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Heparin-like glycosaminoglycans prevent the infection of measles virus in SLAM-negative cell lines

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ABSTRACT

The wide tissue tropism of the measles virus (MV) suggests that it involves ubiquitously expressed molecules. We have constructed a recombinant MV expressing the enhanced green fluorescent protein (EGFP) (rMV-EGFP) and demonstrated that the rMV-EGFP infected several cell types (HEK-293, HepG2, Hep3B, Huh7, and WRL68 cells) that do not express the human signalling lymphocyte activation molecule (SLAM), which is known as a cellular receptor for morbilliviruses. MV infection of HEK-293 and HepG2 cells was not inhibited in an infectivity–inhibition assay using an anti-SLAM monoclonal antibody, indicating that MV could infect cells without using SLAM. Soluble heparin (HP) inhibited the rMV-EGFP infectivity in SLAM-negative cell lines in a dose-dependent manner. Direct interaction between purified virions and HP was detected in a surface plasmon resonance assay. We also demonstrated that the hemagglutinin (H) protein, but not the fusion (F) protein is responsible for the interaction between the virions and HP. Taken together, our results suggest that HP-like glycosaminoglycans bind to the H protein of MV and play a key role in the infection of SLAM-negative cells.

1. Introduction

Measles virus (MV) is a single-stranded, negative-sense RNA virus that is classified in the genus *Morbillivirus* of the family *Paramyxoviridae*, which includes canine distemper virus (CDV), rinderpest virus (RPV), and peste des petits ruminants virus. MV propagates in lymphoid organs throughout the body. In addition, it also spreads to a wide variety of other organs, including skin, conjunctivae, kidney, lungs, gastrointestinal tract, respiratory mucosa, genital mucosa, and liver (Griffin, 2007). The two MV envelope glycoproteins H and F work in combination to elicit the fusion of virus with the cell membrane. The signalling lymphocyte activation molecule (SLAM) (also known as CD150), which is a receptor involved in T cell activation (Cocks et al., 1995), is a cellular receptor for MV (Tatsuo et al., 2000) and can be used by both the vaccine strains and wild-type strains. Vaccine strains of MV also efficiently infect cells via CD46 (Dörig et al., 1993; Naniche et al., 1993), a ubiquitously expressed regulator of complement activation. Binding of the virus to the target cells is a critical factor for determining the tissue tropism and pathogenesis of the virus. However, it has been shown that SLAM is specifically expressed only in some T and B cells, thymocytes and dendritic cells (DCs) (Yanagi et al., 2002). Increasing evidence suggests the presence of receptors other than SLAM and CD46 for MV, and considering the wide tissue tropism of MV, ubiquitous molecules on the cell surface are expected to be involved in the attachment of MV (Hashimoto et al., 2002; Andres et al., 2003; Takeuchi et al., 2003; Takeda et al., 2007; Tahara et al., 2008).

Glycosaminoglycans are unbranched polysaccharides that are ubiquitously present on cell surfaces. Apart from their diverse functions in biologic processes by binding to various proteins including growth factors, chemokines, extracellular matrix proteins and cell adhesion molecules, they have been shown to play important roles in the cell-surface binding of pathogens such as bacteria, parasites and viruses (DeAngelis, 2002; Vogt et al., 2003; Lee et al., 2006). Recent studies demonstrated the involvement of heparin (HP)-like molecules in RPV and CDV infections (Baron, 2005; Fujita et al., 2007). However, it has been reported that infection by the Edmonston strain of MV was not inhibited by HP (Feldman et al., 2000). In the present study, we constructed a recombinant MV (HL strain) expressing the enhanced green fluorescent protein (EGFP) (rMV-EGFP), and examined whether HP-like molecules are involved in the infection of wild-type MV.

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2. Materials and methods

2.1. Cells and viruses

B95a cells (Kobune et al., 1990) were cultured in RPMI 1640 medium (SIGMA) containing 10% fetal calf serum (FCS). Human embryonic kidney (HEK)-293 cells, 293/SLAM cells expressing marmoset SLAM (Sato et al., 2008), and four liver cell lines (HepG2, Hep3B, Huh7, and WRL68) were grown in Dulbecco's modified minimum essential medium (Life Technologies Inc.) supplemented with 10% FCS. The HL strain of MV, a wild-type strain that was originally isolated from a patient, were grown in B95a cells. Recombinant viruses were obtained by using the methods described below. MV-HL and the recombinant viruses were propagated in B95a cells that were grown in 2% FCS-supplemented RPMI 1640 medium.

