Cells and supernatants of wells infected with MV-HL were harvested 12, 24 and 48 h later, and carried out the freezing–thawing cycle three times. Infectivity was determined by $TCID_{50}$ titration using the standard method. The experiment was repeated two times.

Generation of V knockout MV

To create V protein knockout MV, a previously established plasmid encoding the cDNA of the full-length genome of wild-type HL strain of MV was used (Yoneda et al., unpublished data). Two nucleotide substitutions (antigenome ^{2491}AAAAAGGG^{2498} to ^{2491}AAGAAGG^{2498}) were introduced into the editing site in the P gene of the plasmid to ensure that no editing would occur; the result was designated pMV-ΔV. The recombinant V knockout MV-HL was generated from pMV-ΔV. Briefly, 293 cells were placed in a 6-well culture dish, inoculated with a recombinant vaccinia virus (MVA-T7) for 1 h, and then transfected with 1 μg of pMV-ΔV, 1 μg of pKSN1, 1 μg of pKSP, and 0.3 μg of pGEML per well, which expressed N, P, and L proteins, respectively, under the control of a T7 promoter, using FuGENE6. After incubation for 3 days, the cells were co-cultivated with B95a cells at a concentration of 2×10^6 cells per well, and further incubated until extensive cytopathic effects were noted. To confirm the absence of V-expression, total RNA was reverse transcribed with oligo-dT primer, and cDNA of the P gene fragment containing the editing site (antigenome position 2401–3342) was amplified by PCR. No G residue insertion in the editing site was identified by direct sequencing (data not shown).

Preparation and purification of MVs

To eliminate EBV in the stock solution of MV-HL and recombinant V knockout MV, which had been passaged on B95a cells, primary virus solution was passaged twice in 293SLAM cells. The MV solution was inoculated into COBL-a cells cultured in a Cell Factory (Nunc) and propagated. After 3 days, the cells and culture medium were harvested, subjected to three cycles of freeze–thawing, and then centrifuged at 1,500 ×*g* for 10 min. The prepared virus solution was centrifuged in a type19 fixed angle rotor (Beckman Inc.) at 19,000 rpm for 120 min at 4 °C. Pelleted virions were resuspended in 5 ml of RPMI1640.

Preparation of RNA samples for microarray analysis

COBL-a cells and 293SLAM cells (3×10^7) were infected with MV-HL or V knockout MV at an MOI of 2. After 1 h of adsorption at 37 °C, the inocula were replaced with warm spent medium. Alternatively, both cells were treated with 1,000 IU/ml of human universal type I IFN (PBL Biomedical Laboratories). At the predetermined time after addition of the virus or IFN, the medium was removed and the cells were lysed with an ISOGEN reagent. Total RNA was prepared from the lysate in accordance with the manufacturer's instructions. Poly(A)+RNA was prepared from total RNA with a MicroPoly (A)Purist Kit (Ambion), in accordance with the manufacturer's instructions.

Microarray preparation and expression profile acquisition

DNA microarray analysis was performed as previously described (Ito et al., 2003; Kobayashi et al., 2004; Miura et al., 2006; Sakamoto et al., 2005; Yanagisawa et al., 2006). A set of synthetic polynucleotides (80-mers) that represented 22,272 human transcripts mostly originating from the RefSeq clones deposited in the NCBI database was purchased (MicroDiagnostic, Japan) and arrayed on a slide glass (coated slide glass for the microarray, type I; Matsunami, Kishiwada, Japan) with a custom-made arrayer (designated the 22K array) (Ito et al., 2003; Kobayashi et al., 2004). Poly(A)+RNA (2 μg) was labeled with SuperScript II and Cyanine 5-dUTP or Cyanine 3-dUTP (PerkinElmer). A pair of the Cyanine 5- and Cyanine 3-labeled samples was hybridized onto a single microarray. All pairs of target and control samples were labeled and hybridized to microarrays along with color flip of Cyanine 5 and Cyanine 3. Labeling, hybridization, and subsequent washes of microarrays were performed with a Labeling & Hybridization Kit (MicroDiagnostic), in accordance with the manufacturer's instructions. Hybridization signals were measured with a GenePix 4000A scanner (Axon Instruments, CA, USA) and then processed into primary expression ratios ([Cyanine 5-intensity obtained from each target or control sample]/[Cyanine 3-intensity obtained from each control or target sample], which are indicated as 'median of ratios' in GenePix Pro 3.0 software (Axon Instruments)). Normalization was performed for the median of ratios (primary expression ratios) by multiplying normalization factors calculated for each feature on a microarray by the GenePix Pro 3.0 software (designated normalized ratios). After conversion of the normalized ratios into $\log_2$ values (designated log ratios), log ratios derived from pairs of Cyanine 3-labeled target and Cyanine 5-labeled control samples were converted into reciprocals. Eventually, log ratios derived from pairs of Cyanine 5-labeled target and Cyanine 3-labeled control samples and the reciprocal log ratios derived from pairs of Cyanine 3-labeled target and Cyanine 5-labeled control samples were subjected to calculation of mean averages for individual genes (designated mean log ratios). Data processing and subsequent hierarchical clustering analysis were performed with an MDI gene expression analysis software package (MicroDiagnostic). Genes exhibiting the mean log ratio in all data sets were extracted. These genes were further extracted with mean log ratio greater than 1 or lower than −1, and the resulting genes were assembled in the order obtained from the results of hierarchical clustering analysis. All the data in accordance with the MIAME guideline were deposited at DDBJ via CIBEX (http://www.cibex.nig.ac.jp/cibex/HTML/index.html) under accession numbers CBX32.

