Novel Strategy for Inhibiting Viral Entry by Use of a Cellular Receptor-Plant Virus Chimera†

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The plant virus cowpea mosaic virus (CPMV) has recently been developed as a biomolecular platform to display heterologous peptide sequences. Such CPMV-peptide chimeras can be easily and inexpensively produced in large quantities from experimentally infected plants. This study utilized the CPMV chimera platform to create an antiviral against measles virus (MV) by displaying a peptide known to inhibit MV infection. This peptide sequence corresponds to a portion of the MV binding site on the human MV receptor CD46. The CPMV-CD46 chimera efficiently inhibited MV infection of HeLa cells in vitro, while wild-type CPMV did not. Furthermore, CPMV-CD46 protected mice from mortality induced by an intracranial challenge with MV. Our results indicate that the inhibitory CD46 peptide expressed on the surface of CPMV retains virus-binding activity and is capable of inhibiting viral entry both in vitro and in vivo. The CD46 peptide presented in the context of CPMV is also up to 100-fold more effective than the soluble CD46 peptide at inhibiting MV infection in vitro. To our knowledge, this study represents the first utilization of a plant virus chimera as an antiviral agent.

Global control of infectious diseases is dependent upon the development of novel, inexpensive and easily produced vaccine and antiviral reagents. In regions where infections are endemic, the use of appropriate antimicrobial reagents is largely limited by cost and the lack of equipment and facilities for appropriate delivery of the reagents. The use of plant viruses such as the cowpea mosaic virus (CPMV) as biomolecular platforms for designing antiviral agents and vaccines overcomes many of these obstacles. CPMV provides the advantages of inexpensive production of large quantities of material, with up to 1 to 2 g of virus produced per kilogram of infected cowpea plants. In addition, plant virus particles are generally stable to extremes of temperature and pH; for example, CPMV can withstand temperatures up to 60°C and pH levels as low as 1.0 to 2.0 (18), and this stability is an advantage for use in tropical and/or rural areas, where continuous refrigeration is not readily available. Finally, the lack of contaminating animal cells and viruses in plant reagent preparations is beneficial.

The CPMV chimera technology for presenting heterologous peptides is based on the crystal structure of the virus capsid (3, 9). The CPMV capsid consists of 60 copies each of a large (L) and small (S) protein that are arranged with icosahedral symmetry. One L protein consisting of two jelly roll β -barrels and one S protein consisting of a single jelly roll β -barrel make up

the asymmetric unit of the capsid. There are three copies of the L protein arranged around each threefold axis of symmetry, and five copies of the S protein are arranged around each fivefold axis of symmetry. In the S protein, surface-exposed loops that do not appear to be involved in intersubunit contact exhibit high variability among different members of the comovirus family of plant viruses, of which CPMV is a member. In particular, the β B- β C loop is highly exposed on the surface of CPMV and has been shown to be amenable to the insertion of heterologous sequences (19). The insertion of a foreign peptide into a loop on the S protein has the added advantage of presenting five copies of the peptide at each fivefold axis of symmetry, allowing a high effective concentration of the peptide to be achieved.

As a proof of concept for the development of a CPMV-based antiviral, we turned to the problem of measles virus (MV) infection. MV continues to cause widespread morbidity and mortality, with ca. 40 million cases occurring annually, mostly in the developing world. MV infects a wide variety of cell types, including those of the immune system and central nervous system. Such infection leads to immunosuppression and attendant opportunistic infections such as pneumonia and diarrhea, as well as rare but serious neurologic complications. The measles vaccine is a live-attenuated vaccine that is susceptible to heat inactivation and is administered to infants at 12 to 18 months and 5 years of age. Maternal antibody inhibits the success of the vaccine in children younger than 18 months, creating a window of time when children are susceptible to infection. This window of susceptibility, combined with the heat sensitivity of the current vaccine, creates the need for inexpensive supplementary agents such as antiviral agents that could be administered both in conjunction with the vaccine and in the period before the vaccine becomes effective in young infants.