2.2. Rescue of rMV-EGFP

A full-length cDNA of the MV-HL genome was constructed from clones containing each of the individual genes. MV RNA was extracted from infected cells as previously described (Radecke et al., 1995). RT-PCR was performed using Superscript II reverse transcriptase (Invitrogen). All cloning procedures were conducted following standard protocols. PCR amplifications were performed using LA-Taq DNA polymerase (TAKARA) or Pfu DNA polymerase (STRATAGENE). The leader and trailer sequences were amplified using primers including EagI and EcoRI sites (leader), and EcoRI and BsmI sites (trailer). Each fragment was digested by EcoRI and EagI or BsmI, and cloned into pMDB1 using the EcoRI and EagI or BsmI sites, respectively. Amplification of the six MV genes was performed with primers containing unique restriction sites: N (NotI–FseI), P (FseI–PmeI), M (PmeI–MluI), F (MluI–SgfI), H (SgfI–AscI) and L (AscI–BsiWI). Each site was introduced immediately after the terminal codon, except that the NotI site was introduced immediately before the initiation codon of the N gene. We obtained at least 4 clones from each amplification and confirmed that the sequence in each was identical. The fragments were joined using the unique enzyme site and the sequence of the joined fragments was confirmed. Finally, the assembled genes were excised with NotI and BsiWI, and were inserted into the pMDB1 plasmid with the leader and trailer sequences. The resulting plasmid was designated pMV-HL (7+).

For construction of the full-length genome plasmid to express the enhanced green fluorescent protein, the EGFP gene was amplified from pEGFP-N1 (Clonetech) using the following primers EGFP-F; 5′-TATA*GGCCGGCC*ATCATTGTTATAAAAAACTTAGGAACCAGGTTCATCCACAATGGTGAGCAAGGGCGAGGAGCT-3′ and EGFP-R; 5′-AA*GGCCGGCCC*TACTTGTACAGCTCGTCCA (FseI site in italic). The EGFP fragment was cloned into the FseI site of the pMV-HL (7+).

For recovery of rMV-EGFP from cDNA, HEK-293 cells were seeded into 6-well plates (8×10^5 cells per well) 1 day before infection and transfection. The cells were infected with replication-deficient MVAGKT7 vaccinia virus in DMEM supplemented with 2% FCS 1 h before transfection. Prior to transfection, 9 μl of Fugene 6 (Roche) were mixed and incubated with 300 μl serum-free DMEM for 5 min. Plasmids (1 μg of pMV-HL (7+), pKS-N and pKS-P, and 0.03 μg of pGEM-L (Baron and Barrett, 1997)) were mixed into 100 μl. The plasmid mixtures were then carefully pipetted into the diluted Fugene 6. MVAGKT7 was removed from the cells and replaced with 2 ml of maintenance medium containing the plasmids and Fugene 6. After 4 days' incubation, the medium was removed and 1×10^6 cells/well of B95a were added with 2 ml of RPMI1640 containing 2% FCS. When an advanced cytopathic effect was observed (usually 2–4 days after co-cultivation), the cells and medium were harvested and stored at −80 °C.

2.3. Inhibition of infection by antibodies

B95a, HEK-293, and 293/SLAM cells (1×10^4 cells) were grown in 96-well plates overnight. They were then incubated at 37 °C in medium containing 10 μg/ml of an anti-human CD46 monoclonal antibody (MAb) (clone M75: HyCult biotechnology), or an anti-human SLAM MAb (clone IPO-3: Kamiya Biomedical). At 1 h after treatment with MAbs, the cells were infected with rMV-EGFP ($TCID_{50}$ 1×10^4) at a multiplicity of infection (MOI) of 1 $TCID_{50}$/cell. At 40 h after the infection, the number of EGFP-expressing cells was counted at 20 fields under a fluorescence microscope (Olympus, IX70), and the percentage of EGFP-positive cells was calculated.

2.4. Inhibition of infection by soluble HP

For the infection–inhibition assay, rMV-EGFP ($TCID_{50}$ 1×10^5) at an MOI of 2 $TCID_{50}$/cell was inoculated at various concentrations into HEK-293 or 293/SLAM cells (2.5×10^4) in the presence of HP (Sigma). Following incubation for 40 h at 37 °C, the cells were harvested and analyzed by performing FACS using FACScan (Beckton Dickinson). In this analysis, 1×10^4 cells were counted for each sample, and the infected cells were detected by fluorescence intensity set on a log scale. Percentage of inhibition of infection was calculated relative to the extent of infection in control assays performed in the absence of HS.

2.5. Purification of the rMV-EGFP

The B95a cells infected with rMV-EGFP were subjected to 3 cycles of freezing and thawing, followed by centrifugation to remove the cell debris. The supernatant was concentrated by using a size exclusion (100-K) membrane filter (Amicon) and was loaded onto a 20–60% sucrose density gradient. Following centrifugation at 100,000 × *g* and 4 °C for 2 h, the virus layer was collected and applied onto a gel-filtration column (PD-10; Pharmacia) to exchange the buffer with 20 mM sodium phosphate buffer (pH 7.4).

2.6. HP affinity chromatography

For affinity chromatography, purified rMV-EGFP obtained from the infected B95a cells was used. Following equilibration of HP-agarose beads or bovine serum albumin (BSA)-agarose beads (Sigma) in phosphate-buffered saline (PBS), the concentrated virus supernatant was added to the beads, and the mixture was incubated for 30 min at 4 °C and subsequently centrifuged at 1000 × *g* for 1 min. The beads were washed 7 times in 1 ml PBS, followed by elution in PBS containing 2 M NaCl. The final wash and elution fractions were concentrated by using a 100-K membrane filter (VIVASPIN; Sartorius) and analyzed by SDS–PAGE followed by western blot analysis with an MAb against the N protein of MV.

