused complementary portions of two collectins (MBL, surfactant protein D, or bovine serum conglutinin) or one collectin (surfactant protein D) fused to anti-CD89 (an anti-Fc-αRI) had superior antimicrobial and opsonic properties compared with the parent collectins (11, 14). In certain instances, the degree of molecular oligomerization contributed to these observations, but it is also possible that conformational changes in protein quaternary structure played a role.

We selected two proteins that are able to activate the lectin complement pathway, MBL and L-ficolin (ficolin/P35; ficolin-2). Both MBL and L-ficolin (L-FCN) have similar structural templates comprising collagen-like and ligand binding domains. Each parent molecule consists of three identical polypeptides that form a helical homotrimeric structure that is further organized into higher order multimers stabilized by N-terminal disulfide bonds (9, 15–17). However, these molecules differ in a number of important ways. Unlike MBL, L-FCN forms stable tetramers of trimers that represent a simpler structure (17). Whereas MBL is a C-type lectin defined by its ability to bind certain N-glycosylated carbohydrates via calcium-dependent CRDs, L-FCN binds acetylated compounds (such as GlcNAc and GalNAc), elastin, corticosteroids, lipoteichoic acid, and 1,3-β-d-glucan via fibrinogen-like domains (18–22). Both molecules form complexes with an MBL-associated serine protease (MASP-2) dimer stabilized by Ca2+ ions and initiate the lectin complement pathway by cleaving complement component 4 (C4) and C2 to form C4b2a, the C3 convertase, thereby triggering the complement cascade (23). One important difference is that several candidate receptors for MBL have been proposed including calreticulin (cC1qR) (24), whereas L-FCN apparently functions only as an opsonin.

Our objective was to generate novel recombinant chimeric fusion proteins derived from human MBL and L-FCN with enhanced effector functions. We compared the relative function of three recombinant L-FCN/MBL structural variants with recombinant human MBL (rhMBL). To model infection, we selected enveloped RNA viruses and viral derivatives from the order Mononegavirales and families Filoviridae (Ebola virus) in the first instance and Paramyxoviridae (Hendra and Nipah viruses), which are Centers for Disease Control and Prevention Category C agents, because all these viruses have N-glycosylated mannose-rich glycoproteins on their surfaces, high mortality, and no effective therapies (25, 26). We report that L-FCN/MBL76 had superior effector activity that appeared to be mediated by greater structural flexibility and should be considered as a potential therapeutic agent.

EXPERIMENTAL PROCEDURES

L-FCN/MBL Plasmids

Expression plasmids encoding fusion proteins containing the respective polypeptide sequences from L-FCN and human MBL have been described (Ref. 46; NatImmune A/S, Copenhagen, Denmark) and are schematically shown in Fig. 1. Briefly, the parental vector pcDNA2003-cintMBLcDNA was derived from a high copy number plasmid and contained the cDNA for human MBL, a human cytomegalovirus immediate early promoter, an ampicillin resistance gene, and a neomycin resistance gene driven by the SV40 early promoter. The L-FCN/MBL constructs were cloned by fusing the linearized insert derived from Gene Storm clone RG000632 (Invitrogen) containing the L-ficolin sequence to the linearized MBL vector. Correct recombinant plasmid sequences were verified with restriction analyses and DNA sequencing by the Massachusetts General Hospital DNA Sequencing Core with four overlapping primers per plasmid. The proteins are referred to as L-FCN/MBL126, L-FCN/MBL76, and L-FCN/MBL64 based on the identity of the first amino acid of the MBL polypeptide (Fig. 1). Specifically, 1) L-FCN/MBL126 (251 amino acids) consisted of the entire L-FCN N-terminal and collagenous region ending at amino acid 104; the collagen stalk contained 17 Gly-X-Y triplet repeats (where X and Y represent any amino acid). 2) L-FCN/MBL76 (255 amino acids) contained components of the collagen stalk from both L-FCN and MBL including 21 Gly-X-Y triplet repeats and the MBL coiled coil neck region; the transition was at a lysine residue that corresponded to the MASP-binding site. 3) L-FCN/MBL64 (247 amino acids) comprised a proportionately shorter fragment of L-FCN (to amino acid 45) and a larger portion of the MBL collagenous region including 21 Gly-X-Y triplet repeats in total and the MBL “kink” (supplemental figure). In summary, each chimera had the identical L-FCN signal peptide, varying lengths of the L-FCN and MBL collagenous domains, variations in the protein sequences flanking the MASP-binding site, and the same entire MBL CRD. Complete protein sequences, based on their DNA sequences, are available in the supplemental figure.

