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Host range and receptor utilization of canine distemper virus analyzed by recombinant viruses: Involvement of heparin-like molecule in CDV infection

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Abstract

We constructed recombinant viruses expressing enhanced green fluorescent protein (EGFP) or firefly luciferase from cDNA clones of the canine distemper virus (CDV) (a Japanese field isolate, Yanaka strain). Using these viruses, we examined susceptibilities of different cell lines to CDV infection. The results revealed that the recombinant CDVs can infect a broad range of cell lines. Infectivity inhibition assay using a monoclonal antibody specific to the human SLAM molecule indicated that the infection of B95a cells with these recombinant CDVs is mainly mediated by SLAM but the infection of 293 cell lines with CDV is not, implying the presence of one or more alternative receptors for CDV in non-lymphoid tissue. Infection of 293 cells with the recombinant CDV was inhibited by soluble heparin, and the recombinant virus bound to immobilized heparin. Both F and H proteins of CDV could bind to immobilized heparin. These results suggest that heparin-like molecules are involved in CDV infection.
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Keywords: Canine distemper virus; Host range; Receptor; Heparin; Glycosaminoglycan

Introduction

Canine distemper virus (CDV) possesses a non-segmented single stranded RNA genome with negative polarity and belongs to the genus *Morbillivirus* within the family *Paramyxoviridae*. CDV infection induces such symptoms as fever, diarrhea, immunosuppression and encephalitis in dogs and other canids along with high mortality rates. For many decades, animals susceptible to CDV infection had been thought to be limited to those in the family *Canidae*. However, CDV infections in seals, lions, tigers and leopards were recently reported (Appel et al., 1994; Grachev et al., 1989; Roelke-Parker et al., 1996). Moreover, CDV infection is prevalent in domestic dogs which have already been vaccinated (Blixenkrone-Moller et al., 1993; Gemma et al., 1996; Kai et al., 1993; Maes et al., 2003). Since recent isolates were serologically distinct from the vaccine strain (Gemma et al., 1996), it is

suspected that vaccination with the current vaccine strain can no longer completely protect dogs from CDV infection.

It has been reported that SLAM (CD150) is a cellular receptor for morbilliviruses including CDV (Tatsuo et al., 2000; Tatsuo et al., 2001). However, most of the reverse genetics systems for morbilliviruses are based on their vaccine strains which have been adapted to Vero cells (Baron and Barrett, 1997; Gassen et al., 2000; Parks et al., 2002; von Messling et al., 2001). SLAM molecules are expressed only on the cells of the immune system (immature thymocytes, activated lymphocytes, activated monocytes and mature dendritic cells), and not on Vero cells (Seki et al., 2003). It is not easy to isolate wild-type CDVs using Vero cells (Seki et al., 2003). Furthermore, the Vero-adapted CDV isolates have exhibited biased hypermutations and have been attenuated (Nielsen et al., 2003). Recently, we have succeeded in recovering an infectious CDV from a cDNA clone of a field isolate (Yanaka strain) (Kai et al., 2000). The Yanaka strain of CDV, which was obtained from a dog clinically diagnosed with distemper, was isolated and maintained in B95a cells (Gemma et al., 1996) which are derived

from marmoset B cells (Kobune et al., 1990) and express SLAM molecules on their surface. Although several reverse genetics systems have been reported for CDV Onderstepoort strain (Gassen et al., 2000; Parks et al., 2002; von Messling et al., 2001) and Vero-adapted A75/17 strain (Plattet et al., 2004), these viruses were maintained in Vero cells and may be inappropriate to be used for the investigation of CDV host range.

Glycosaminoglycans (GAGs) are linear (unbranched) heteropolysaccharides consisting of repeated disaccharide units that are variably N- and O-sulfated (Jackson et al., 1991). GAGs are covalently attached to the protein cores of proteoglycans, which are ubiquitously expressed as integral membrane proteins, glycerol phosphatidyl inositol-linked membrane proteins and proteins of the extracellular matrix. In vivo, GAGs bind to a diverse group of growth factors, chemokines, enzymes, and matrix components (Jackson et al., 1991). In certain viruses, including human herpes simplex virus (WuDunn and Spear, 1989), human immunodeficiency virus type 1 (Patel et al., 1993), vaccinia virus (Chung et al., 1998), adenovirus types 5 and 2 (Dechecchi et al., 2000), hepatitis C virus (Barth et al., 2003), respiratory syncytial virus (Krusat and Streckert, 1997) and human parainfluenza type 3 (Bose and Banerjee, 2002), heparan sulfate is involved in the process of viral entry. The latter two viruses are the members of the family *Paramyxoviridae* to which CDV belongs.