Western blotting

293SLAM cells and COBL-a cells in 3.5 cm-diameter dish were infected with MV-HL or V knockout MV at an MOI of 2.

After 6 h of infection, cells were washed with PBS, lysed with the SDS sample buffer, and then subjected to a 30-s sonication with a Sonifier 450 (BRANSON). Cell lysate was boiled for 5 min, and then resolved on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were incubated with a 1:1000 dilution of anti-STAT1 rabbit polyclonal antibody (Santa Cruz Biotechnology), a 1:200 dilution of anti-pY-STAT rabbit polyclonal antibody (Santa Cruz Biotechnology), a 1:1000 dilution of anti-N rabbit polyclonal antibody, or a 1:1000 dilution of anti-GAPDH mouse monoclonal antibody (CHEMICON) at 4 °C overnight. The membranes were washed three times with PBS, then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated rabbit anti-mouse, or goat anti-rabbit, IgG (Dako) at room temperature for 1 h. Proteins that bound antibodies were detected by ECL Plus western blotting detection reagents (Amersham).

Luciferase reporter assays

To construct expression plasmids encoding the MV-V gene, the P gene of MV-HL was amplified with specific primer pairs containing the *Eco*RI sequence flanking its 5' terminus and inserted into the *Eco*RI site of the pCAGGS eukaryotic expression vector, and then introduced specific insertion of G residues at the editing site by a PCR-based mutagenesis technique using the QuikChange site-directed mutagenesis kit (Stratagene) (pCAG-V). 293SLAM cells were seeded at 2×10^5 cells per well of a 24-well tissue culture plate and then transiently cotransfected with 0.1 μg of pISRE-Luc, 10 ng of pRL-CMV (Promega) with or without 1.2 μg of pCAG-V using FuGENE6. COBL-a cells were seeded at 4×10^5 cells per well of a 24-well plate and transiently cotransfected with 1 μg of pISRE-Luc, 20 ng of pRL-CMV, and with or without 2.4 μg of pCAG-V using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. After 42 h, cells were infected with mock or MV at an MOI of 2 for 6 h, and then treated with 1,000 IU/ml of human universal type I IFN and incubated for 24 h. Cells were lysed with passive lysis buffer (Promega), and luciferase activity was measured using Dual-luciferase reporter assay system (Promega). Experiments were performed in triplicate.

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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′-*AAGGCCGGCCC*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

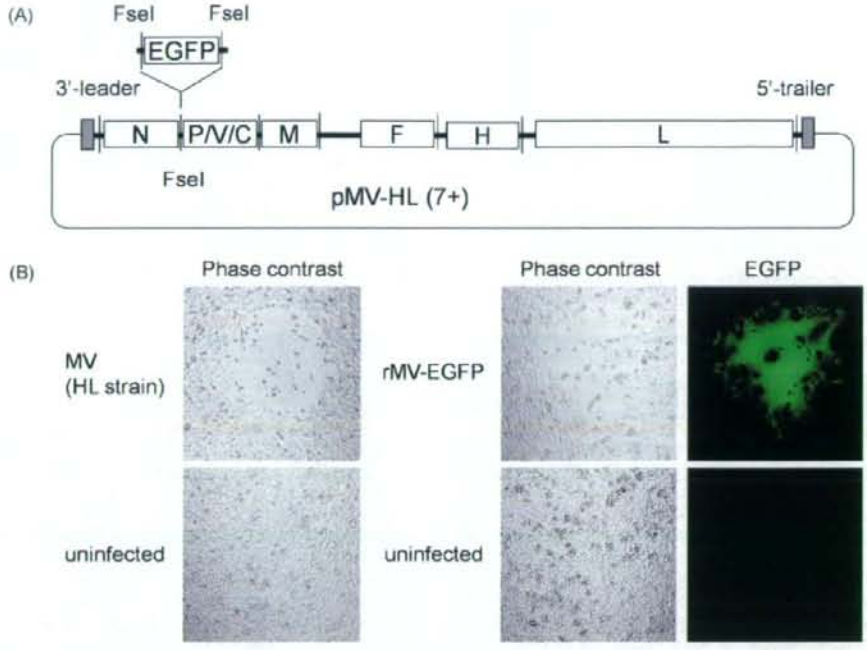

Fig. 1. (A) Construction of rMV-EGFP. The coding regions for MV structural proteins are indicated by opened boxes. The EGFP open-reading frame (ORF) fragment flanked by restriction sites for FseI was introduced into the pMV plasmid. (B) Syncytium formation in B95a cells infected with wild-type MV (HL-strain) (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.