Chimeric Protein and rhMBL Production

We selected stable transfectants of each of three L-FCN/MBL plasmids using the Freestyle 293 Expression System (Invitrogen). Freestyle 293-F cells (1 × 10^6/ml) were transfected with 30 μg of each plasmid DNA and 40 μl of 293fectin in Opti-MEM I. Cells were incubated in Freestyle Expression Medium and 300 μg/ml G418 (Geneticin, Invitrogen), and individual transfectants were selected with limiting dilutions. Pro-
tein expression in the supernatant of at least 10 expanded clones per plasmid was quantified by mannan binding and C4 cleavage assays (see below) using anti-hMBL antibody HYB 131-01 (BioPorto, Gentofte, Denmark). One high expressing clone per plasmid was selected and propagated in T175 flasks (BD Biosciences) with Freestyle Expression Medium, 2% fetal bovine serum (Invitrogen), and 300 μg/ml G418 at 37 °C with 5% CO2. The supernatant was harvested twice weekly and centrifuged to remove cell debris.

Filtered aliquots of supernatant were incubated with an equal volume of 10 mM Tris buffer, 50 mM CaCl2, 1.25 mM NaCl, pH 7.8 and 1 ml of d-mannose, 4% agarose beads (Sigma-Aldrich) overnight at 4 °C in a rocking flask. Beads were washed with the same buffer, and bound proteins were eluted with 10 mM Tris buffer, 50 mM EDTA, 1.25 mM NaCl, pH 7.8 in 0.5-ml fractions. Buffer was exchanged with the same as that used for rhMBL (10 mM Tris, 1 mM CaCl2, 140 mM NaCl, pH 7.4) using a Vivaspin 20 (Vivaproducts, Littleton, MA). Protein suspensions were filter-sterilized using a 0.22-μm Millipore syringe filter (Millipore Corp., Bedford, MA); all batches contained 0.05% Tween 20, pH 7.4; and blocked with 0.1% BSA in Tris-buffered saline, 5 mM CaCl2, pH 7.4 at 37 °C for 1 h with a variety of acetylated and non-acetylated hexose monosaccharides (1–100 mM) and then added to mannan-coated wells. After washing, detection was performed using 1 μg/ml anti-hMBL HYB-131-01 together with 1:7500 alkaline phosphatase-conjugated anti-mouse antibody (Promega Corp., Madison, WI) diluted in blocking solution and 0.5 mg/ml p-nitrophenyl phosphate (Sigma-Aldrich). Absorbance at A405 nm was measured with a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). Chimeric protein titers were interpolated from the rhMBL standard curve.

C4 cleavage activity of chimeric proteins was measured by a modification of previously reported methods (28). In brief, triplicate samples of diluted chimeric proteins were added to mannan-coated microtiter plates with 1% BSA-null mouse serum (5) as a source of MASP (animal protocol approved by the Massachusetts General Hospital Subcommittee on Research Animal Care). Normal human serum complement standard (Quidel, San Diego, CA) containing native MBL was used to generate a standard curve. After incubation at 37 °C for 1 h and rinsing, deposited human C4 fragments (Sigma-Aldrich) were detected with anti-human C4c antibodies (Dako Denmark A/S) followed by biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), avidin-containing Vectastain ABC-alkaline phosphatase reagent (Vector Laboratories, Burlingame, CA), and p-nitrophenyl phosphate measured at A405 nm.

**Amino Acid Analysis**

To quantify the protein content of L-FCN/MBL chimeric fusion proteins and rhMBL, aliquots were hydrolyzed with 6 N hydrochloric acid, 0.2% phenol for 16 h at 115 °C. This standard technique does not accurately quantitate tryptophan (degrades; reports low) and cysteine (detected as cysteine and cystine peaks; cysteine can coelute with proline), which were excluded from the final estimation of protein concentration. The hydrolysates were analyzed with a Hitachi L-8900 Amino Acid Analyzer (Hitachi High Technologies America, Inc.) that separates the amino acids with an ion exchange column and then derivatizes them with ninhydrin for detection at 570 and 440 nm. Data analysis was performed with EZChrom Elite software for the Hitachi Amino Acid Analyzer. Results are accurate within a 10% margin of error. Amino acid analyses were performed by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT) by J. M. Crawford and F. M. Pineda.

**Surface Plasmon Resonance and Apparent Affinity Kinetics**

Real time surface plasmon resonance spectroscopy was performed using a BiaCore 3000 biosensor and BIAevaluation 3.1 software (GE Healthcare) to determine the apparent equilibrium dissociation constants (Kd) between rhMBL or L-FCN/MBL chimeric fusion proteins and N-acetyl-D-glucosamine-BSA (Sigma-Aldrich) or mannan, a mannose polymer, as described previously (29). Briefly, for mannan binding experiments, all the lectins were covalently bound to CM5 biosensor...
chips as ligands according to the manufacturer’s instructions. Because the analyte \( N \)-acetyl-\( D \)-glucosamine-BSA did not bind to immobilized L-FCN/MBL76, all the proteins were then tested as analytes using immobilized \( N \)-acetyl-\( D \)-glucosamine-BSA as a ligand. Only L-FCN/MBL76 and rhMBL bound to immobilized \( N \)-acetyl-\( D \)-glucosamine-BSA. Therefore, \( K_d \) values were measured using different methods as indicated in Fig. 3A. Analytes (rhMBL, L-FCN/MBL76, or carbohydrates where appropriate) diluted in running buffer (143 mM NaCl, 10 mM HEPES, 10 mM CaCl\(_2\), pH 7.4) were injected over the control (immobilized BSA) and the experimental flow cells with non-specific binding subtracted. For affinity analyses, the compounds were diluted in duplicate using five different concentrations injected at 20 \( \mu l/min \) for 2.5 min. Data were analyzed by global fitting to a 1:1 Langmuir binding model.