In the present study, we report the construction of recombinant CDVs harboring an enhanced green fluorescent protein gene or a firefly luciferase gene. Using these recombinant CDVs, we investigated the susceptibilities of various cells to infection with the recombinant CDV infection and examined cellular surface molecules implicated in CDV infection.

Results

Recovery of recombinant CDVs expressing EGFP or firefly luciferase

The plasmid pCDV containing the full-length cDNA of CDV was manipulated to contain the unique restriction enzyme sites in the non-coding regions between adjacent genes of the genome. The FseI site, which was inserted immediately downstream of the CDV N gene, was chosen to introduce the additional genes. The coding regions of EGFP and luciferase genes were attached to transcription signal (transcription termination/polyadenylation signal of N gene and transcription start signal of H gene) units and cloned into the FseI site of pCDV. The resulting plasmids, pCDV-EGFP and pCDV-Luc, were co-transfected into 293 cells preinfected with the recombinant vaccinia virus MVA-T7 expressing phage T7 RNA polymerase, together with supporting plasmids which supply the N, P and L proteins of rinderpest virus. The reason why we used a set of supporting plasmids of rinderpest virus is that our supporting plasmids of CDV did not work in our hands. The cells were incubated for 3 days followed by co-cultivation with B95a cells. After incubation of several days, syncytia

induced by the recombinant CDVs were observed. The sizes of syncytia produced by three recombinant viruses were similar (Fig. 1C). The expression of EGFP in rCDV-EGFP-infected cells was verified under the confocal microscopy (Fig. 1C), and that of luciferase in rCDV-Luc-infected cells was confirmed by the measurement of luciferase activity in cell lysates (data not shown; see also Table 1). Growth of the rCDV-EGFP and rCDV-Luc was slightly slower than that of the rCDV, however, the maximum titers were similar to that of the rCDV (data not shown).

### Host range of CDV characterized using recombinant viruses

Using rCDV-EGFP, susceptibilities of several primary tissue cultures and cell lines to CDV infection were analyzed. Cells of canine origin such as primary-cultured dog embryo brain cells, stimulated PBMC, 3132 cells and MDCK cells, and cells derived from other species, namely, CRFK cells (cat), stimulated phocine PBMC, stimulated lion PBMC, 293 cells (human), HeLa cells (human), Vero cells (African green monkey), COS-7 cells (African green monkey), NIH-3T3 cells (mouse), CPK cells (pig), MDBK cells (cattle), RK13 cells (rabbit) and BHK-21 cells (baby hamster), were infected with rCDV-EGFP at an MOI of 2 $TCID_{50}$/cell (determined in B95a cells). Forty hours after infection, the cells were harvested and the expression of EGFP was analyzed by flow cytometry (the results are summarized in Table 1). All of the tested cells derived from dog or non-dog species were shown to be susceptible to infection by the recombinant CDV, although the susceptibilities of individual cell lines were variable. Unexpectedly, NIH-3T3, a mouse fibroblast cell line, was found to be susceptible to rCDV-EGFP infection, while it has been reported that the Onderstepoort strain, a vaccine strain of CDV, cannot infect these cells (Loffler et al., 1997). The infectivities of rCDV-EGFP for cell lines were generally higher than those for primary cells except BHK-21 cells.

We also infected these cells with rCDV-Luc at the same MOI, and luciferase activities in these cells were measured (Table 1). Consistent with the results of rCDV-EGFP infection, all of the cell lines infected with rCDV-Luc showed luciferase activities. Relative luciferase activities per infected cell were calculated for different cell types using data obtained from rCDV-EGFP and rCDV-Luc infections, revealing that the relative activities varied among these cells ranging from 3.42 RLU/cell (HeLa cells) to 145 RLU/cell (BHK-21 cells).