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To determine whether a CPMV-based antiviral could be developed that inhibits MV infection, we utilized previous studies that extensively characterized the interaction of MV with cell surface receptors. One cellular receptor for MV has been identified as CD46 or membrane cofactor protein (13, 17). CD46 is a member of the family of regulators of complement activation and functions to inactivate complement components C3b and C4b and prevent the formation of the membrane attack complex. MV binds to CD46 within two 60-amino-acid extracellular domains or short consensus repeats (SCRs) known as SCR1 and SCR2. Within these SCRs, specific CD46-derived peptides that interact with MV have been identified. We have previously shown that two peptides, peptide 12 in SCR1 and peptide 24 in SCR2, inhibited MV infection of HeLa cells in vitro by 50 and 80%, respectively (12). In MV entry and infection of cells, the MV hemagglutinin (H) glycoprotein has been postulated to bind to receptors such as CD46 as a trimer (2, 7). Thus, in presenting CD46 peptides on the CPMV capsid, the clustering of CD46 peptides at each fivefold axis might lead to an increased binding affinity for the H protein, allowing the CPMV-CD46 chimera to be a more effective antiviral than the soluble CD46 peptides. In order to test this hypothesis, we constructed CPMV-CD46 chimeras by inserting sequences encoding inhibitory CD46 peptides into the CPMV genomic cDNA. Viable chimeras were generated and tested for antiviral activity both in vitro and in a transgenic mouse model for MV infection in vivo.

MATERIALS AND METHODS

Preparation of chimeric CPMV RNA2 infectious clone. The creation of plasmids containing full-length cDNA copies of the two RNA moieties of CPMV, RNA-1 and RNA-2, has been previously described (6). These cDNA plasmids are termed pCP1 and pCP2, respectively. A CPMV-CD46 chimera was made by cloning an oligonucleotide coding for a partial sequence of CD46 peptide 12, called 12.2B, with the peptide sequence ATHTIADRNHT, into the *AatII-NheI* site in pCP2. The ATHTIADRNHT peptide sequence was encoded by the oligonucleotide sequence 5'-GCTACTCACACTATAGCTGATAGAAATCAC ACG-3'. Another chimera was designed with the sequence encoding CD46 peptide 12 (IPPLATHITICDRNHTWLPVS) inserted into the pCP2 plasmid.

Production and purification of CPMV-CD46 chimeric virus particles. Directly infectious double-stranded cDNA clones of the CPMV genome (RNA-1 and RNA-2) were utilized in the production of chimeric virus. These constructs consist of the cauliflower mosaic virus 35S promoter sequence linked to the 5' ends of the viral cDNAs to generate infectious transcripts in the plant (6). The pCP2 plasmid containing the oligonucleotide encoding the CD46 peptide insert was linearized by digestion with *EcoRI* while wild-type (WT) pCP1 plasmid was linearized by digestion with *MluI*. Kentucky cowpea plants (*Vigna unguiculata*) were inoculated as 10-day-old seedlings, bearing two primary leaves and with secondary leaves just beginning to show. Carborundum was first dusted onto the leaves to aid in the wounding process and then 10 µg of the linearized recombinant pCP2 in 100 µl of MilliQ water and 10 µg of the linearized WT pCP1 in 100 µl of MilliQ water were combined and inoculated into each plant by rubbing the plasmids directly onto the leaves. At ca. 3 weeks postinoculation, when a systemic CPMV infection was observed as characterized by lighter-colored punctate lesions on the primary and secondary leaves, the infected leaves were harvested, weighed, and frozen at -20°C or on dry ice for 30 min. The leaves were homogenized in a Waring blender, and chimeric virus was purified from the infected leaves by a method described elsewhere (1). The purified virus or an extract from infected plants was used as an inoculum for passaging through fresh 10-day-old cowpea seedlings, with each plant receiving an ~0.2 optical density (at 260 nm) concentration of purified CPMV-CD46 or CPMV-HRV-II in 0.1 M phosphate buffer (pH 7.0). The yield of purified virus obtained from infected leaves was 0.33 g/kg (wet weight) of leaves.