Calreticulin Binding Assay

Microtiter wells (Immulon 2HB) were coated with tricryptic samples of 10 \( \mu g/ml \) chimeric proteins, BSA (negative control), and rhMBL (positive control) in carbonate-bicarbonate buffer, pH 9.6 at room temperature for 1 h. Washing and blocking methods were the same as for mannan binding assays. Wells were incubated with 50 \( \mu g/ml \) biotinylated human placental calreticulin or BSA at room temperature for 1 h (24). Detection was achieved with the same reagents as for the C4 cleavage assay.

Viral Binding and Inhibition Assays

**Ebola Experiments**

HIV–Ebola (Zaire) glycoprotein (GP) virions that express luciferase were produced as described previously (4). Virus concentration was determined by ELISA for the HIV p24 core protein according to the manufacturer’s instructions (SAIC-Frederick, National Cancer Institute, Frederick, MD).

**Binding Assay**—Binding characteristics of chimeric fusion proteins to HIV–Ebola GP and the negative control, HIV-env neg (pNL 4–3 E+; AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, Bethesda, MD), were determined as described previously (4) with the following modifications: the chimeras and rhMBL were diluted to 10 \( \mu g/ml \) in Veronal-buffered saline (VBS; 12.5 mM barbituric acid, 7.3 mM sodium barbital, 0.72 M NaCl, pH 7.3) with 5 mM CaCl\(_2\), and the final virus concentration was 10 ng of p24/ml diluted in VBS-CaCl\(_2\). The percentage of viral binding relative to virus input was calculated after subtracting background binding.

**Infection Inhibition Assay**—HepG2 cells (ATCC HB-8065) were grown to \( \sim \)80% confluence in 96-well cell culture microplates (Costar) in Eagle’s minimum essential medium (EMEM; ATCC) with 10% fetal bovine serum (Invitrogen) and 50 \( \mu g/ml \) gentamicin (Sigma-Aldrich). HIV–Ebola GP virus diluted to 400 pg/ml in VBS and 10 mM CaCl\(_2\) was preincubated at 37 °C for 1 h with 0, 0.1, or 1 \( \mu g/ml \) L-FCN/MBL proteins or rhMBL diluted in MBL-deficient (MASP-replete) serum (1:1) from a consenting healthy volunteer (protocol approved by the Massachusetts General Hospital Institutional Review Board) with MBL haplotype LYPB/LYPB and undetectable serum MBL (<2.5 ng/ml). The adherent cells were washed with serum-free Dulbecco’s modified Eagle’s medium (Invitrogen), the virus/MBL mixture was added, and the plates were centrifuged at 1000 \( \times \) g for 2 h to facilitate infection followed by incubation at 37 °C in 5% CO\(_2\) for 3 h. Thereafter, the cells were washed with Dulbecco’s modified Eagle’s medium and incubated with fresh Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum at 37 °C in 5% CO\(_2\) for 48 h. The cells were lysed with 10% Triton X-100, and chemiluminescence was measured with a luciferase assay according to the manufacturer’s instructions (Promega Corp.). Experiments were performed in triplicate and repeated twice.

Wild type-like recombinant Ebola virus Zaire (Mayinga strain)–eGFP (30) was used for infection inhibition experiments in a BSL4 laboratory as described above with the following modifications: 3 \( \times \) 10\(^4\) HepG2 cells/well were plated in 96-well microplates and incubated at 37 °C in 5% CO\(_2\) for 72 h before infection, and virus (multiplicity of infection of 0.1) was preincubated with 0, 1, or 10 \( \mu g/ml \) L-FCN/MBL proteins or rhMBL diluted in MBL-deficient serum (1:1) as above. The virus/MBL mixture was replaced with fresh EMEM, and the cells were incubated for 48 h at which time fluorescence was read with a spectrophuorometer (Molecular Devices; excitation, 485 nm; emission, 515 nm).

**Hendra Virus (HeV) and Nipah Virus (NiV) Experiments**

HeV was isolated in Vero cells from the lung of a horse infected in Brisbane, Australia in 1994 as described previously (31). NiV was isolated in Vero cells from the brain of a human fatally infected in the 1998–1999 Malaysian outbreak as described previously (32).

**Binding Assays**—Glutaraldehyde-inactivated HeV or NiV (1 \( \times \) 10\(^4\) TCID\(_{50}\)/ml) or recombinant soluble NiV G protein (36 \( \mu g/ml \); provided by Benhur Lee, UCLA, Los Angeles, CA) in carbonate-bicarbonate buffer, pH 9.6 was coated on Nunc MaxiSorp microplates. After washing and blocking with 1% BSA, 2-fold serial dilutions of chimeric fusion proteins, rhMBL, or BSA (negative control) in a 5 mM CaCl\(_2\)-containing buffer were incubated for 30 min at 37 °C. After intervening wash steps, binding was detected with 1:5000 biotinylated anti-HMBL antibody (HYB 131-01, BioPorto) and then 1:5000 streptavidin-conjugated HRP-conjugated antibody (ICN). Tetramethylbenzidine substrate (Sigma-Aldrich) was added, and the absorbance read at 450 nm.