Inhibition of rCDV-EGFP infection by monoclonal antibodies

Field isolates of CDV use SLAM (also known as CD150) expressed on B95a cells as their receptor, as measles virus does (Tatsuo et al., 2001). To investigate whether the Yanaka strain of CDV also uses SLAM as its receptor, B95a cells were incubated with a monoclonal antibody (clone IPO-3) specific against human SLAM prior to infection with rCDV-EGFP. In the absence of the antibodies, approximately 90% of B95a cells were EGFP-positive (Fig. 2A; panel a). The presence of an anti-SLAM antibody significantly inhibited the infection of B95a
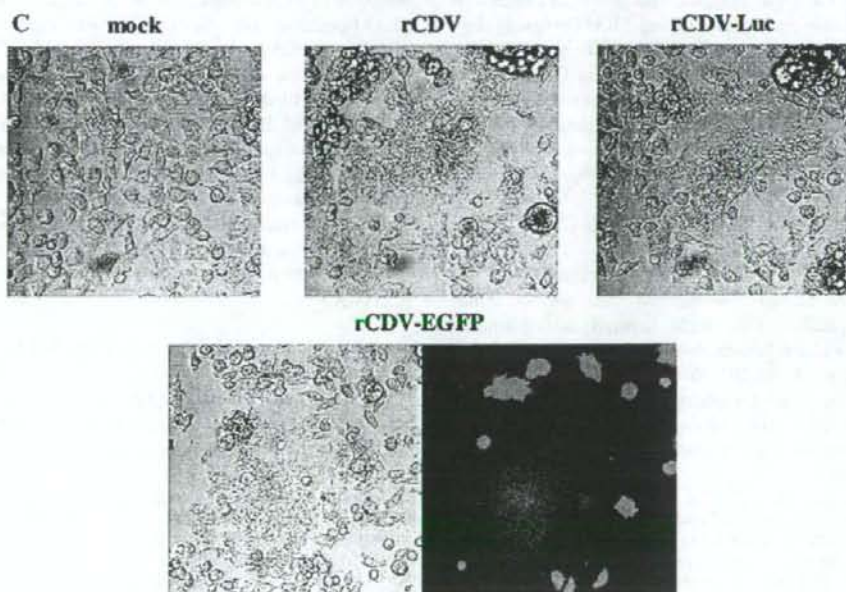
A

B

C

mock   rCDV   rCDV-Luc
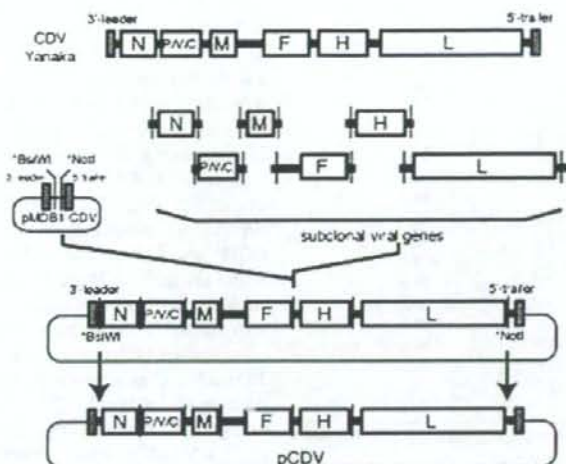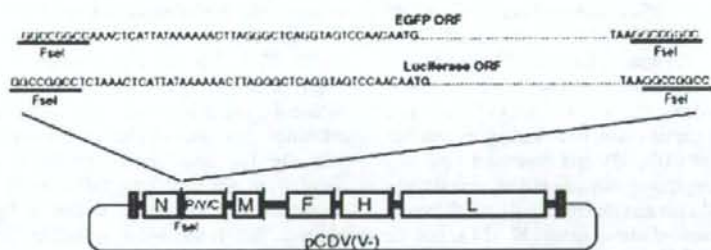
rCDV-EGFP

Table 1
Comparison of rCDV-EGFP infectivities and luciferase activities in infected cells with rCDV-Luc