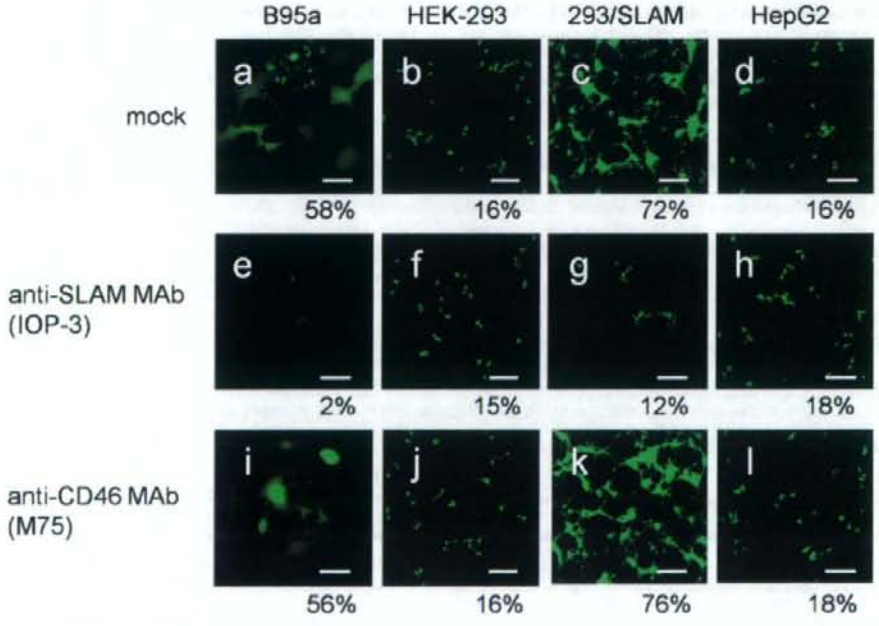

Fig. 2. (A) Effects of the anti-SLAM and anti-CD46 MAbs on rMV-EGFP infection of each cells. B95a (a, e, and i), HEK-293 (b, f, and j), 293/SLAM (c, g, and k) and HepG2 (d, h, and l) cells were pretreated with either an anti-SLAM MAb (IPO-3) (e–h) or an anti-CD46 MAb (M75) (I–l) and were subsequently 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.

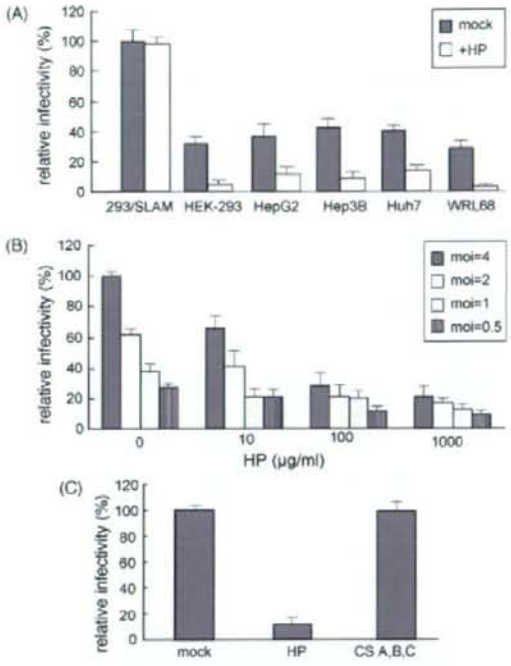

Fig. 3. Inhibition of rMV-EGFP infection by soluble HP. 293/SLAM, HEK-293, and human liver cells (HepG2, Hep3B, Huh7, and WRL68) were infected with rMV-EGFP at an MOI of $2 TCID_{50}$/cell in the presence of soluble HP at a concentration of 100 μg/ml (A). Effect of the concentration of HP and MOI of 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.

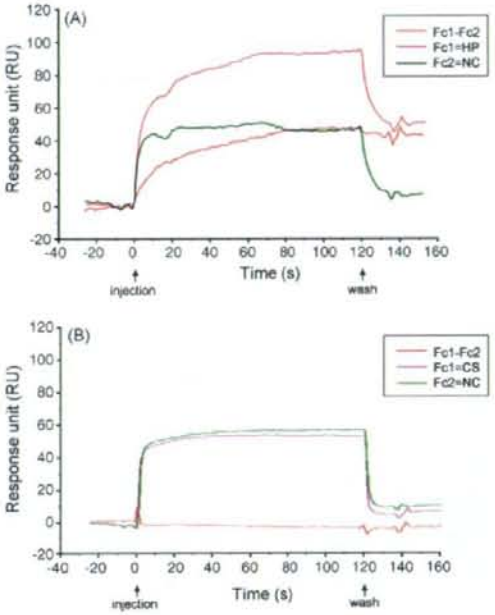

Fig. 4. Interactions between HP and MV analyzed by performing an SPR assay. HP (A) or CS (B) was covalently immobilized onto the SPR analysis. Interaction with the HP or CS were examined in real time by using 10 μg/ml purified MV. Associations among ligands occurred for 120 s following injection of the 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.