To express F or H of MV, open-reading frames (ORFs) of F and H were amplified by RT-PCR and cloned into pCAGGS and pCMV-myc, respectively. The H protein expressed in pCMV-myc vector contained the N-terminal myc epitope tag. The resulting plasmids were transfected into HEK-293 cells using Fugene 6 as a transfection reagent according to the Manufacturer's instructions. At 48 h after transfection, the cells were lysed in lysis buffer (20 mM sodium phosphate (pH 7.4) containing 150 mM NaCl and 1% Triton X-100). Then, the lysates were mixed with the beads equilibrated in the lysis buffer and incubated for 1 h at 4 °C and beads were washed in the lysis buffer (7 times, with 1 ml each time), and the bound materials were eluted with increasing concentrations of NaCl in

the lysis buffer (stepwise gradient: 250 mM, 500 mM, 1 M, and 2 M). The eluted fractions were concentrated by using a 5-K membrane filter (VIVASPIN, Sartorius) and were then analyzed by SDS–PAGE followed by western blot analysis with an anti-myc or anti-MV-F MAb (see below).

2.7. Surface plasmon resonance binding assays performed using BIAcore technology

Surface plasmon resonance (SPR) measurements were performed on a BIAcore X instrument (BIAcore AB). HP (16 kDa, derived from porcine intestinal mucosa (Sigma); 100 μg/ml in 10 mM acetate buffer (pH 4.0)) was covalently immobilized to the dextran matrix of a CM5 sensor chip (Fc1) via its primary amine groups (amine coupling kit, BIAcore AB) at a flow rate of 5 μl/min (Fc1-HP). No protein was immobilized on the other side of CM5 sensor chip (Fc2) for negative control (Fc2-NC). After coating on sensor chips, we performed the masking treatment with ethanol-amine for blocking non-specific binding on sensor chip. rMV-EGFP that had been purified over a sucrose density gradient was diluted in Hanks' balanced salt (HBS) buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20; BIAcore AB) and duplicate analyses were performed using 10 μg/ml of the virus. All affinity measurements were performed at room temperature in HBS with a constant flow rate of 20 μl/min and an injected sample volume of 40 μl. Between measurements, the chip surface was regenerated by using 100 mM HCl. Kinetic constants were determined by using the BIAevaluation 2.1 software (BIAcore X). The association, dissociation, and regeneration phases were tracked in real time by monitoring the changes in the signal expressed in resonance units (RU).

2.8. Generation of monoclonal antibodies

To produce recombinant F protein in *E.coli*, the ORF of MV-F was amplified using the following primers: MV-F F; 5′-ATGGGTCTCAAGGTGAACGT-3′ and MV-F R; 5′-TCAGAGCGACCTTACATAGG-3′, and the DNA fragment was inserted into pQE-30 (QIAGEN). Protein expression was carried out in the *E.coli* strain M15. The N-terminally His-tagged F protein was purified by Ni–NTA column (QIAGEN) according to the manufacturer's protocols with AKTA prime FPLC (Amersham Bioscience).

BALB/c mice were immunized three times with the purified F protein mixed with RIBI adjuvant (Corixa). The anti-MV-F antibody titer was assessed by performing an ELISA using the recombinant F protein as the antigen. When high anti-F antibody titers were detected in their sera, the mice were euthanized, and spleen cells were obtained. The spleen cells were fused with myeloma PAI cells, using PEG1500 (Roche). Hybridoma cells were selected by using hypoxanthine–aminopterin–thymidine (HAT) medium (Gibco BRL), and the culture supernatants were examined by immunofluorescence assay (IFA), using B95a cells infected with the HL strain of MV. Hybridomas producing anti-F antibodies were propagated and inoculated into the peritoneal cavity of BALB/c mice that had been treated with pristan (Sigma–Aldrich). At approximately 10 days after inoculation, ascites fluid was collected.

3. Results

3.1. Recovery of rMV-EGFP

The pMV plasmid containing the full-length cDNA of MV was manipulated to include unique restriction sites in the noncoding regions between adjacent genes in the MV genome. Coding regions of EGFP gene were attached to the transcription signal units (the transcription termination/polyadenylation signal of the N gene and the transcription start signal of the H gene), and cloned into the FseI site of pMV, which was inserted immediately downstream to the N gene of MV (Fig. 1A). The resulting plasmid, pMV-EGFP, was cotransfected along with supporting plasmids carrying the N, P, and L proteins of RPV into HEK-293 cells that were preinfected with the recombinant vaccinia virus MVAGKT7 expressing phage T7 RNA polymerase. The cells were incubated for 3 days, followed by cocultivation with B95a cells. Following incubation for several days, syncytia induced by the recombinant MVs were observed. The sizes of the syncytia produced by the wild-type (wt) MV (HL strain) and rMV-EGFP were similar (Fig. 1B). The growth kinetics of rMV-EGFP was comparable to that of wt MV (data not shown).