	% infectivity	Luciferase activity ($RLU/10^4$ cells)	Relative activity (RLU/infected cell)
HeLa	38.0	1.3×10^6	3.4
293	40.0	1.9×10^7	47.5
Vero	8.4	1.3×10^6	15.5
MDCK	20.3	2.2×10^6	10.8
CRFK	50.5	7.7×10^6	15.2
RK-13	22.6	3.4×10^6	15.0
BHK-21	2.0	2.9×10^6	145.0
MDBK	21.4	2.6×10^6	12.1
CPK	15.3	1.9×10^6	12.4
NIH-3T3	17.5	7.2×10^5	4.1
COS-7	32.8	4.9×10^6	14.9
B95a	92.6	1.9×10^7	20.5
3132	32.2	2.0×10^7	62.1
DEB	2.5	2.0×10^6	80.0
Dog PBMC	5.0	4.5×10^6	90.0
Phocine PBMC	4.4	2.6×10^6	59.1
Lion PBMC	1.8	5.3×10^5	29.4

Cells were infected with rCDV-EGFP or rCDV-Luc at an MOI of 2 $TCID_{50}$/cell (determined in B95a cells) and analyzed at 40 h.p.i.

cells (approximately 26% of B95a cells were infected) (Fig. 2A; panel c), whereas the infection was not inhibited by a monoclonal antibody (M177) against human CD46 (a receptor for the Edmonston strain of measles virus (MV) (Fig. 2A; panel b)). Thus, it was revealed that the Yanaka strain of CDV also uses the SLAM molecule expressed on B95a cells as its receptor like other field isolates of CDV, MV and rinderpest virus. As SLAM is believed to be expressed only on cells of limited types *in vivo*, such as lymphoid cells and dendritic cells, the infection of other cell types as described above means SLAM is not the only receptor for CDV. Actually, the infection of 293 cells with rCDV-EGFP was blocked by neither anti-SLAM antibody (Fig. 2B; panel c) nor anti-CD46 antibody (Fig. 2B; panel b). The negative inhibition of our field isolate strain of CDV by anti-CD46 antibody is consistent with the results for a vaccine strain (Loffler et al., 1997). Thus, it is suspected that the entry of CDV into 293 cells and other non-lymphoid tissues is mediated by one or more unknown surface molecules.

Inhibition of rCDV-EGFP infection by soluble GAGs

CDV can infect a large variety of cells regardless of the expression of the SLAM molecules on their surface. Since infections of cultured cells with heparan sulfate-binding viruses are blocked by heparin, we investigated the effect of heparin treatment on CDV infection. rCDV-EGFP was incubated with heparin at a concentration of 1 µg/ml prior to the inoculation to B95a cells or 293 cells, and then the EGFP-positive cells were analyzed by flow cytometry.

Treatment of rCDV-EGFP with heparin had little effect on the infection of B95a cells (Fig. 2A; panel d), although a slight decrease in infectivity (from 88% (mock-treated) to 81% (heparin-treated)) was apparent (Fig. 2A; panels a and d). When SLAM-dependent infection was inhibited by the anti-SLAM antibody, the inhibitory effect of heparin was more pronounced (26% for anti-SLAM antibody treatment vs. 15% for anti-SLAM antibody plus heparin treatment) (Fig. 2A; panels c and e). On the other hand, infection of 293 cells with rCDV-EGFP was dramatically inhibited in the presence of heparin (Fig. 2B; panel d). These results suggest that heparin-like molecules are involved in infection of rCDV-EGFP via SLAM-independent pathway. The major GAGs found on most cells are heparan sulfate and the chondroitin sulfates (Kjellen and Lindahl, 1991). Thus, rCDV-EGFP was also treated with heparan sulfate, chondroitin sulfate A, B (also known as dermatan sulfate) or C prior to inoculation to 293 cells. Consistent with the results shown in Fig. 2A, treatment of rCDV-EGFP with heparin inhibited the infection of 293 cells in a dose-dependent manner (Fig. 3A). Treatment of rCDV-EGFP with heparan sulfate also inhibited the infection of 293 cells (Fig. 3C), however, the effect was smaller than that of heparin. Chondroitin sulfate A and C did not significantly inhibit the infection of 293 cells with rCDV-EGFP (Figs. 3D and F), although a slight inhibition was observed at a high concentration. In the case of chondroitin sulfate B, infectivity was reduced by the treatment and the inhibition rate was similar to that by heparan sulfate (Fig. 3E). On the other hand, treatment of rCDV-EGFP with heparin did not inhibit the infection of B95a cells (Fig. 3B) consistent with the results shown in Fig. 2A. These results indicate that heparin-like molecules are involved in CDV infection of SLAM-negative cells to more extent than that of SLAM-positive cells. Since rCDV-EGFP is a recombinant virus from cDNA, it still remains possible that the results obtained above do not reflect the native characteristics of CDV. To confirm the involvement of heparin-like molecules in the entry of the parental CDV strain, the Yanaka strain of CDV was treated with heparin before inoculation to 293 cells and the infectivity was determined by the use of a monoclonal antibody specific to the H protein of CDV. The results were consistent with the results obtained with rCDV-EGFP (data not shown). Therefore, those characteristics mentioned above were not acquired through a recovery of rCDV-EGFP.