**Infection Inhibition Assay**—Vero cells were grown in EMEM, 2 mM l-glutamine, 100 units/ml penicillin, 10 \( \mu g/ml \) streptomycin, and 10% fetal calf serum. Cells were seeded into white 96-well cell culture plates at 2 \( \times \) 10\(^4\) cells/well and incubated at 37 °C with 5% CO\(_2\) overnight. Log dilutions of the chimeric fusion proteins and rhMBL (0.1 ng/ml–10 \( \mu g/ml \)) were prepared in MBL-deficient (MASP-replete) human serum diluted 1:5 in complete EMEM containing 5 mM CaCl\(_2\). Under BSL4 conditions, 10,000 TCID\(_{50}\)/ml NiV and HeV were incubated 1:1 with each protein mixture for 45 min at 37 °C, then medium was removed from wells, and the virus/protein mixture was added to wells in triplicate and incubated for 60 min at 37 °C. The inoculum was then replaced with fresh EMEM and incubated for 24 h at 37 °C. Viral protein immunoassays were performed as described previously (33).
tion was performed with 1:1000 anti-HeV and anti-NiV antibody (rabbit polyclonal anti-N protein (34) provided by Brian Shiel), 1:2000 HRP-conjugated anti-rabbit antibody, and 10% chemiluminescent peroxidase substrate-3. A Luminoskan Ascent luminometer (Thermo Fisher Scientific, Waltham, MA) was used, and non-linear regression analysis was performed using SigmaPlot software to determine the concentration exhibiting 50% inhibition (IC50).

**Atomic Force Microscopy (AFM)**

To visualize the molecular architecture of L-FCN/MBL chimera and rhMBL in the native phase, proteins diluted in physiological buffer were subjected to AFM. Ten microliters of solution with a 500 ng/ml concentration of each L-FCN/MBL chimera and rhMBL, respectively, was deposited on cleaved mica surfaces modified with 2-amino-2-deoxy-D-mannose (Sigma-Aldrich) as described previously (35). Samples were allowed to settle for 2 min, then a liquid cell was mounted, and imaging buffer (10 mM Tris, 1 mM CaCl2, 140 mM NaCl, pH 7.4) was gently injected into the chamber to simulate physiological conditions of binding. Topographies were recorded using tapping mode AFM. More than 300 molecules of each lectin from separate locations across the mica surfaces were analyzed to ensure reproducibility. AFM images were acquired using a commercial atomic force microscope (Veeco Instruments, Santa Barbara, CA) with a NanoScope V controller, "J" scanner, and NP-S probe (spring constant, k = 0.15 newton·m−1; tip curvature radius, R < 10 nm). The driving frequency was chosen at 5% lower amplitude than the amplitude at the cantilever free resonance frequency. The amplitude set point was adjusted such that the interaction between probe and sample was minimal during each oscillation cycle, yet the microscope performed stably. All the images were first flattened using the NanoScopeTM software (version 7.0, Veeco Instruments) and then further analyzed with the commercial Scanning Probe Image Processor (SPIP™) software (version 4.5.7, Image Metrology ApS, Lyngby, Denmark).

**Statistics**

Normally distributed data were compared with analysis of variance tests, and skewed data were compared with Mann-Whitney U or Kruskal-Wallis tests where appropriate. All tests were two-tailed. p values <0.05 were considered statistically significant.

**RESULTS**

**L-FCN/MBL Chimeric Proteins Multimerize to Form Smaller Oligomers than rhMBL**—To evaluate the effector activity and relative contributions of the structural domains of L-FCN and MBL, three L-FCN/MBL chimeric fusion proteins were constructed (Fig. 1). These chimeric molecules comprised progressively increasing portions of the MBL neck and collagenous regions. Recombinant MBL and the three chimeric fusion proteins were produced and purified as described under “Experimental Procedures.” We first compared their relative sizes and oligomerization using reducing and non-reducing polyacrylamide gel electrophoresis (Fig. 2). Proteins were compared on the basis of 1) equivalent capacity to bind mannan (ELISA) relative to a standard curve generated with rhMBL (Enzon Pharmaceuticals Inc.) and 2) equivalent protein composition measured by amino acid analysis. Overall, SDS-PAGE resolved similar quantities of protein by both methods. We found that reduced chimeric monomers and rhMBL were ~26 kilodaltons. Under non-reducing conditions, the chimeras formed a range of nona-, dodeca-, and pentadecameric oligomers (trimers, tetramers, and pentamers of trimeric subunits, respectively). Recombinant hMBL formed octadecameric and larger oligomers, which is consistent with a previous report in which rhMBL formed large homogenous oligomeric structures very similar to the oligomers found in human plasma (27). We confirmed the identity of the proteins with Western blots using an MBL-specific monoclonal antibody that specifically recognizes MBL CRDs contained in all three fusion proteins (not shown).