Characterization of chimeric virus particles. The CPMV-CD46 chimeric virus was analyzed by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), matrix-assisted laser desorption ionization (MALDI) mass spectrometry, and DNA sequencing. For the SDS-PAGE, 15 µg of the CPMV-CD46 chimera was loaded onto a 16% reducing Tris-glycine polyacryl-

amide gel and electrophoresed at 125 V for 2 h. Then, 15 µg of WT CPMV was electrophoresed in parallel for comparison, and protein bands were visualized by silver stain. Nucleic acid sequencing was also performed on cDNA generated from RNA isolated from the CPMV-CD46 chimera to confirm that the sequences encoding the CD46 peptides had been successfully incorporated into the viral genomic RNA. Total RNA was extracted from CPMV-CD46 virus particles by homogenizing 0.1 mg of chimeric virus in 100 µl of RNA extraction buffer (0.1 M glycine, 10 mM EDTA, 0.1 M NaCl, 20% SDS, 50 mg of bentonite/ml). RNA was extracted with 100 µl of a 1:1 mixture of phenol and chloroform, followed by precipitation with 95% ice-cold ethanol. RNA (0.5 µg) was converted to cDNA by reverse transcription with MMLV-Reverse Transcriptase (Promega, Madison, Wis.) at 37°C for 1.5 h, followed by treatment at 70°C for 5 min, with oligo(dT) as a primer. The cDNA was then amplified by PCR with 12.5 µl of cDNA, 0.5 µl of *Taq* polymerase (Roche, Mannheim, Germany), and 50 pmol each of the primers 5'TCCCGCTTGCTTGAGAC3' and 3'ACCTTTAGACCTTGATA5'. Forty cycles of denaturing at 95°C, annealing at 45°C, and amplification at 72°C were carried out in a Perkin-Elmer 2400 thermocycler, and the PCR products were sequenced by using a Thermo Sequenase Terminator Cycle Sequencing Kit (USB, Cleveland, Ohio). Finally, MALDI mass spectrometry was conducted on CPMV-CD46 (dialyzed against MilliQ water) by using a Perceptive Biosystems Voyager Elite apparatus in 0.5 µl of 3,5-dimethoxy-4-hydroxycinnamic acid (Aldrich) in a saturated solution of acetonitrile-water (50:50)-0.25% trifluoroacetic acid.

In vitro virus inhibition assays with CPMV-CD46 chimera. HeLa cells were plated on glass coverslips in 24-well plates at a concentration of 5×10^4 HeLa cells/well. Purified MV (multiplicity of infection [MOI] of 2) was incubated for 30 min on ice with WT CPMV or CPMV-CD46, at 5 mg/ml (5.4 µM peptide) and 0.5 mg/ml (0.54 µM peptide), or with CD46 peptides 12 (IPPLATHITICDRNHTWLPVS) or 12.2B (ATHTIADRNHT) diluted to 100 µM in ice-cold phosphate-buffered saline (PBS). Medium (100 µl) was then added, and the MV-peptide mixtures were divided between duplicate wells of HeLa cells and incubated for 1 h at 37°C. The cells were washed three times in PBS, fresh medium was added, and the cells were incubated for 20 h at 37°C to allow for the expression of MV proteins in infected cells. After this incubation, the cells were fixed and stained for MV proteins with a human subacute sclerosing panencephalitis (SSPE) antiserum and visualized with a fluorescein isothiocyanate-labeled goat anti-human secondary antibody. Fluorescent cells were viewed at $\times 200$ magnification under an Olympus BH-2 fluorescent microscope. The percentage of fluorescent cells for each treatment was calculated by quantitating a minimum of three independent fields per sample, and each sample was assayed in duplicate. Values are reported as the mean $\pm$ the standard error. The 50% effective concentrations (EC_{50} s) for CPMV-CD46 and peptide 12.2B were calculated by using Graph Pad Prism (Graph Pad Software, Inc., San Diego, Calif.).