Heparin-binding ability of rCDV-EGFP and its glycoproteins

To investigate whether CDV particles directly bind to heparin-like molecules, concentrated supernatant of rCDV-EGFP-infected B95a cells was subjected to heparin affinity

Fig. 1. (A) Strategy for construction of the full-genomic cDNA clone of CDV. (B) Construction of recombinant rCDV-EGFP and rCDV-Luc. Coding regions for viral structural proteins of CDV are shown as filled boxes. Additional sequences of T7 promoter, terminator and ribozyme are shown as open boxes. Artificial fragments which contain CDV GE signal, GS signal and EGFP or Luc ORF flanked by *Fse*I restriction enzyme sites (underlined) were introduced into pCDV (V⁻) plasmid. (C) Syncytia formation in B95a cells infected with the rCDV, rCDV-Luc or rCDV-EGFP. rCDV-EGFP-infected B95a cells were also analyzed by confocal microscopy to detect the expression of EGFP.

chromatography. Heparin-agarose or BSA-agarose was mixed with the rCDV-EGFP, and the bound materials were eluted with high concentrations of salt. The presence of virus was determined by western blot analysis using an anti-N monoclonal antibody. Despite the finding that no virion was recovered from BSA-agarose (Fig. 4A, lane 5), the rCDV-EGFP virions were eluted from heparin-agarose (Fig. 4A, lane 3). Thus, the recombinant CDV directly binds to heparin at a physiological salt concentration.

To investigate which CDV glycoprotein (F or H) binds to heparin, 293 cells were independently transfected with expression plasmids for CDV F and H. Proteins were metabolically labeled by ^{35}S-methionine and cysteine, and the cell lysates were subjected to heparin affinity chromatography, followed by immunoprecipitation using specific antibodies against F and H proteins (Fig. 4B). Both of the F and H proteins were found to bind to the heparin-agarose. However, the H protein was only faintly detected in the elution fractions (Fig. 4B, right panel), whereas the F protein gave strong signals (Fig. 4B, left panel). Because of the nature of the experiments, we could not compare the binding strength between the F and H proteins. Nevertheless, our results strongly suggest that not only H protein but also F protein of CDV is involved in the attachment to cell surface molecules.

Discussion

Reverse genetics system for CDV was recently established (Gassen et al., 2000; Kai et al., 2000; Parks et al., 2002; von Messling et al., 2001). Recombinant CDVs established by other groups are based on a vaccine strain (the Onderstepoort strain; isolated and passaged in Vero cells) or a Vero-adapted field isolate (Plattet et al., 2004) while ours are based on a recent field isolate (the Yanaka strain; isolated in B95a cells from an affected dog in Japan in 1994). In measles virus (MV), it has been reported that MV propagated in Vero cells loses its pathogenicity, while MV propagated in B95a cells maintains virulence in vivo (Parks et al., 2002). It has also been reported that field isolates of MV passaged in B cell lines use their entry receptors different from that used by a vaccine strain passaged in Vero cells (Bartz et al., 1998). Therefore, it is important to establish a reverse genetics system based on field isolates especially when investigating the native characteristics of CDV such as its host cell specificity.