**Repertoire of Carbohydrate Binding Is Similar among L-FCN/MBL Chimeric Fusion Proteins and rhMBL**—Various enveloped viruses such as Ebola virus have highly glycosylated surface glycoproteins that display a variety of carbohydrate side chains (25). C-type lectins bind high mannose residues of N-linked glycans in a calcium-dependent manner. To compare the repertoire of carbohydrate binding, we used a broad selection of hexoses to competitively inhibit binding of the chimeras and rhMBL to immobilized mannan. Both acetylated and non-acetylated hexose monosaccharides inhibited binding in a specific and dose-dependent manner (as defined by >50% reduction) in the following order of descending magnitude for the three chimeras: d-mannose (half-maximal inhibitory concentration (IC50), 10–13 mM) > GlcNAc (16–17 mM) > l-fucose (17–19 mM) > N-acetyl-d-mannosamine (20–24 mM) > d-galactose (35–43 mM) > d-glucose (50 mM) except for L-FCN/MBL76, which was not inhibited by d-glucose. Recombinant hMBL was inhibited by the same carbohydrates but in a slightly different order and at slightly higher IC50 values: GlcNAc (IC50, 17 mM) > d-mannose = N-acetyl-d-mannosamine (24 mM) >
Novel Chimeric Molecule Active against Ebola Virus

**FIGURE 2.** Chimeric L-FCN/MBL proteins and rhMBL form oligomeric structures. Proteins were resolved with SDS-PAGE using 4–20% gels under non-reducing (2000 ng) (A) and reducing (800 ng) (B) conditions and visualized with Imperial Protein Stain. Equivalent quantities of proteins were determined by amino acid analysis. Molecular masses were compared with protein standards using Bio-Rad Quantity One 1-D analysis software. Under non-reducing conditions, chimeras formed nona-, dodeca-, and pentadecameric oligomers (234–390 kDa), whereas rhMBL formed predominantly octadecameric and larger oligomers (≥468 kDa). Reduced chimeric monomers were ~26 kilodaltons and ran slightly faster than rhMBL, which could not be explained by differences in the protein length (MBL, 248 amino acids versus chimeric proteins, 247–255 amino acids). The observed sizes were consistent with those of MBL previously reported to be 25.3 kilodaltons (27). In C, the observed molecular masses of protein bands under non-reducing conditions (mean of all chimeras ± S.E.) correlated with numbers of peptide subunits (linear regression, \( r^2 = 0.997 \)), confirming that the chimeric proteins were in fact multimers of the monomers shown in A.

**FIGURE 3.** Equilibrium dissociation constants \( (K_D) \) of chimeric proteins and rhMBL. Real time surface plasmon resonance spectroscopy was used to measure binding affinity between the lectins and N-acetyl-D-glucosamine-BSA (A) and mannan (B). For mannan binding experiments, all the lectins were covalently bound to CM5 biosensor chips as ligands, and mannan was used as the analyte. For N-acetyl-D-glucosamine-BSA binding experiments, rhMBL and L-FCN/MBL76 were tested as analytes (\(*)\) refers to analytes), whereas L-FCN/MBL126 and -64 were tested as ligands because lack of binding to sugars precluded comparing all the lectins with the same method. The analytes (rhMBL, L-FCN/MBL76, and carbohydrates) diluted in running buffer containing 10 mM CaCl\(_2\) were injected over immobilized BSA (control) and the experimental flow cells. For affinity analyses, the analytes were diluted in duplicate using five different concentrations (rhMBL, L-FCN/MBL76, and mannan, 0.6–1 µg/ml; N-acetyl-D-glucosamine-BSA, 2–12 nM) injected at 20 µl/min for 2.5 min. In each case, L-FCN/MBL76 had the lowest \( K_D \) of the chimeras (\( p < 0.05 \)). In addition, compared with rhMBL, it had significantly greater affinity to mannan (\( p = 0.004 \)).

L-fucose (27 mM) > D-galactose = D-glucose (54 mM). None of the proteins bound D-fucose, D-mannosamine, D-glucosamine, D-galactosamine, or N-acetyl-D-galactosamine. These patterns suggest that ligand recognition by the MBL CRD in each case is minimally affected by substituted ficolin structural domains.

The apparent binding affinities of the chimeric fusion proteins and rhMBL for GlcNAc-BSA or mannan were compared using surface plasmon resonance. L-FCN/MBL76 had the lowest equilibrium dissociation constants \( (K_D) \) for these carbohydrates indicating significantly greater apparent binding affinities (Fig. 3, A and B).

L-FCN/MBL76 Had Superior Functional Activity as Demonstrated by Complement Activation and Cognate Receptor Binding—To determine whether the chimeric proteins were functionally active, L-FCN/MBL proteins and rhMBL were coated on microwells, washed, and incubated with 1:100 MBL-null mouse serum as a source of MASP. Activation of complement via the lectin pathway was measured by assaying deposition of C4c, the end product of C4. As shown in Fig. 4A, L-FCN/MBL76 induced significantly greater C4c deposition compared with the other chimeric fusion proteins and rhMBL. MBL-null mouse serum alone did not cleave C4.