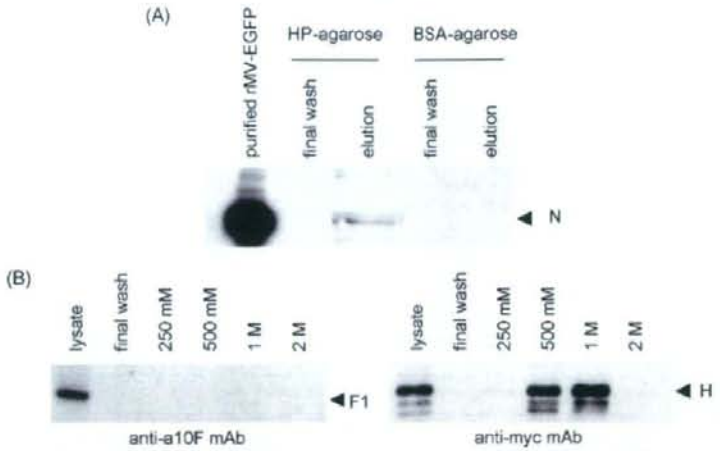

Fig. 5. HP affinity chromatography of virus particles and MV glycoproteins. (A) HP affinity chromatography of rMV-EGFP. Total MV proteins prior to before chromatography (lane 1), the final wash fraction (lane 2) and bound proteins (lane 3) eluted from the HP column, the final wash fraction obtained from 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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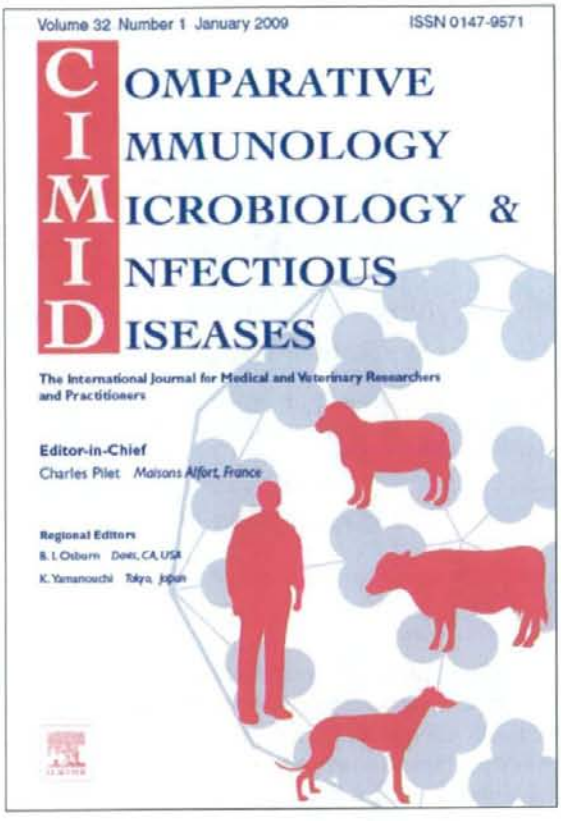

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Inhibition of host protein synthesis in B95a cells infected with the HL strain of measles virus

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Abstract

The shut-off of host protein synthesis in virus-infected cells is one of the important mechanisms for viral replication. In this report, we showed that the HL strain of measles virus (MeV-HL) as well as other field isolates, which were isolated from human blood lymphocytes using B95a cells, induce the shut-off in B95a cells. Since the Edmonston strain of MeV failed to induce the shut-off in B95a cells, the ability to induce the shut-off was considered to be dependent on virus strains. Although, the modification of eukaryotic translation initiation factors (eIF) including eIF4G, eIF4E, and 4E-BP1 was reported for shut-off by various viruses, the involvement of these eIFs was not observed in MeV-HL-infected B95a cells. Instead, the accumulation of phosphorylated eIF2α was found to coincide to the decrease of host protein synthesis, suggesting the involvement of phosphorylation of eIF2α in inhibition of translation as one of the mechanisms of the shut-off.

Keywords: Measles virus; Shut-off; eIF2α

Résumé

La suppression de la synthèse protéique de la cellule hôte au cours de l'infection est un des mécanismes majeurs de la réplication virale. Dans cette étude nous avons montré que la souche HL du virus de la rougeole (MeV-HL) ainsi que d'autres souches sauvages du virus, isolées dans

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des cellules B95a à partir de lymphocytes sanguins humains, induisent ce type de suppression dans les cellules B95a. Comme la souche Edmonston du virus de la rougeole est incapable d'induire cette suppression dans les cellules B95a, cette propriété a été considérée comme dépendante de la souche virale. Bien qu'il ait été observé une extinction de l'expression des facteurs d'initiation de la traduction eucaryote (eIF) dont eIF4G, eIF4E et 4E-BP1 par de nombreux virus, il n'a pas été vu d'implication de ces facteurs dans les cellules B95a infectées par MeV-HL. Par contre, dans ces cellules on a montré que l'accumulation de la forme phosphorylée de eIF2a est concomitante à la diminution de la synthèse protéique, suggérant l'inhibition de la traduction par phosphorylation de eIF2a dans pourrait être un des mécanismes de la suppression de la synthèse protéique.

Mots clés : virus de la rougeole ; eIF2α ; suppression de la synthèse protéique

1. Introduction

One of the most striking changes observed in the cells infected with certain viruses is the almost complete inhibition of the translation of host mRNAs in the presence of effective translation of viral mRNAs [1]. Such inhibition of host protein synthesis (shut-off) have been reported in the infection with picornaviruses, adenovirus, influenza virus and vesicular stomatitis virus (VSV) and considered to occur at the stage of host translational level, as cellular mRNAs are recovered as an intact and functionally active form from the virus-infected cells [2–5]. Modification of eukaryotic initiation factors (eIFs) including the subunit of eIF4F complex (e.g., eIF4G and eIF4E) is observed in these virus-infected cells [6], and the modification of eIF4F by the viral infection resulting in the inhibition of cap-dependent translation is proposed as one of the mechanisms for shut-off.

Measles virus (MeV) belongs to the genus *Morbillivirus* within the family Paramyxoviridae, the genome of which is a single-stranded RNA with negative polarity. The MeV mRNA has a cap structure at 5′ end of the mRNA and is thought to be translated in a cap-dependent manner [7]. The Edmonston strain of MeV (MeV-Ed) has been reported not to induce the host shut-off of host protein synthesis [8,9]. Although most information on MeV–cell interaction has been obtained from the studies on MeV-Ed in epithelial or epithelial-like cells such as CV-1, Hela and Vero cells, accumulating evidence obtained by the use of lymphoblastoid B95a cells has suggested that MeV circulating in human is heterogenous and MeV-Ed represent minor subpopulation of the virus selected during long passage in cell cultures [10].