Inoculation of NSE-CD46 transgenic mice with chimeric virus particles. The transgenic mouse model (NSE-CD46) for human MV infection has been previously described (20). Eight 1-day-old NSE-CD46 transgenic mice were injected intracranially (i.c.) with 30 µl of a 1:1 mixture of CPMV-CD46 (diluted to 21 mg/ml in sterile PBS) and MV (4.4×10^5 PFU/ml) of the Edmonston strain (MV-Ed)/animal that had been passaged twice through Vero (African green monkey kidney) cells. Five control NSE-CD46 transgenic mice were injected with a 1:1 mixture of a control CPMV chimera, CPMV-HRV-II (21 mg/ml), and 4.4×10^5 PFU of MV-Ed/ml, prepared as described above. The CPMV-HRV-II chimera has the N1m-1A epitope of human rhinovirus 14 (HRV14), with the sequence KDATGIDNHREAKL inserted into the β B- β C loop of the CPMV capsid S protein (22, 23). Mice were monitored daily for morbidity and mortality over a period of 2 months. Disease symptoms included roughness of coat and ataxia, leading to paralysis, severe weight loss, and wasting in the end stages. Occasionally, animals that were immobile or moribund were euthanized according to Institutional Animal Care and Use Committee (IACUC) guidelines. The brains of mice that died from both groups were removed for Northern analysis to confirm that death occurred from viral replication in the brain. Briefly, RNA was isolated by homogenizing brains in TRI reagent (MRC, Cincinnati, Ohio), followed by chloroform extraction and isopropanol precipitation according to the manufacturer's instructions. The 10 µg of RNA from each brain was electrophoresed at 125 V for 1 h on a 1.5% NuSieve GTG agarose (BioWhittaker, Rockland, Maine) gel containing 1.81 M morpholinepropanesulfonic acid (MOPS) and 5% formaldehyde in 1.81 M MOPS running buffer. The gel was then incubated in a solution of 0.5 M NaOH and 1.5 M NaCl for 30 min to denature the RNA followed by neutralization in a solution of 0.5 M Tris-HCl and 1.5 M NaCl (pH 7.0) for 30 min. The separated denatured RNA bands were transferred to a Nytran (Schleicher & Schuell, Keene, N.H.) membrane in 20 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by using a Turbo-blotter device (Schleicher & Schuell). RNA was UV cross-linked to the nitrocellulose and probed for 3 h at 55°C with 4.10⁶ cpm of an oligonucleotide repre-

FIG. 1. Schematic showing the site of insertion of the CD46 peptide in the β B- β C loop of the CPMV S protein. The loop is well exposed on the virus surface, resulting in the presentation of 60 copies of the CD46 peptide (shown as red dots on figure on the right) per virion.

senting a partial sequence of the MV nucleoprotein (MV-N) gene end labeled with ^{32}P . The radiolabeled probe was prepared by incubating a 10 μM concentration of the MV-N oligonucleotide with 0.2 mCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (ICN, Costa Mesa, Calif.) and 20 U of T4 kinase at 37°C for 45 min and then at 68°C for 10 min to inactivate the kinase. Membranes were exposed to Kodak Biomax film overnight at -80°C . Two previous experiments were conducted to test the in vivo efficacy of CPMV-CD46 as an antiviral reagent. Combining the two additional experiments, 11 mice were injected i.c. with a 1:1 mixture of CPMV-CD46 and MV and 5 mice were injected i.c. with a 1:1 mixture of CPMV-HRV-II and MV.

The CPMV-CD46 chimera (30 μl of a 10.5-mg/ml solution/animal) was also injected i.c. into 3 1-day-old NSE-CD46 mice, followed a day later by an i.c. injection of 30 μl of MV-Ed (4.4×10^5 PFU/ml)/animal prepared as described above. Four 1-day-old NSE-CD46 control mice were injected with 30 μl of PBS/animal, followed a day later by an i.c. injection of 30 μl of MV-Ed (4.4×10^5 PFU/ml)/animal. The brains of mice that died were removed for Northern analysis as described above to confirm that death occurred from viral replication in the brain. At ca. 40 days postinfection the surviving mice were euthanized and their brains were removed for Northern analysis to ascertain the amount of brain viral replication.

RESULTS

Creation of the CPMV-CD46 chimera. To generate a CPMV chimera designed to inhibit MV infection, recombinant CPMV particles expressing CD46 peptides were produced by using the infectious cDNA clones pCP1 and pCP2, encoding the full-length RNA-1 and RNA-2 genomes of CPMV, respectively (6). RNA-1 codes for viral protease and polymerase, and

RNA-2 codes for the viral capsid proteins and the movement protein. Initial attempts to generate chimeras displaying full-length peptide 12 were not successful in rescuing infectious viruses (data not shown). Such a phenomenon occasionally occurs when chimeras are designed with peptides that have significant secondary structure or hydrophobic content. We had previously shown that a smaller version of peptide 12, containing the sequence ATHTICDRNHTW (CD46 peptide 12.2), was also an effective inhibitor of MV infection in vitro (12). Thus, we generated a chimera expressing the CD46 peptide 12.2B, with the sequence ATHTIADRNHT. When we designed peptide 12.2B, the terminal tryptophan of peptide 12.2 was omitted in order to reduce the hydrophobicity of the insert and to increase the likelihood that it would be presented on the surface of the hydrophilic CPMV capsid. In addition, the cysteine in the sixth position was changed to alanine in order to minimize the possibility of formation of disulfide bonds and secondary structure in the insert. Oligonucleotides encoding peptide 12.2B were ligated into the pCP2 plasmid so that, upon translation of pCP2 in the host plant, one copy of the 12.2B peptide would be inserted between Ala22 and Pro23 in the β B- β C loop on each S protein of the CPMV capsid (Fig. 1). The WT pCP1 and recombinant pCP2 plasmids were com-