Although the most critical MBL receptor, coreceptor, or chaperone has not yet been defined, calreticulin is a leading candidate (24). Because enhanced receptor binding may up-regulate opsonophagocytosis, it could be an important attribute of a therapeutic molecule. Therefore, we compared the relative receptor binding of the chimeric proteins and rhMBL. L-FCN/MBL76 had significantly greater binding to calreticulin than either the other chimeric fusion proteins or rhMBL (\( p < 0.005 \); Fig. 4B).

L-FCN/MBL76 Demonstrated Strongest Inhibition of Wild Type-like Ebola Zaire Virus in Cell-Based Infection Model Compared with rhMBL and Other Candidate Fusion Proteins—Given that the fusion protein L-FCN/MBL76 had similar carbohydrate binding but increased C4 deposition and calreticulin binding compared with the other chimeras and rhMBL, we tested its relative
binding to Ebola GP using an HIV-Ebola GP pseudotyped virus. Immobilized chimeric fusion proteins and rhMBL bound viral particles in serum-free buffer to a similar extent after adjustment for nonspecific binding except for L-FCN/MBL64, which bound less virus compared with rhMBL (p < 0.01; Fig. 5A). These experiments reflected the complement- and MASP-independent activities of the chimeric fusion molecules. To evaluate the effector functions of the recombinant lectins in the presence of MASP and complement components in human serum, cell-based infection models using HIV-Ebola GP (Fig. 5B) and wild type-like Ebola Zaire (Mayinga strain) virus (Fig. 5C) were used. The chimeric fusion proteins and rhMBL all inhibited HIV-Ebola GP infection by 78–92%. In addition, L-FCN/MBL76 reduced infection by wild type-like Ebola virus by 92%, which was significantly better than the next recombinant proteins, which inhibited infection by 56–79% (L-FCN/MBL76 versus the next most effective protein, p = 0.008).

To broaden the scope of our findings, we explored the effectiveness of the novel chimeric fusion proteins and rhMBL against two additional emerging infectious agents, Hendra and Nipah viruses. L-FCN/MBL76 and -126 bound Nipah virus surface glycoproteins and inactivated Nipah and Hendra viruses significantly greater than rhMBL and L-FCN/MBL64 (p ≤ 0.001; Fig. 5, D–F). All recombinant lectins inhibited Nipah infection of Vero cells by 25–35%, and all had similar IC_{50} values; Hendra infection was inhibited by ~40% by all lectins, but IC_{50} values were lowest for L-FCN/MBL76 and rhMBL (Fig. 5, G and H).

Finally, in an attempt to explain the enhanced effector functions of L-FCN/MBL76 against Ebola virus, we used AFM to analyze potential differences in the biophysical properties of rhMBL and the chimeric fusion proteins. The recombinant lectins were diluted in physiological buffer and allowed to bind to D-mannose on a mica surface so they could assume their native conformations. Topographies were recorded using tapping mode AFM. In general, rhMBL formed hexameric molecules, whereas the chimeras typically formed trimeric or tetrameric molecules (Fig. 6). These observations confirmed the overall pattern of protein oligomerization predicted by SDS-PAGE (Fig. 2). Average height measurements of ~300–500 molecules of each lectin revealed that L-FCN/MBL76 was significantly less tall than the other two chimeras as well as rhMBL (p < 0.05; Fig. 7). However, observed differences in height could not be explained by differences in protein lengths based on amino acid sequences.

**DISCUSSION**

Ebola viruses pose a potential bioterrorist threat to civilians and the military. We sought to exploit their conserved surface carbohydrates as targets for antiviral molecules. Mannose-binding lectin and the ficolins possess intrinsic antimicrobial activity and modulate the immune response through complement-dependent or phagocyte-driven mechanisms. These functions are an integral part of the process of innate immunity, which shares many of the hallmarks of successful anti-infective therapy, namely rapid onset of action and a broad spectrum of antimicrobial activity against viruses, bacteria, fungi, and parasites (2, 17, 36). Industrial scale production of rhMBL is limited by its expense and complexity of its oligomeric structure. Therefore, we investigated whether we could manufacture highly active yet architecturally simpler chimeric molecules derived from MBL and L-ficolin. Here we report the antiviral activities of three L-FCN/MBL chimeric fusion proteins and rhMBL against Ebola virus. We extended the scope of our studies to two additional emerging life-threatening and untreatable viral pathogens that contain high mannose on their surfaces, Hendra and Nipah viruses. Here we report that the chimeric fusion protein with the greatest overall activity (L-FCN/MBL76) appeared to be the molecule with the most structural flexibility.