In the present study, we examined the effect of the HL strain of MeV (MeV-HL), which was isolated using B95a cells from a measles patient and maintains virulence in monkeys [10], on the host protein synthesis in B95a cells and found that MeV-HL induces marked shut-off of host protein synthesis. As the mRNA level of host proteins was not altered in MeV-HL-infected B95a cells, we focused on possible modification of translation factors involved in the cap-dependent translation initiation to clarify the mechanisms involved in the induction of the shut-off of host protein synthesis by MeV-HL infection.

2. Materials and methods

2.1. Cell and viruses

B95a cells [11] were grown in RPMI1640 supplemented with 5% fetal calf serum (FCS). As a typical virus isolated from measles patient, MeV-HL and two other field isolates, 9106 and 9301 strains [12] were used. MeV-Ed that was passed twice in B95a cells was also used. Virus infectivity titers were determined in B95a cells and expressed as a 50% tissue culture infectious dose ($TCID_{50}$). For examining shut-off of host protein synthesis, a monolayer culture of B95a cells was infected with MeV with a multiplicity of infection (MOI) of 1 $TCID_{50}$.

2.2. Metabolic labeling of cells

B95a cells were mock-infected or infected with MeV and then labeled with [^{35}S] EXPRESS (PerkinElmer, MA, USA) in methionine- and cystein-free RPMI1640 with 2% FCS for 1 h at 0, 11, 17, 23 and 35 h post-infection (hpi). Cells were lysed in lysis buffer C (125 mM NaCl, 20 mM Tris–HCl pH 8.0, 0.5% NP-40). Cell lysates were centrifuged and the supernatants were collected. Proteins were electrophoresed in SDS-PAGE gels, and ^{35}S-labeled proteins were visualized with autoradiography using X-ray film or quantitated with a phosphoimager plate in BAS 2000 (Fujifilm, Tokyo, Japan).

2.3. Real-time RT-PCR

Real-time RT-PCR was used to determine the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and 18S rRNA in accordance with the method described by Sato et al. [13].

2.4. Antibodies

Rabbit polyclonal antibodies against eIF4G and eIF2α, goat polyclonal antibodies against phospho-eIF4E, 4E-BP1 and β-actin and mouse monoclonal antibody against eIF4E were purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Rabbit polyclonal antibody against phospho-eIF2α was purchased from Cell Signaling Technology (MA, USA). Horseradish peroxidase-conjugated secondary antibodies against rabbit, goat or mouse immunoglobulin were purchased from DAKO (Glostrup, Denmark).

2.5. Western blotting assay

Mock- or MeV-HL-infected B95a cells were lysed with lysis buffer C containing 1 mM PMSF, 1 mM benzamidine, 1 μg/ml aprotinine, 100 μM NaF and 1 μM Na_3VO_4. Equal amounts of protein extracts were subjected to SDS-PAGE and the proteins were transferred onto Hybond-N(+) nitrocellulose membrane (GE Healthcare UK Ltd., Buckinghamshire, UK). Detection of 4E-BP1 was performed as described previously [14]. Western blotting assay was performed with each antibody according to the recommendations of

manufacturer. Bands were visualized using an ECL plus detection reagent (GE Healthcare UK Ltd.). The intensity of bands was quantitated by scanning with LAS-1000 mini and Imagegauge software (Fujifilm).

2.6. Establishment of cell lines stably expressing eIF2α

Human eIF2α cDNA was obtained with PCR using the sense primer (5′-GCGGGAATCACACACATACCTCAGAA-3′) and antisense primer (5′-TCAAGTCTAG-GATTTACAGCCAGGAAGCGC-3′) with reverse transcription (RT) products from the mRNA of HeLa cells and was then subcloned into pCR2.1-TOPO vector (Invitrogen, CA, USA). Phosphorylation site at serine 51 of eIF2α cDNA was mutated to alanine (S51A) using a PCR-based mutagenesis strategy with Pfu turbo polymerase (Stratagene, CA, USA) to obtain the cDNA of S51A mutant eIF2α. The eIF2α cDNA was inserted into a pCMV-myc expressing vector (Clontech, CA, USA). The myc-tagged eIF2α (wild type or S51A mutant) expression vector (1.5 μg) and 0.5 μg of pCDNA3.1 (Invitrogen) were co-transfected to B95a cells with DMRIE-C reagent (Invitrogen) according to the recommendations of manufacturer. After incubation for 24 h, the cells were replated to 150-mm dishes and cultured in RPMI1640 with 5% FCS and 500 μg/ml G418 (bioactive; Invitrogen). G418-resistant colonies were selected approximately 2 weeks later. Expression of myc-tagged proteins was confirmed by Western blotting assay using a monoclonal antibody against myc tag (Clontech).

3. Results

3.1. Effect of several strains of MeV on host protein synthesis in B95a cells

Effect of MeV-HL-infection on protein synthesis was shown in Fig. 1a. The rate of host protein synthesis was determined by quantitation of the total radioactivity of one lane except four bands of N, P, M and H derived from MeV-HL. The ratio of host protein synthesis in MeV-HL-infected B95a cells to that in mock-infected cells was shown in Fig. 1b. The relative rate of viral protein synthesis to host protein synthesis, which was determined by sum of the radioactivity of four viral proteins and that of host proteins, was also shown in Fig. 1b. A marked decrease of host protein synthesis was observed between 18 and 36 hpi. On the other hand, relative viral protein synthesis to host protein synthesis increased and reached a peak at 24 hpi. This result indicates that MeV-HL induces shut-off of host protein synthesis in B95a cells.