In the present study, we used a V-knockdown version of cDNA clone of CDV to generate recombinant viruses expressing foreign genes. V protein of measles virus has been reported to be non-essential in vitro but act as a virulent factor in vivo (Patterson et al., 2000; Tober et al., 1998; von Messling et al., 2006), probably due to its ability to counteract host's interferon system (Horvath, 2004). The purpose of this study is not only to analyze the characteristics of CDV but also to obtain basic information for production of multivalent vaccines and viral vectors based on CDV. For the use of such recombinant CDVs in vivo, pathogenicity of the vector itself should be as little as possible. Therefore, for expressing foreign genes, we constructed recombinant CDVs in which the V protein

production is abrogated. The parental strain of CDV and the V(−) recombinant CDV grew similarly in vitro (data not shown), and it seems unlikely that knockdown of V results in altered host cell specificity in cell culture.

In this study, using recombinant CDVs expressing EGFP or firefly luciferase, we investigated susceptibilities of cells derived from non-dog species to CDV infection using the recombinant viruses. A broad range of cells was shown to be susceptible to CDV infection. It has been reported that the Onderstepoort strain of CDV can infect various cell lines (Loffler et al., 1997). rCDV-EGFP in the present study was able to infect the NIH-3T3 cell line, although it has been reported that the Onderstepoort strain cannot infect this cell line (Loffler et al., 1997). This is not surprising because some strains of CDV can be propagated in mouse brain. In this study, we found no cells that are completely resistant to CDV infection.

Using the recombinant rCDV-Luc, we determined levels of CDV transcription in infected cells (Table 1). Since luciferase is a very sensitive marker, rCDV-Luc facilitates detection of infection at low levels. Actually, we detected considerably high luciferase activities in cells which were associated with low rCDV-EGFP infectivities. Surprisingly, the luciferase activity in BHK-21 cells was almost equivalent to those in other epithelial cell lines, while the rCDV-EGFP infectivity in BHK-21 cells was much lower than those in other cell lines. The relative luciferase activity per single infected BHK-21 cell calculated by dividing the luciferase activity by the infectivity was higher than those of other cell lines, including B95a cells which are highly susceptible to CDV infection. It might be possible that the transcription of CDV is upregulated in BHK-21 cells. Alternative possibility is that the duration of mRNA or translation efficiency is different between EGFP and luciferase within BHK-21 cells, although the 5'- and 3'-non-coding regions within those recombinant viruses are the same. It is also possible that duration of one of these proteins is somewhat different in BHK-21 cells compared to that in other types of cells.

It has been reported that field isolates of CDV use SLAM as a receptor (Lan et al., 2005; Seki et al., 2003; Tatsuo et al., 2001). In this study, infection of B95a cells with rCDV-EGFP was inhibited by treatment with an anti-SLAM monoclonal antibody. However, it is also known that SLAM is expressed at high levels only on lymphoid cells and dendritic cells, a fact that seems to be inconsistent with the infection of other cells by CDV. The question of whether CDV infects primarily SLAM+ cells in vivo remains to be answered. Infection of 293 cells with rCDV-EGFP was not inhibited by the aforementioned antibody (Fig. 4) because of the lack of SLAM expression on 293 cells. Thus, CDV seems to have the intrinsic capacity to use more than one receptor. To investigate whether CDV can utilize the CD46 molecule as an alternative receptor like measles virus does, we performed an infection inhibition assay based on 293 cells using an anti-human CD46 monoclonal antibody. This antibody did not inhibit infection of 293 cells with CDV (Fig. 2B; panel b). So far, no CDV strain that uses CD46 as a receptor has been reported. Therefore, there may be one or more cellular receptors for CDV in addition to SLAM, and such unknown

receptor(s) might be ubiquitous considering the broad host range of CDV infection.