bined and inoculated into cowpea plants as described in Materials and Methods. Chimeras expressing peptide 12.2B formed viable virus particles, as evidenced by a systemic infection accompanied by punctate lesions on the primary and secondary leaves. This CPMV chimera was harvested 3 to 4 weeks later, and a yield of 0.33 g of purified chimeric virus was obtained per kg of infected cowpea leaves. The chimera was termed CPMV-CD46 (containing the 11-residue insert ATHTIADRNT) and was used in the subsequent experiments.

Biochemical characterization of the CPMV-CD46 chimera. Display of the CD46 sequence ATHTIADRNT on the CPMV-CD46 chimera was confirmed by three separate methods. First, the CPMV-CD46 chimera was analyzed by reducing SDS-PAGE alongside WT CPMV for comparison (Fig. 2). As expected, both the WT CPMV and the CPMV-CD46 chimera had L proteins of similar molecular mass (~40 kDa). SDS-PAGE of WT CPMV (Fig. 2, lane 1) revealed two species of S protein typically seen in WT CPMV with molecular masses of 23 and 21 kDa corresponding to previously characterized slow- and fast-form S protein (21). The slow-form particles are full-length S protein, while the fast-form particles result from cleavage of the WT CPMV S protein at its C terminus. In addition, it has previously been shown that for many CPMV chimeras, the recombinant S protein containing the peptide insert is cleaved between the last two carboxy-terminal residues of the insert by an unidentified plant protease (24). SDS-PAGE of the CPMV-CD46 chimera (Fig. 2, lane 2) revealed three S protein bands. The largest chimeric S protein band migrated slower (higher) than the WT S protein bands and represents the uncleaved S protein containing the peptide insert. The two smaller S protein bands (S' and S'') have molecular masses of 20 and 6 kDa, respectively, corresponding to cleavage products from the proteolytic cleavage of the chimeric S protein at the carboxy-terminal end of the CD46 peptide insert (24). These results are consistent with SDS-PAGE profiles observed with other CPMV chimeras containing peptides from HRV14 and human immunodeficiency virus type 1 (HIV-1) (10, 14, 18). Second, MALDI mass spectrometry of CPMV-CD46 revealed species confirming that the additional ATHTIADRNT sequence was present in the CPMV-CD46 chimera (data not shown). Finally, sequence analysis of RNA isolated from the CPMV-CD46 chimera showed that the sequence encoding the ATHTIADRNT insert was retained even following three successive passages in plants (data not shown).

Inhibition of MV infection in vitro by CPMV-CD46. We previously showed that CD46 peptides 12 (IPPLATHTICDRNHTWLPVS) and 12.2 (ATHTICDRNHTW) inhibited MV infection of susceptible HeLa cells in vitro (12). To determine whether the CPMV-CD46 chimera was an effective inhibitor of MV infection, we compared the abilities of CPMV-CD46, WT CPMV, and the free CD46 peptides 12 and 12.2B (ATHTIADRNT; the same sequence as that present in the CPMV-CD46 chimera) to inhibit MV infection of HeLa cells. Infection was monitored by immunofluorescence with a polyclonal antibody specific for MV. Figure 3 shows that infection of HeLa cells by MV was inhibited by 5 mg of CPMV-CD46/ml (comparing Fig. 3B to C). This concentration of CPMV-CD46 contains a 0.54 μ M concentration of the ATHTIADRNT peptide. In contrast, 5 mg of WT CPMV/ml had no

FIG. 2. Reducing SDS-PAGE of WT CPMV and the CPMV-CD46 chimera. A total of 15 μ g of WT CPMV and 15 μ g of CPMV-CD46 chimera were electrophoresed on a 16% Tris-glycine polyacrylamide gel and then visualized by silver stain. The S protein bands in the WT CPMV lane correspond to slow-form (23-kDa) and fast-form (21-kDa) WT S proteins. The slower-migrating S protein band in the CPMV-CD46 lane corresponds to the uncleaved chimeric S protein, while the two faster-migrating bands correspond to the cleavage products obtained upon cleavage of the chimeric S protein at the C-terminal end of the CD46 insert in the β B- β C loop.