Subsequently, other field isolates of MeV, 9106 strain and 9301 strain were also examined for their ability to induce the shut-off (Fig. 2a right). The autoradiograph gel was stained with Coomassie brilliant blue before drying the gel to confirm that total protein levels are equal (Fig. 2a left). As shown in Fig. 2a, 9106 and 9301 strains also induced the inhibition of host protein synthesis at 24 hpi similar to MeV-HL. The effect of the MeV-Ed, which was reported not to induce shut-off in CV-1 or Hela cells, was tested in B95a cells. Host protein synthesis in MeV-Ed-infected B95a cells was not inhibited until 24 hpi when the inhibition was clearly observed in MeV-HL-infected cells (Fig. 2b). These results

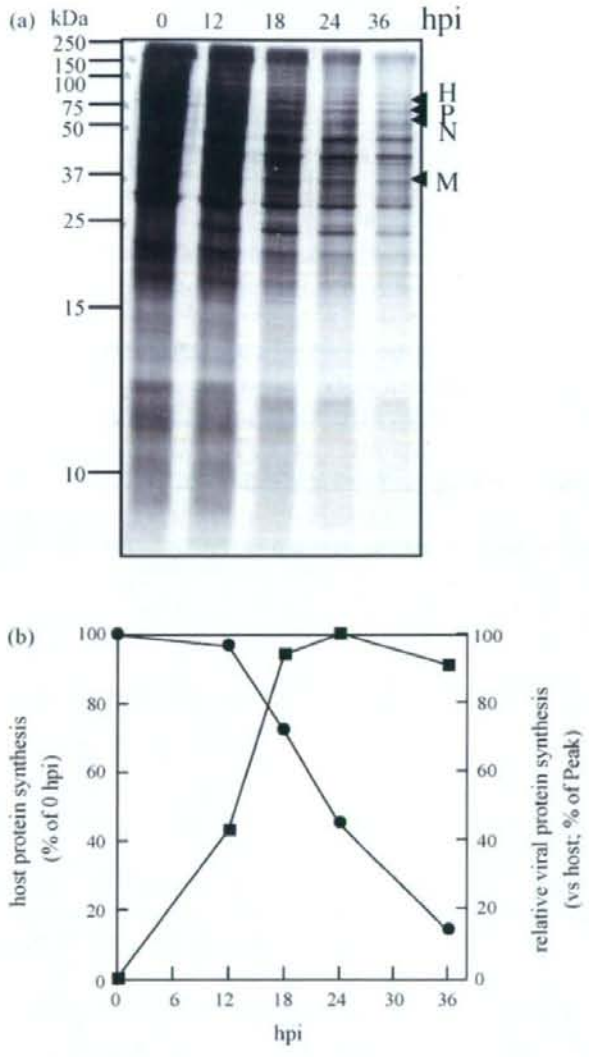

Fig. 1. Protein synthesis in B95a cells infected with MeV-HL. (a) B95a cells infected with MeV-HL were labeled with a mixture of [^{35}S] methionine/cysteine for 1 h and collected at the indicated time. Labeled proteins were separated by 12% SDS-PAGE gel. The proteins derived from MeV-HL are indicated to the right of the image. (b) Quantitation of host protein synthesis (closed circle) and relative viral protein synthesis to host protein synthesis (closed square). The rates of protein synthesis were determined from images as described in the text.

indicate that field isolated MeVs, which maintain their virulence, have an ability to induce shut-off of host protein synthesis, whereas only the MeV-Ed does not.

3.2. *Effect of MeV-HL infection on GAPDH mRNA*

The expression level of the GAPDH gene, one of house keeping genes, was measured as a representative to determine whether MeV-HL-induced inhibition of host protein synthesis occurs at the transcription stage (Fig. 3). The relative expression level of GAPDH