3.2. Infection of SLAM-negative cells with rMV-EGFP

We analyzed the permissibility of many cell lines to MV by using rMV-EGFP, and observed that most of these cell lines are infected with rMV-EGFP, although the levels of infection were different among cell lines (data not shown). Representatively, the HEK-293 and HepG2 cells, which do not express SLAM, exhibited specific fluorescence (Fig. 2b and d). Since wt MV does not enter cells via CD46, which is a receptor for the vaccine strain MV-Ed, our results indicate that wt MV (HL strain) can infect cells independently of SLAM and CD46. To examine whether these two known receptors, namely, SLAM and CD46, were involved in entry of the virus into these cells, we treated the cells with antibodies against these receptors followed by infection with rMV-EGFP. Pretreatment of B95a and 293/SLAM cells, which constitutively express marmoset SLAM, with a SLAM-specific MAb (IPO-3) strongly suppressed the rMV-EGFP infection (Fig. 2e and g). In contrast, pretreatment with IPO-3 did not inhibit the rMV-EGFP infection in HEK-293 and HepG2 cells (Fig. 2f and h). Furthermore, pretreatment with an MAb against CD46 (M75) did not influence the infectivity of rMV-EGFP in any of the cell lines tested (Fig. 2i–l). Therefore, it was suggested that rMV-EGFP utilize SLAM but not CD46 as a receptor, and rMV-EGFP infects SLAM-negative cells via unknown receptor(s). These results are consistent with the previous studies demonstrating the presence of alternative entry pathway(s), independent of SLAM and CD46 (Hashimoto et al., 2002; Takeuchi et al., 2003; Takeda et al., 2007; Tahara et al., 2008).

3.3. The effect of soluble HP on rMV-EGFP infection

To test whether HP-like molecules are involved in the alternative entry pathway of MV, cells were infected with rMV-EGFP in the presence of soluble HP (100 μg/ml). Not only HEK-293, 293/SLAM and HepG2 cells but also Hep3B, Huh7, and WRL68 cells were tested since MV exhibited high infectivity of MV in these cells. The number of EGFP-positive cells was counted by performing fluorescence-activated cell sorting (FACS) analysis, and the infectivity relative to that in 293/SLAM in the absence of HP was calculated. HP treatment dramatically decreased the infectivity of rMV-EGFP in HEK-293, HepG2, He3B, Huh7 and WRL68 cells by 83%, 72%, 87%, 71% and 97%, respectively (Fig. 3A). The effect of HP treatment on infection of 293/SLAM cells was small (3% reduction), probably because SLAM is a strong receptor and enables the maximum infection of MV. Thus, HP is capable of inhibiting MV infection in SLAM-negative cells.

To analyze the dose dependence, HEK-293 cells were infected with different MOIs of rMV-EGFP in the presence of increasing concentrations of HP (10, 100, and 1000 μg/ml). The infectivity at MOI 4 in the absence of HP was set as 100%. The treatment of rMV-EGFP with HP even at a low concentration of 10 μg/ml significantly inhibited the infection of the HEK-293 cells (by ∼25%) and the percent inhibition increased in a dose-dependent manner (Fig. 3B). The