effect on MV infection (Fig. 3A). The percentage of MV-infected HeLa cells was determined for each treatment group by quantifying infected cells in a minimum of three independent fields per sample under a fluorescence microscope (Fig. 3D). A dose-dependent inhibition of MV infection was observed. In addition, 5.4 μ M CD46 peptide 12.2B presented on CPMV was found to have the same efficacy as 100 μ M free CD46 peptide, an 18.5-fold enhancement. The EC_{50} s for the peptide presented on CPMV-CD46 and for the free peptide 12.2B were 0.33 and 60 μ M, respectively (data not shown). Thus, in vitro experiments indicate that CPMV-CD46 specifically inhibits MV infection of HeLa cells and that, in multiple experiments, CPMV-CD46 was between 18- and 180-fold more effective an inhibitor than the same sequence presented as the soluble peptide 12.2B.

CPMV-CD46 as an antiviral agent in a transgenic mouse model for MV infection. To determine whether the CPMV-CD46 chimera could effectively inhibit viral infection in vivo, a transgenic mouse model of human MV infection, the NSE-CD46 transgenic mouse (20), was used. NSE-CD46 transgenic mice express the BC1 isoform of human CD46 under control of the neuron-specific enolase promoter. This promoter directs expression of the CD46 receptor to neurons of the central nervous system. NSE-CD46 mice are susceptible to infection by MV-Ed and develop a course of disease that includes encephalitis, ataxia (loss of muscle coordination), weight loss, paralysis, and death by between 8 and 20 days postinfection (20). MV-induced encephalitis is characterized by viral replication in the brain (particularly in the hippocampus, cortex, and thalamus), lymphocytic infiltration (CD4⁺ and CD8⁺ T cells, B cells), major histocompatibility complex upregulation, gliosis, and neuronal apoptosis (11). In a representative experiment, eight NSE-CD46 transgenic mice were injected i.c. with CPMV-CD46 (0.32 μ g/animal), along with 1.3×10^4 PFU of

FIG. 3. The CPMV-CD46 chimera inhibits MV infection of HeLa cells bearing the CD46 receptor. HeLa cells were inoculated with MV at an MOI of 2 in the presence of 5 mg of WT CPMV/ml (A), 5 mg of CPMV-CD46/ml (B), or no CPMV (C). Cells were stained 24 h postinfection for MV proteins by indirect immunofluorescence with a human polyclonal antiserum as the primary antibody and fluorescein isothiocyanate-labeled goat anti-human immunoglobulin as the secondary antibody. (D) Quantification of the inhibitory effect of CPMV-CD46 on MV infection of HeLa cells. Purified MV (MOI = 2) was incubated for 30 min with CPMV, with CPMV-CD46 (CPMV chimera containing the sequence ATHTIADRNHT), or with 100 μ M peptide 12 (IPPLATHTICDRNHTWLPVS) or 100 μ M peptide 12.2B (ATHTIADRNHT) and then added to duplicate wells containing 5×10^4 HeLa cells/well and incubated for 1 h at 37°C. Molar concentrations of the ATHTIADRNHT peptide in the context of the CPMV-CD46 chimera are noted in parentheses.

MV per animal. Five NSE-CD46 transgenic mice were injected in parallel with a control CPMV chimera, CPMV-HRV-II (0.32 μ g/animal), along with 1.3×10^4 PFU of MV per animal. Animals were observed daily for signs of infection including ataxia, paralysis, weight loss, and death. Survival curves for mice that received CPMV-CD46 compared to those that received the control chimera are shown in Fig. 4A. One hundred percent of the mice that had received MV in combination with the control chimera (CPMV-HRV-II) died by day 11 postinfection. In contrast, only 25% of the mice that had received MV in combination with CPMV-CD46 were dead by day 12 postinfection, and no further deaths occurred over a 2-month period (Fig. 4A). The MV N fragment (MV-N) RNA was identified in the brains of mice from both groups that died during the first 2 weeks postinfection by Northern blot with an oligonucleotide probe specific for MV-N (Fig. 4B), confirming that the mortalities observed were due to MV replication in the brain. Similar survival rates were obtained in two additional independent experiments. Taking the results of the three experiments together, 14 of 19 mice injected with a 1:1 mixture of CPMV-CD46 and MV survived till day 55 postinfection (74% survival), while 2 of 10 control mice injected with a 1:1 mixture of CPMV-HRV-II and MV survived (20% survival). As previously demonstrated, nontransgenic animals were not susceptible to MV infection and did not suffer any ill effects due to the CPMV inoculation.