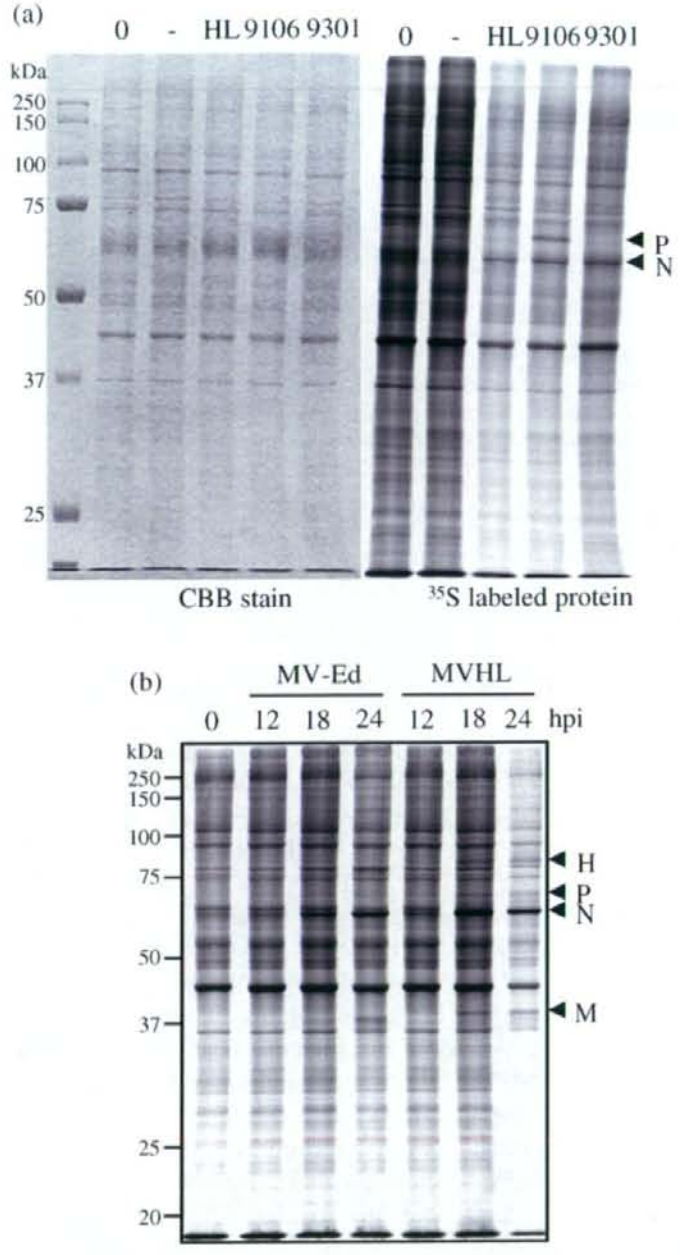

Fig. 2. The shut-off effect of other strain of MeV (a) Protein synthesis at 24 hpi in B95a cells infected with field isolates of MeV was examined similar to Fig. 1a (right panel) (0: 0 hpi; - :mock-infected B95a cells). To confirm the equivalence of protein, the autoradiograph gel was stained with Coomassie brilliant blue (left panel). (b) The protein synthesis in MeV-Ed- or MeV-HL-infected B95a cells was examined similar to Fig. 1a.

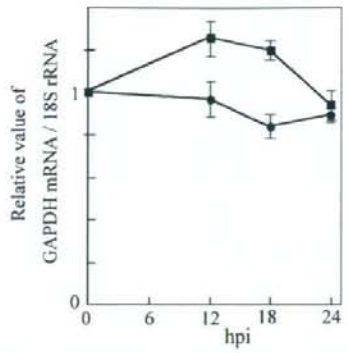

Fig. 3. The mRNA levels of GAPDH in B95a cells infected with MeV-HL. The expression levels of GAPDH mRNA in mock- (closed circle) or MeV-HL- (closed square) infected B95a cells were determined using one-step real-time RT-PCR and shown as means of three experiments.

mRNA to 18S rRNA was not suppressed by MeV-HL infection. Since the transcription level of GAPDH did not decrease during the period of shut-off, the inhibition of host protein synthesis in MeV-HL-infected B95a cells is suggested to occur at a post-transcription stage.

3.3. Modification of eIF4G, eIF4E and 4E-BP1 by MeV-HL infection

Previous reports on other viruses indicated that cap-binding proteins such as eIF4G, eIF4E, and 4E-BP1 are major targets for the virus-induced shut-off of host protein synthesis [6]. Picornavirus (except for cardiovirus) cleaved the eIF4G, which is one of the subunits of cap-binding complex eIF4F, by viral protease, $2A^{pro}$ [15–17]. This cleavage results in inhibition of binding of eIF4F to cap of host mRNA. In adenovirus- or influenza virus-infected cells dephosphorylation of eIF4E, which is a cap-binding protein, is observed [18–20]. Phosphorylation of eIF4E increases its affinity for the cap of mRNA [21]. Therefore, dephosphorylation of eIF4E by viral infection results in decrease of the affinity for the cap and may inhibit cap-dependent translation. The eIF4E is also regulated by eIF4E-binding protein-1 (4E-BP1). Encephalomyocarditis virus (EMCV), poliovirus and VSV dephosphorylate 4E-BP1 [22,23]. Dephosphorylated 4E-BP1 binds to eIF4E strongly, resulting in the suppression of cap-dependent translation. Considering these functions of cap-binding proteins, we first examined the characteristics of these three proteins in B95a cells at intervals after inoculation with MeV-HL. As shown in Fig. 4a, eIF4G was not cleaved throughout the course of MeV-HL-infection. Moreover, dephosphorylation was not observed for eIF4E and 4E-BP1 until 36 hpi (Fig. 4b and c). These results indicate that eIF4G and eIF4E are not involved in MeV-HL-induced shut-off of host protein synthesis as their function appear to be intact.

3.4. Accumulation of phosphorylated eIF2α in MeV-HL-infected B95a cells

Given that the modification of eIF4F was not detected in MeV-HL-infected B95a cells, we then focused on phosphorylation of eIF2α. It was reported that the interferon-inducible