First, we found that the oligomerization pattern of three variant recombinant L-FCN/MBL chimeric fusion proteins differed from that of rhMBL (Fig. 2). This observation reflected the fact that although both MBL and L-FCN polypeptides are covalently cross-linked by disulfide bonds at their N-termini they typically form different orders of multimers. Under non-reducing conditions, the chimeras formed predominantly nona- and dodecameric molecules, which approximate the native ficolin structural domains (15, 17). On the other hand, rhMBL formed octadecameric and larger oligomers (27). Studies of both plasma-derived MBL and rhMBL have shown that larger oligomers are more potent activators of complement than smaller oligomers (27). Similarly, higher order surfactant protein-D oligomers exhibit greater influenza-aggregating activity and mediate enhanced opsonization by neutrophils compared with lower order surfactant pro-
tein-D multimers (14). Importantly, we found that lower order L-FCN/MBL chimeric fusion proteins actually had equivalent or superior complement-activating activity and capacity to bind human calreticulin compared with rhMBL (Fig. 4). These findings are supported by those of White et al. (11) and Meschi et al. (13), who documented that the N-terminal and collagenous domains of dodecameric surfactant protein-D (four trimers) in surfactant protein-D/MBL chimeric fusion proteins conferred greater anti-influenza and -HIV activity, respectively, than that of octadecameric MBL (six trimers).

The carbohydrate binding specificity of the L-FCN/MBL chimeras was dictated by the MBL CRDs as expected. They all similarly bound a variety of acetylated and non-acetylated hexoses. L-FCN/MBL76, however, had the greatest binding affinity for GlcNAc-BSA and mannan as determined by surface plasmon resonance (Fig. 3). The chimeric fusion proteins bound to and inhibited infection by HIV-Ebola GP pseudotypes to similar degrees within a physiological range of concentrations (Fig. 5) as defined by median human levels of MBL and L-FCN (1.2 and 3.7 μg/ml, respectively (37)). As a proof of concept, we modeled native infection with Ebola virus Zaire and showed that L-FCN/MBL76 mixed with human serum resulted in the greatest inhibition of infection of human cells (92% inhibition), which was significantly greater than the next most effective chimeric protein (p = 0.008) although perhaps by as little as 13% (Fig. 5). To broaden the scope of these experiments, we tested other glycosylated enveloped viruses with no known cure and also showed enhanced interaction with Hendra and Nipah viruses, although the antiviral effect was moderate (Fig. 5).

Considering that all three chimeras had the same CRDs, we investigated other possible explanations for differences in carbohydrate binding affinities and antiviral activity. It was notable that L-FCN/MBL76 activated complement and bound calreticulin to a significantly greater extent than the other chimeras and rhMBL (Fig. 4). Calreticulin (cC1qR) is a candidate human MBL receptor that is thought to function in complex with the endocytic receptor CD91 (38, 39). Therefore, we considered the possibility that an altered quaternary structure of this molecule facilitated presentation of the shared MASP- and calreticulin-binding motif (Fig. 1) that contains amino acids OGKXGP (where O is hydroxyproline and X is an aliphatic residue other than glycine). Greater exposure to their cognate ligands could
Novel Chimeric Molecule Active against Ebola Virus

FIGURE 6. Topographic features of L-FCN/MBL chimeras differ from those of rhMBL. Proteins were deposited on cleaved mica surfaces modified with 2-amino-2-deoxy-D-mannose. Physiological buffer containing 1 mM CaCl₂ was gently injected into the imaging chamber to simulate physiological conditions of binding. Topographies were recorded using tapping mode atomic force microscopy. More than 300 molecules of each lectin from separate locations were analyzed to ensure reproducibility. The images (A–D) are representative of each of the chimeras and rhMBL. The chimeras typically formed oligomers containing four or five subcomponents presumably representing carbohydrate recognition domains and a collagen stalk. rhMBL formed larger oligomers usually consisting of six or seven subcomponents, a finding that is consistent with the SDS-PAGE results under non-reducing conditions.

atomic force microscopy in liquid phase under physiological conditions. Based on the crystal structure of human MBL (neck region and CRD), the optimal binding between the CRD complex and a ligand is achieved when the distance between CRDs is ~45 Å (8). This precise distance may contribute to the preference of MBL for microbial rather than mammalian carbohydrates (8, 9, 43). Importantly, Dong et al. (35) observed a conformational change in MBL upon binding to ligand-coated surfaces with separation of the CRDs. This phenomenon was hypothesized to arise from unfolding of the flexible neck/CRD region of MBL or a simple unbending of the collagen stalk curvature, thereby underscoring the inherent flexibility of MBL. Using AFM, we observed that L-FCN/MBL76 was significantly less tall than the other molecules (Fig. 7). The possible mechanism for this unexpected difference is conjectural, but considering that the peptide lengths of these lectins are similar, the current evidence suggests that L-FCN/MBL76 is more flexible, allowing it to “collapse” on the mica surface either spontaneously or because of compression of the tapping mode AFM. Enhanced flexibility may arise from quaternary structural changes in the collagenous or neck region. Consequently, greater molecular flexibility may enhance cooperativity between CRDs when they bind carbohydrates on viral surfaces regardless of surface topography and density of target ligands, and it may change MASP and calreticulin binding dynamics.