To determine whether the CPMV-CD46 chimera could inhibit measles infection when administered prophylactically, CPMV-CD46 (0.32 μ g/animal) was i.c. injected into 3 1-day-old NSE-CD46 mice, followed a day later by an i.c. injection of 1.3×10^4 PFU of MV per animal. Four 1-day-

old NSE-CD46 control mice were injected with 30 μ l of PBS/animal, followed a day later by an i.c. injection of 1.3×10^4 PFU of MV per animal. By day 12 postinfection with MV, three of the control animals were exhibiting signs of typical measles-induced ataxia (loss of muscle coordination), severe wasting, and megacephaly. These animals were euthanized, and their brains were removed for Northern analysis. The fourth control animal became moribund at day 17 postinfection and was euthanized. One hemisphere of the brain of this animal was found to be collapsed upon dissection, an occurrence that can be attributed to severe encephalitis caused by MV infection of the brain. The animals that received CPMV-CD46, on the other hand, remained healthy for a month and a half postinfection with MV (Fig. 4A). These animals were then euthanized, and their brains were removed for Northern analysis. When the RNA extracted from the brains of animals from both groups was probed with a 32 P-labeled oligonucleotide specific for MV nucleoprotein, MV RNA was observed in the brains of all of the

FIG. 4. Efficacy of the CPMV-CD46 chimera as an inhibitor of MV infection in vivo. (A) Percent survival of NSE-CD46 transgenic mice injected i.c. as neonates with a 1:1 mixture of CPMV-CD46 (21 mg/ml) and MV at an MOI of 2 (solid diamond; $n = 8$) or CPMV-HRV-II (21 mg/ml) and MV at an MOI of 2 (shaded square; $n = 5$). Mice were monitored for mortality from measles over a period of 2 months. (B) Northern blot of RNA isolated from brains of NSE-CD46 transgenic mice that died from a typical MV-induced disease during the experiment. Lanes 1 to 3 contain RNA extracted from brains of mice that died in the 2 weeks after inoculation with CPMV-HRV-II and MV. Lane 4 contains RNA extracted from a mouse that died in the 2 weeks after inoculation with CPMV-CD46 and MV. The RNA samples were probed with a ^{32}P -labeled oligonucleotide representing a partial sequence of the MV-N (nucleoprotein) gene. (C) Percent survival of NSE-CD46 transgenic mice injected i.c. with CPMV-CD46 (10.5 mg/ml) at an MOI of 2 (solid square; $n = 3$) or PBS (shaded circle; $n = 4$) 1 day prior to i.c. injection with MV at an MOI of 2. (D) Northern blot of RNA isolated from brains of mice from the experiment depicted in panel C. Lanes 1 to 4 contain RNA extracted from brains of mice that received PBS a day prior to infection with MV and subsequently died during the course of the experiment. Lanes 5 to 7 contain RNA extracted from brains of mice that received CPMV-CD46 a day prior to infection with MV; these mice survived up to the end of the experiment (day 40 postinfection).

control animals but not in those of any of the CPMV-CD46-treated animals (Fig. 4D).

DISCUSSION

The present study utilizes CPMV peptide presentation technology to create a unique antiviral agent that effectively inhibits MV infection in vitro and in vivo. We previously showed that an 11-mer peptide derived from the SCR1 domain of the human MV receptor CD46 inhibited MV infection of HeLa cells (12). We now show that this inhibitory peptide, when presented on the surface of CPMV, effectively inhibits MV infection in vitro and also prevents mortality in an in vivo model of MV infection, both when administered to mice concomitantly with MV and when injected 1 day prior to MV infection. Northern data also show that viral replication was inhibited in mice from the latter experiment that were treated with CPMV-CD46, whereas viral replication occurred in control animals. These results suggest that the CPMV-CD46 chimera can efficiently block MV entry into neurons in the brains