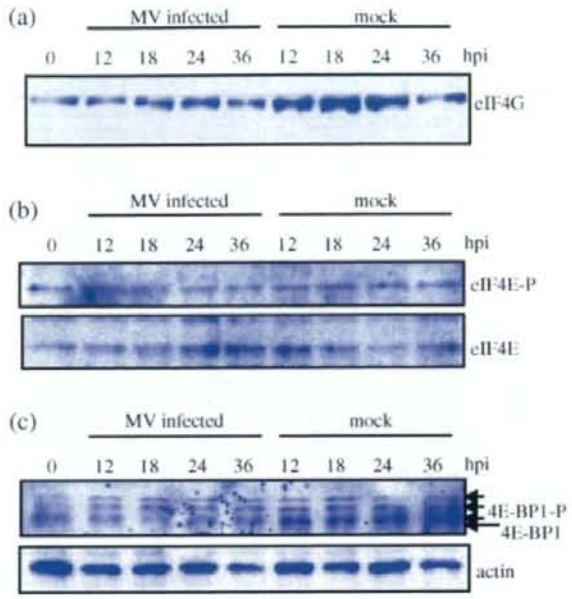

Fig. 4. Modification of the components of the eIF4F complex in MeV-HL-infected B95a cells. Expression levels of eIF4G and the phosphorylated states of eIF4E and 4E-BP1 in mock- or MeV-HL-infected B95a cells were determined by Western blotting assay. (a) Cell lysates were subjected to 6% SDS-PAGE and the proteins were transferred onto nitrocellulose membrane. The eIF4G was detected by Western blotting assay using rabbit antibody against eIF4G. (b) Detection of phosphorylated eIF4E by Western blotting assay using antibodies against phospho-eIF4E at serine 209 (upper panel) or eIF4E (lower panel) antibody. (c) The phosphorylation state of 4E-BP1 in the mock- or MeV-HL-infected B95a cells was examined by Western blotting assay using goat antibody against 4E-BP1. The quantity of protein was normalized to that of β-actin determined by goat antibody against β-actin.

PKR, known as a kinase that phosphorylate eIF2α at serine 51 [24], is activated by dsRNA during the infection with RNA viruses and involved as a host defense in preventing the translation of viral transcripts, concomitantly with the inhibition of host mRNA translation [25]. Considering such function of eIF2α, we analyzed the phosphorylation state of eIF2α by Western blotting assay with an antibody against phospho-eIF2α or eIF2α (Fig. 5). The ratio of phospho-eIF2α in MeV-HL-infected B95a cells increased after 12 hpi and reached a maximum (3.9-fold increase) at 18 hpi, although the effect was lower than that observed in the control with thapsigargin that induces eIF2α phosphorylation through ER-stress [26]. Thereafter, the ratio was sustained until 36 hpi. Phosphorylation of eIF2α occurred at a relatively early stage of infection, prior to the clear inhibition of host protein synthesis. The acceleration of host shut-off was accompanied by an increase in phosphorylation of eIF2α.

3.5. Suppression of MeV-HL-induced phosphorylation of eIF2α in B95a cells stably expressing S51A mutated human eIF2α

Involvement of phosphorylation of eIF2α in shut-off of host protein synthesis in MeV-HL-infected B95a cells was examined using B95a cells that stably express

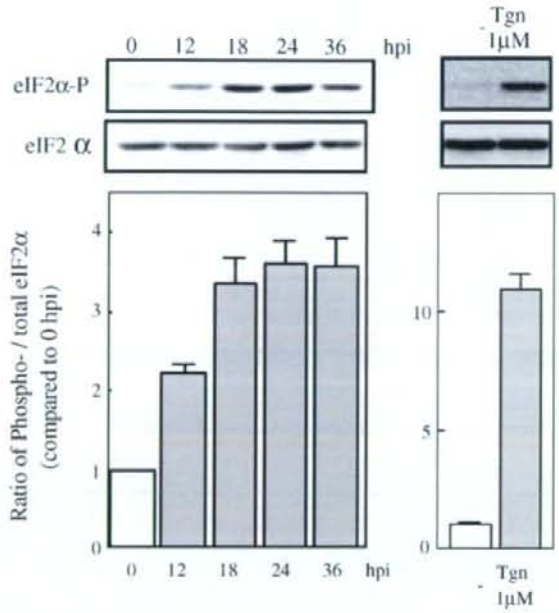

Fig. 5. Phosphorylation of eIF2α in MeV-HL-infected B95a cells. Lysates of mock- or MeV-HL-infected B95a cells were analyzed by Western blotting assay using antibodies against eIF2α-P (phosphorylated form at serine 51) or eIF2α on the same membrane (left, top). Quantitation of the relative amounts of phospho-eIF2α and the total eIF2α was measured and the ratio of phosphorylated eIF2α to total eIF2α (vs. 0 hpi) is shown as a bar graph (left, bottom). As a control experiment, B95a cells were treated with 1 μM thapsigargin for 1 h and shown as the same way as left column (right). The values are means ± standard errors of triplicate determinations.

eIF2α mutant, of which phosphorylation site serine 51, was replaced to alanine (B95a-2αS51A) and is able to inhibit the phosphorylation of endogenous eIF2α [27]. As a control experiment, the B95a cells that stably express wild type of eIF2α (B95a-2αWT) were used. The phosphorylation rate of total eIF2α in B95a-2αWT cells apparently increased at 18 hpi (Fig. 6a), whereas that in B95a-2αS51A cells was significantly inhibited. Shut-off of host protein synthesis was noted from 12 hpi in B95a-2αWT cells similar to the parental B95a cells. In B95a-2αS51A cells, shut-off of host protein synthesis was suppressed until 18 hpi (Fig. 6b and c) and the rate of host protein synthesis was higher than that of B95a-2αWT cells throughout the test period. These results indicate that the phosphorylation of eIF2α involved in shut-off of host protein synthesis in MeV-HL-infected B95a cells.

4. Discussion

In the present study, we showed that MeV-HL induces the shut-off of host protein synthesis in B95a cells. This shut-off is not specific feature of MeV-HL because other field isolates, 9106 and 9301 strain, also induce the shut-off in B95a cells. On the other hand, MeV-Ed that has been reported not to induce the shut-off in epithelial or epithelial-like cells did not induce the shut-off of host protein synthesis in B95a cells as well. Therefore, the inability of MeV-Ed to induce shut-off is suggested to be a characteristic of this strain