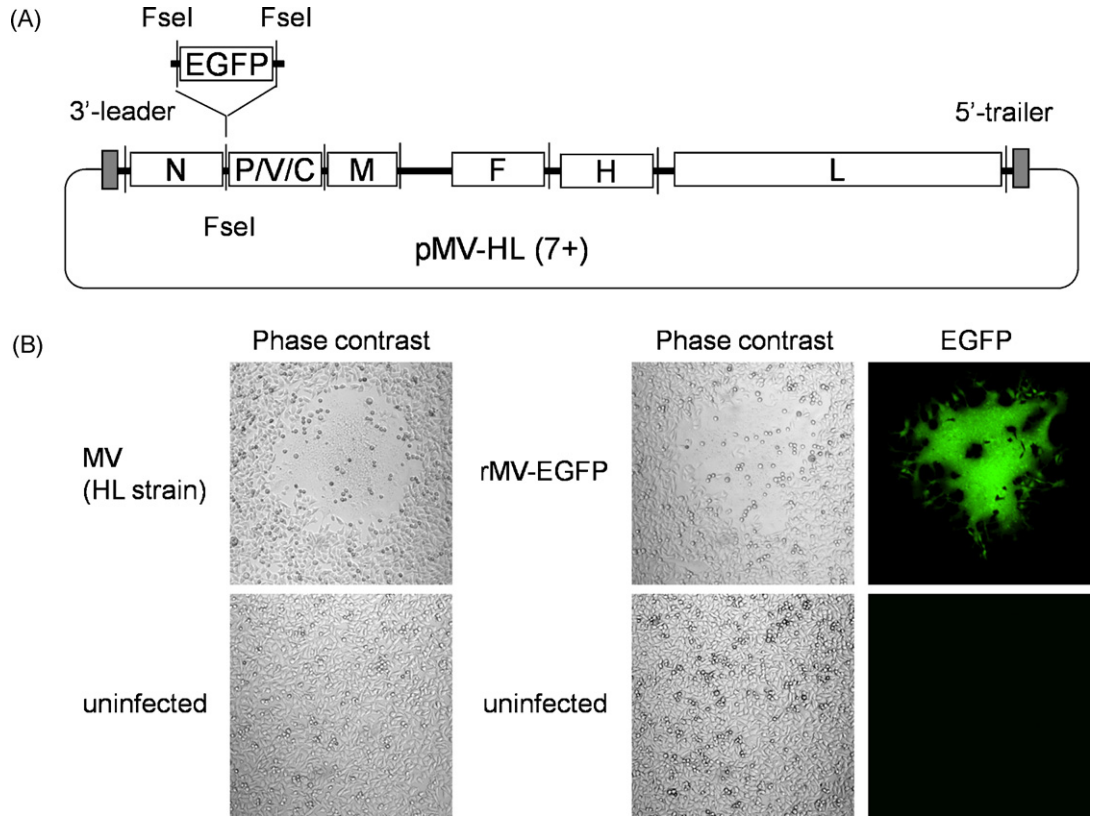

Fig. 1. (A) Construction of rMV-EGFP. The coding regions for MV structural proteins are indicated by opened boxes. The EGFP open-reading frame (ORF) fragment flanked by restriction sites for FseI was introduced into the pMV plasmid. (B) Syncytium formation in B95a cells infected with wild-type MV (HL-strain) (left) or rMV-EGFP (right). EGFP expression in the rMV-EGFP-infected B95a cells was observed under a confocal microscope (200×). Phase contrast or immunofluorescence images of uninfected cells are shown in the bottom.

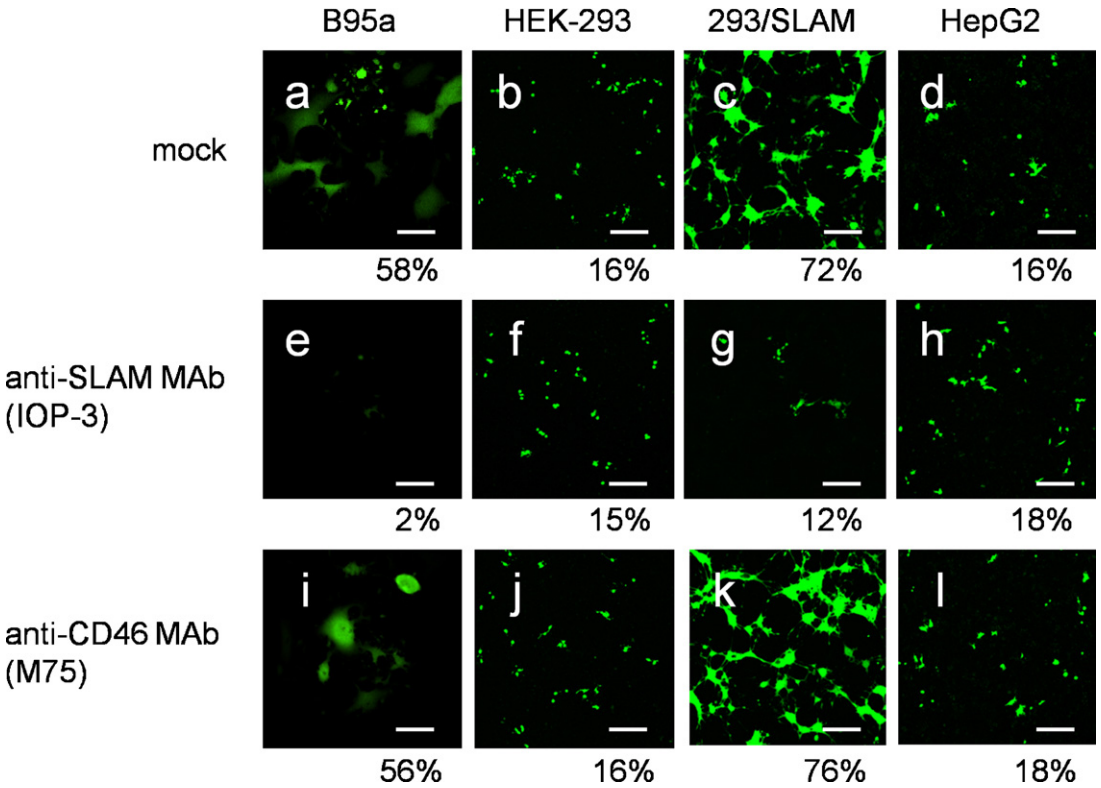

Fig. 2. (A) Effects of the anti-SLAM and anti-CD46 MAbs on rMV-EGFP infection of each cells. B95a (a, e, and i), HEK-293 (b, f, and j), 293/SLAM (c, g, and k) and HepG2 (d, h, and l) cells were pretreated with either an anti-SLAM MAb (IPO-3) (e–h) or an anti-CD46 MAb (M75) (I–l) and were subsequently infected with 10^4 $TCID_{50}$ of rMV-EGFP. At 40 h after the infection, EGFP fluorescence was observed under a fluorescence microscope (100×). The % cells infected is indicated under the panels. Bars, 100 μm.