of the CPMV-CD46 treated animals, thus preventing the establishment of a measles brain infection and facilitating clearance of MV from the brain. Comparative in vitro studies indicate that the chimera is approximately 2 orders of magnitude more potent than the soluble free CD46 peptide. The reasons for this enhancement are not clear, but one possibility is that the presentation of CD46 peptides on CPMV increases their stability. Another nonmutually exclusive possibility is that the increased local concentration of peptides around the fivefold axis of symmetry on the CPMV particle provides an enhancement in inhibitory activity. The effective multimerization of MV-hemagglutinin (H)-binding CD46 peptides in proximity on the fivefold axis might lead to an increased binding affinity for the H protein, which itself has been suggested to function as a trimer (2, 7). Multimerization increases the binding energy and, since the relationship between K_d and binding energy is exponential, doubling the binding energy squares the K_d , i.e., the affinity of the interaction is squared. The CD46 peptides presented on CPMV could thus be more effective at blocking MV-H binding to the cellular receptor. Another possibility is

that the local concentration of MV-H binding peptides might more efficiently trigger conformational changes in the associated MV fusion protein, thus inactivating the virus. Such a scenario has been proposed for a soluble, experimentally octamerized form of the CD46 receptor that has shown a similar ~100-fold enhancement in antiviral activity compared to that of monomeric soluble CD46 (4).

For measles in particular, a CPMV-based antiviral could be an effective companion to the measles vaccine. The current measles vaccine utilizes live attenuated MV derived from the Edmonston laboratory strain, but this vaccine is inhibited by maternal antibody. An inexpensive measles antiviral agent could act as a supplement to the measles vaccine, protecting children against MV infection during the window of time before the vaccine becomes effective. It would be very interesting to determine whether a CPMV-based antiviral agent could be effective after MV infection has been initiated. In the NSE-CD46 transgenic model and in previously published models (4), it has not been possible to do mortality-challenge studies in animals after MV inoculation because once CD46-dependent entry into neurons of the central nervous system has been achieved, subsequent cell-to-cell spread likely occurs transsynaptically and does not require CD46 (8). Thus, to determine whether a CPMV-based antiviral agent will be effective in inhibiting an established infection, other virus-receptor interactions are being explored.

One potential application of the CPMV system to the development of antiviral agents is to display multiple peptides or peptides with structural conformation on the capsid surface. Structural studies on the capsid S protein of CPMV have revealed multiple sites at which heterologous peptides can be inserted. In addition to the βB - βC loop, other sites that have been identified on the S protein include the $\beta\text{C}'$ - $\beta\text{C}''$ site and sites at or near the C terminus of the S protein. Studies are currently under way to express the CD46 peptide 12.2B at the βB - βC site and an MV-neutralizing antibody epitope and/or cytotoxic-T-cell epitope at other sites on the CPMV capsid S protein. CPMV has been previously used to generate vaccines against several viral epitopes with encouraging success. Chimeric CPMV particles expressing epitopes from HIV-1, human rhinovirus, canine parvovirus, foot-and-mouth disease virus, mink enteritis virus, and the bacterium *Staphylococcus aureus* have been created that evoke strong protective antibody responses in experimental animals (5, 14–16, 18, 25). A CPMV chimera that combined potent inhibition of MV infection and the induction of virus-specific antibodies and cellular immune responses could potentially act as both a vaccine and an antiviral agent simultaneously. The CPMV technology also presents the possibility of manipulating the three-dimensional structural presentation of an epitope. This is important in order to evoke optimal epitope-specific immune responses or for presentation of a peptide whose activity is dependent on its three-dimensional conformation. Recently, CPMV chimeras have been created that express the N1m-1A epitope of human rhinovirus, CPMV-HRV14, in the uncleaved form by moving the N1m1A epitope one residue toward the N terminus (23). Since the native N1m1A epitope forms a constrained loop, the uncleaved CPMV-HRV14 chimera induced antibodies of a higher titer and affinity for HRV14 than the

cleaved chimera. This strategy could potentially be applied to the creation of antiviral reagents that display peptides whose antiviral activity depends on a constrained three-dimensional structure. The success of the CPMV-CD46 chimera in inhibiting MV infection in vivo and in vitro paves the way for the creation of antivirals and antiviral agent-vaccine combinations against other important viruses such as HIV-1 whose cellular receptors have been structurally and biochemically characterized.

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