We observed that there were greater differences among chimeric proteins in serum-free, solid phase binding assays compared with serum-replete, soluble phase viral neutralization assays. The enhanced flexibility of L-FCN/MBL76 may favor
optimal binding to immobilized ligands compared with the other chimeric proteins, whereas other neutralizing soluble factors in serum may diminish differences in protein-specific viral inhibition.

Our results provide a conceptual framework for further structure-function analyses to define precisely which domains of L-FCN and MBL determine protein flexibility, degree of oligomerization, binding affinity, and effector functions of lectins. It is plausible that a more pliable molecule would allow optimal cooperativity and binding between the CRDs and their cognate ligands on the one hand or between the MASP-binding site and MASP or calreticulin on the other hand. MBL-based immunotherapy offers several potential advantages particularly against glycosylated enveloped viruses such as Ebola virus. These advantages include the stability of MBL under physiological conditions, prolonged serum half-life, and low toxicity in humans. Lectins have immediate onset of action and broad specificity, which would be critical in the setting of natural epidemics of unidentified infections or a bioterrorist attack. Because lectins activate combinatorial effector mechanisms, they may render development of microbial resistance less likely. Finally, large scale synthesis using recombinant molecular techniques has already been achieved (44, 45). The potential limitations of using chimeric molecule molecules in clinical medicine include induction of antibodies to novel biologi-
Jones, E. Y., and Stuart, D. J. (2008) J. Virol. 82, 11628–11636
27. Vorup-Jensen, T., Sørensen, E. S., Jensen, U. B., Schwaebel, W., Kawasaki, T., Ma, Y., Uemura, K., Wakamiya, N., Suzuki, Y., Jensen, T. G., Takahashi, K., Ezekowitz, R. A., Thiel, S., and Jensenius, J. C. (2001) Int. Immunopharmacol. 1, 677–687
28. Petersen, S. V., Thiel, S., Jensen, L., Steffensen, R., and Jensenius, J. C. (2001) J. Immunol. Methods 257, 107–116
29. Montalto, M. C., Collard, C. D., Buras, J. A., Reenstra, W. R., McClaine, R., Gies, D. R., Rother, R. P., and Stahl, G. L. (2001) J. Immunol. 166, 4148–4153
30. Towner, J. S., Paragas, J., Dover, J. E., Gupta, M., Goldsmith, C. S., Huggins, J. W., and Nichol, S. T. (2005) Virology 332, 20–27
31. Hyatt, A. D., and Selleck, P. W. (1996) Virus Res. 43, 1–15
32. Shiell, B. J., Gardner, D. R., Cramer, G., Eaton, B. T., and Michalski, W. P. (2003) Virus Res. 92, 55–65
33. Aljofan, M., Porotto, M., Moscona, A., and Mungall, B. A. (2008) J. Virol. Methods 149, 12–19
34. Juozapaitis, M., Serva, A., Zvirbliene, A., Slihinskas, R., Staniulis, J., Sausnuska, K., Shiell, B. J., Wang, L. F., and Michalski, W. P. (2007) Virus Res. 124, 95–102
35. Dong, M., Xu, S., Oliveira, C. L., Pedersen, J. S., Thiel, S., Besenbacher, F., and Vorup-Jensen, T. (2007) J. Immunol. 178, 3016–3022
36. Holmsoy, U., Thiel, S., and Jensenius, J. C. (2003) Annu. Rev. Immunol. 21, 547–578
37. Kilpatrick, D. C., Fujita, T., and Matsushita, M. (1999) Immunol. Lett. 67, 109–112
38. Stuart, G. R., Lynch, N. J., Day, A. J., Schwaebel, W. J., and Sim, R. B. (1997) Immunopharmacology 38, 73–80
39. Ogden, C. A., deCathelineau, A., Hoffmann, P. R., Bratton, D., Ghebrehiwet, B., Fadok, V. A., and Henson, P. M. (2001) J. Exp. Med. 194, 781–795
40. Girija, U. V., Dodds, A. W., Roscher, S., Reid, K. B., and Wallis, R. (2007) J. Immunol. 179, 455–462
41. Teillet, F., Lacroix, M., Thiel, S., Weiguny, D., Agger, T., Arlaud, G. J., and Thielens, N. M. (2007) J. Immunol. 178, 5710–5716
42. Cseh, S., Vera, L., Matsushita, M., Fujita, T., Arlaud, G. J., and Thielens, N. M. (2002) J. Immunol. 169, 5735–5743
43. Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., and Ezekowitz, R. A. (1999) Science 284, 1313–1318
44. Petersen, K. A., Matthiesen, F., Agger, T., Kongsrlev, L., Thiel, S., Cornelissen, K., and Axelsen, M. (2006) J. Clin. Immunol. 26, 465–475
45. Jensenius, J. C., Jensen, P. H., McGuire, K., Larsen, J. L., and Thiel, S. (2003) Biochem. Soc. Trans. 31, 763–767
46. Kongsrlev, L., Weiguny, D., and Matthiesen, F. (August 24, 2006) U.S. Patent 20060188963