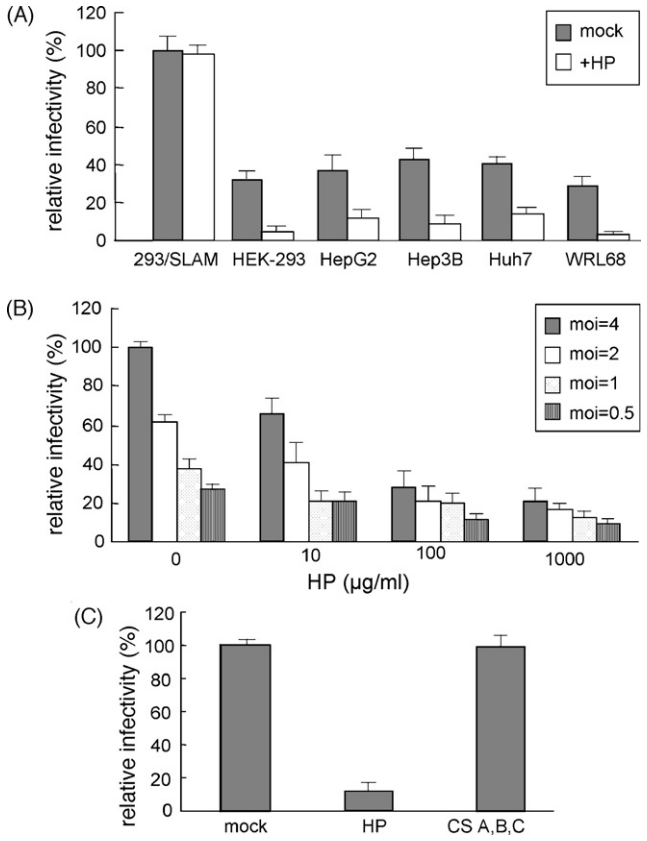

Fig. 3. Inhibition of rMV-EGFP infection by soluble HP. 293/SLAM, HEK-293, and human liver cells (HepG2, Hep3B, Huh7, and WRL68) were infected with rMV-EGFP at an MOI of 2 $TCID_{50}$/cell in the presence of soluble HP at a concentration of 100 μg/ml (A). Effect of the concentration of HP and MOI of the virus on infection of HEK-293 cells (B). Effect of the treatment with 100 μg/ml CS on infection of HEK-293 cells (C). The viral infectivity was measured at 40 h after the infection by FACS analysis.

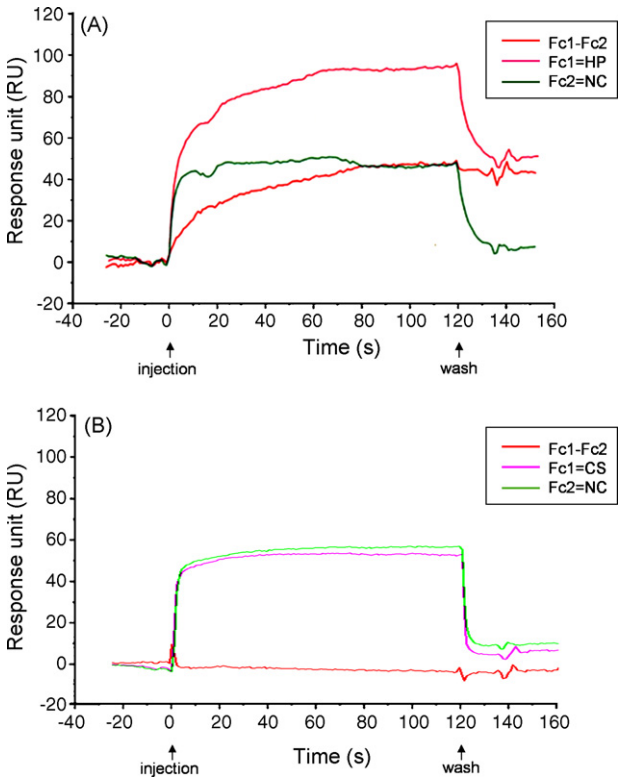

Fig. 4. Interactions between HP and MV analyzed by performing an SPR assay. HP (A) or CS (B) was covalently immobilized onto the SPR analysis. Interaction with the HP or CS were examined in real time by using 10 μg/ml purified MV. Associations among ligands occurred for 120 s following injection of the relevant analyte (Fc1, sensorgram obtained from the HP-coated sensor chip; Fc2, sensorgram obtained from the uncoated sensor chip; Fc1–Fc2, net value of Fc1).

inhibitory effect of HP was also confirmed in the experiments using different MOIs of the virus (Fig. 3B).

Two major types of glycosaminoglycan chains are found in animal cells, namely HS and chondroitin sulphate (CS) chains. To investigate whether the CS is capable of inhibiting the infection, HEK-293 cells were infected with rMV-EGFP in the presence of CS A, B, and C at a concentration of 100 μg/ml, respectively, and the infectivity of rMV-EGFP was analyzed by FACS analysis. We observed that CS A, B, and C did not significantly inhibit the rMV-EGFP infection in HEK-293 cells (Fig. 3C).

3.4. Interaction between HP and MV analyzed by performing SPR measurements

The direct interactions between MV and HP were analyzed based on the SPR (Fig. 4A). Increasing concentrations of purified MV were introduced over the HP surface on an Fc1-coated biosensor. The binding and subsequent dissociation of the MV was monitored in real time by measuring changes in the SPR in terms of response units (RU). Although MV particles bound non-specifically to the uncoated sensor chip surface during the binding phase (Fc2), coating of the sensor chip with HP dramatically increased the binding (Fc1) (Fig. 4A). Furthermore, most of the MV particles bound to the uncoated sensor chip were washed away from the chip surface (Fc2), while approximately 60% of the MV particles remained bound to the HP-coated sensor chip after washing (Fc1). When CS was coated on the Fc1 sensor chip, there was no interaction with MV (Fig. 4B). Thus, these BIAcore experiments revealed the specific interactions between the MV particles and HP *in vitro*.

3.5. HP binding envelope glycoproteins

To determine the viral proteins responsible for HP binding, affinity chromatography was performed using HP- and BSA-agarose beads. First, the concentrated supernatant of the rMV-EGFP-infected B95a cells was subjected to HP affinity chromatography. The bound materials were eluted with 2 M NaCl and analyzed by performing SDS–PAGE and western blotting with an anti-N polyclonal antibody. Although rMV-EGFP virions could not be recovered from the BSA-agarose (Fig. 5A, lane 5), they were eluted from the HP-agarose (Fig. 5A, lane 3). Thus, recombinant MV directly binds to HP at a physiological salt concentration.

Next, the *in vitro* HP binding of solubilized viral membrane glycoproteins, namely, the H and F proteins, was assessed by using HP-agarose beads. The H and F viral glycoproteins were expressed individually in HEK-293 cells by transfecting expression plasmids for these proteins. The detergent-solubilized lysates of transfected cells were subjected to HP affinity chromatography. The F protein in the resulting fractions was identified by immunoblot using an MAb against the F protein, while the H protein was identified by using an anti-myc MAb. The H protein (approximately 75 kDa) was eluted at high salt concentrations (500 mM to 1 M), while the F protein was not recovered even at the highest salt concentration (Fig. 5B), indicating the lack of interaction between HP and the F protein. These results suggest that the H protein, but not the F protein, is responsible for the binding of MV particles to HP.

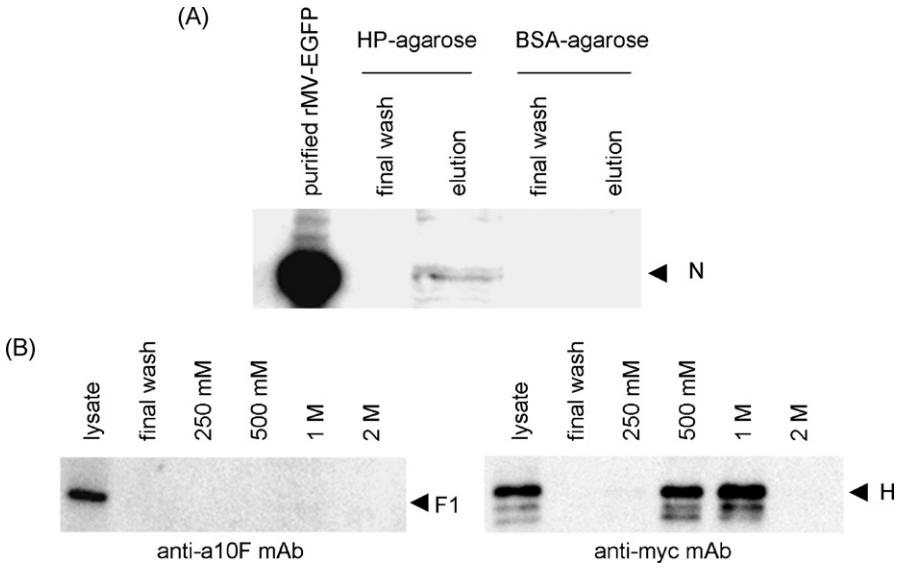

Fig. 5. HP affinity chromatography of virus particles and MV glycoproteins. (A) HP affinity chromatography of rMV-EGFP. Total MV proteins prior to before chromatography (lane 1), the final wash fraction (lane 2) and bound proteins (lane 3) eluted from the HP column, the final wash fraction obtained from the BSA-agarose beads (lane 4), and the elution fraction obtained from the BSA-agarose beads (lane 5) were subjected to western blotting with a polyclonal antibody against the N protein of MV. (B) HP affinity chromatography by using the extract of HEK-293 cells transfected with expression plasmids encoding either the F (left panel) or H gene (right panel) of MV. The proteins were eluted with increasing concentrations of NaCl in a stepwise manner. Lane 1: lysate of HEK-293 cells transfected with the plasmid expressing the corresponding MV gene; lane 2: final wash fraction; lanes 3–6: elution fractions. The NaCl concentrations that were used for elution are indicated at the top of the lanes. The fractions were analyzed by performing SDS–PAGE and detected by western blotting with the anti-MV-F or anti-myc MAb.

4. Discussion

We established and applied the reverse genetics system to wt MV in order to generate a recombinant virus carrying the EGFP marker gene, i.e. an EGFP-expressing recombinant virus. Our initial studies revealed that MV can infect some SLAM-negative cells, and this was consistent with the results obtained in our previous study on CDV (Fujita et al., 2007). Since attachment to a cell surface receptor is a critical step in viral infection and entry into the cell, some important molecules are suggested to be involved in the MV infection of SLAM-negative cells.

In this study, we demonstrated that MV can replicate in various liver cell lines (Fig. 3). However, its infectivity towards the human liver cell lines was considerably lower than that towards B95a and 293/SLAM cells that express SLAM as a receptor.

Griffin have suggested that MV spreads to a wide range of organs including skin, conjunctivae, kidneys, lungs, gastrointestinal tract, respiratory mucosa, genital mucosa, and liver (Griffin, 2007). Furthermore, they reported that the mechanism of MV replication in the liver, particularly in the bile duct epithelium, is common in all age groups; however, clinically evident hepatitis is most frequent in adults (Griffin, 2007). The MV infection spreads to many epithelial surfaces, and this may directly cause gastrointestinal symptoms. Recently, it has been reported that the DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) plays an important role in MV infection of DCs (de Witte et al., 2006). Nevertheless, in contrast to SLAM and CD46, DC-SIGN does not support MV entry, but only functions as an attachment receptor for MV to enhance the SLAM/CD46-mediated infection of DCs. These studies suggest that the broad range of infections caused by MV in SLAM-negative cells may involve interactions with (as yet) unidentified receptor(s).

HP and HS belong to a class of carbohydrates designated as glycosaminoglycans, which are unbranched polymers of repeating disaccharide units. They are found as proteoglycans covalently linked to membrane proteins on the surface of most mammalian cells. They are classified as CS, dermatan sulphate (DS), HP/HS, keratan sulphate (KS), and hyaluronic acid (HA). HP is produced by mast cells, and more than 85% of its glucosamine residues are *N*-sulphated (Jacobsson and Lindahl, 1980). On the other hand, HS is produced by almost all cell types, and only 40–60% of its glucosamine residues are *N*-sulphated. The most prominent physicochemical property of glycosaminoglycans is that they carry a large and varying number of negative charges that are conferred onto the molecule by sulphate residues in most glycosaminoglycan types (Lindahl and Kjellén, 1991). The attachment of a number of viruses to the cell surface is known to involve proteoglycans (Jackson et al., 1991).

Baron reported that HS is important for RPV (RBOK strain) infection (Baron, 2005). It has been shown that wild-type RPV requires CD150 (SLAM) as a receptor, while the cell culture-adapted vaccine strain has acquired the ability to use HS as an alternative receptor. We tested the effect of HP by using the RPV-Lv strain and observed that interactions with HS observed in RPV infection were consistent with this previous report, although the Lv strain is a lapinized (i.e. attenuated by serial passage in rabbits) RPV strain (data not shown). In addition, we previously reported that an HP-like molecule is an important factor for CDV infection and that wt CDV also uses this molecule for infecting SLAM-negative cells (Fujita et al., 2007). In our present study, we showed that an HP-like molecule is also implicated in the entry of MV into cells by competitive inhibition assay using HP and rMV-EGFP (Fig. 3). The rMV-EGFP infection of SLAM-negative HEK-293 cells and liver cell lines was greatly inhibited by treatment with soluble HP. Furthermore, the SPR assay clarified that purified virions interacted with soluble HP; and HP affinity chromatography revealed the binding of the envelope H glycoprotein to immobilized HP (Figs. 4 and 5). Therefore, HP/HS commonly play an important role in the infection of morbilliviruses including MV, CDV, and RPV, and thus HP/HS or a related molecule could be a target for inhibition of morbilliviruses in non-lymphatic organs.

Our observation that MV (HL strain) infection was inhibited by HP is interesting because it has been reported that soluble HP does not inhibit the infection of Vero cells with the Edmonston vac-

cine strain of MV (Feldman et al., 2000). This MV strain can use CD46 as well as SLAM as cellular receptors (Naniche et al., 1993; Dörig et al., 1993) and these receptors are expressed in a wide range of human cells, except for erythrocytes. Vero cells express the high-affinity receptor CD46; therefore, the effect of soluble HP on MV attachment may be inconspicuous. In contrast, wild-type MV strains isolated from the marmoset B cell line B95a or from human B cell lines are unable to use CD46 as a receptor (Ono et al., 2001). Since we used recombinant viruses derived from cDNA clones of wild-type MV strain (HL strain), we could study the implication of HP-like molecules in the natural infection of MV in SLAM-negative human cells. In our experiment, the rMV-EGFP infection of 293/SLAM cells was not significantly inhibited, probably because this infection was mainly mediated by the high-affinity receptor SLAM and the contribution of HP-like molecules was therefore small.

In summary, we demonstrated the involvement of HP-like molecules in MV infection. The rMV-EGFP infection was not completely blocked by the treatment with HP. Thus, other molecule(s) must be involved in the attachment process, and the interaction between MV and HP-like molecules on the cell surface may promote the binding of MV envelope glycoproteins to a receptor that has not (yet) been identified.

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