Since a broad range of cells was susceptible to CDV infection, we tested whether heparin-like molecules are involved in CDV infection. Infection of 293 cells with rCDV-EGFP was inhibited in the presence of heparin. It was also the case with heparan sulfate and chondroitin sulfate B,

although the effects were less pronounced than that of heparin. It has been reported that infection with relevant MV was not inhibited by heparin (Feldman et al., 2000). However, in that report, the authors used Vero cells which express CD46, the high-affinity receptor for laboratory strains of MV (Feldman et al., 2000). Therefore, the effect of soluble heparin on the attachment of MV might be hidden. In our
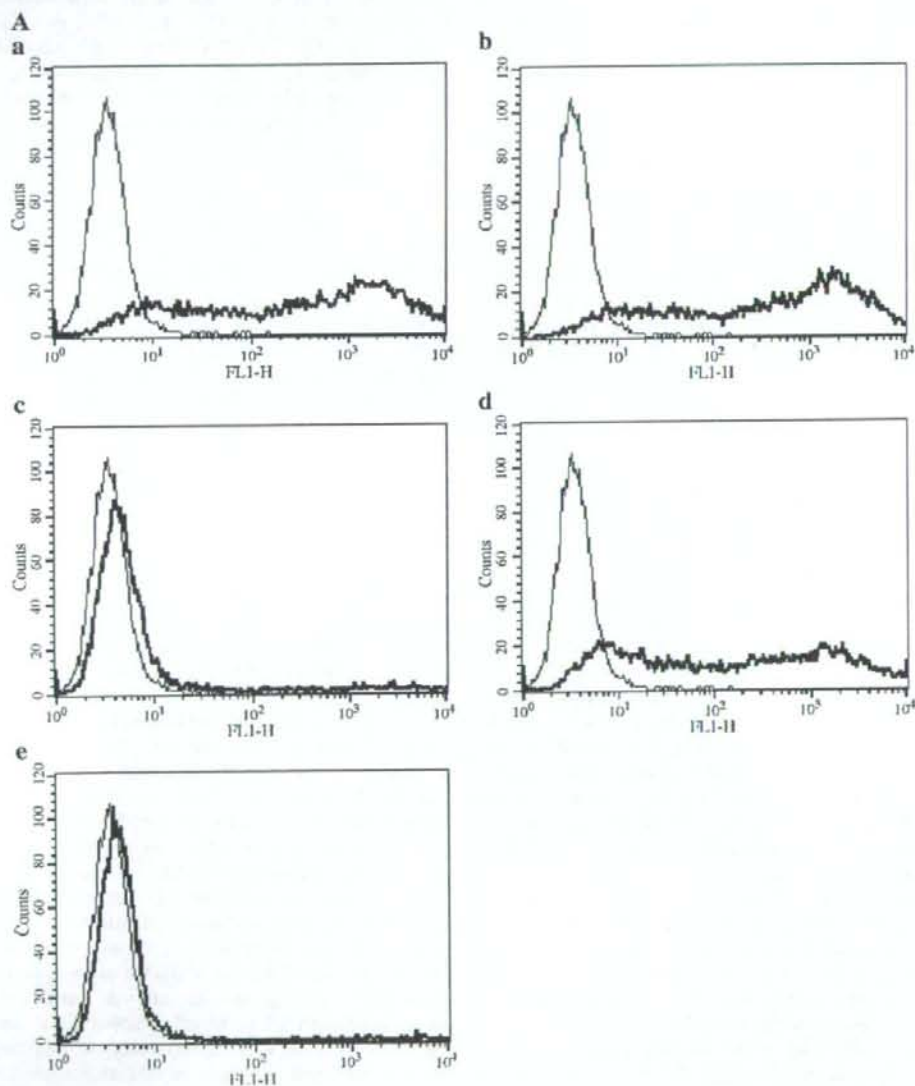

Fig. 2. Effect of monoclonal antibodies against CD46 or SLAM or heparin on rCDV-EGFP infection. Cells were treated with monoclonal antibodies against either CD46 or SLAM prior to infection with rCDV-EGFP. Meanwhile, rCDV-EGFP was treated with heparin prior to inoculation. (A) B95a cells. (B) 293 cells. Panel a, mock-treated. Panel b, treated with anti-CD46 antibody (clone M177). Panel c, treated with anti-SLAM antibody (clone IPO-3). Panel d, treated with heparin. Panel e, treated with anti-SLAM antibody plus heparin. EGFP-positive cells were analyzed by flow cytometry. Thin line: mock-infected cells. Thick line: rCDV-EGFP-infected cells.
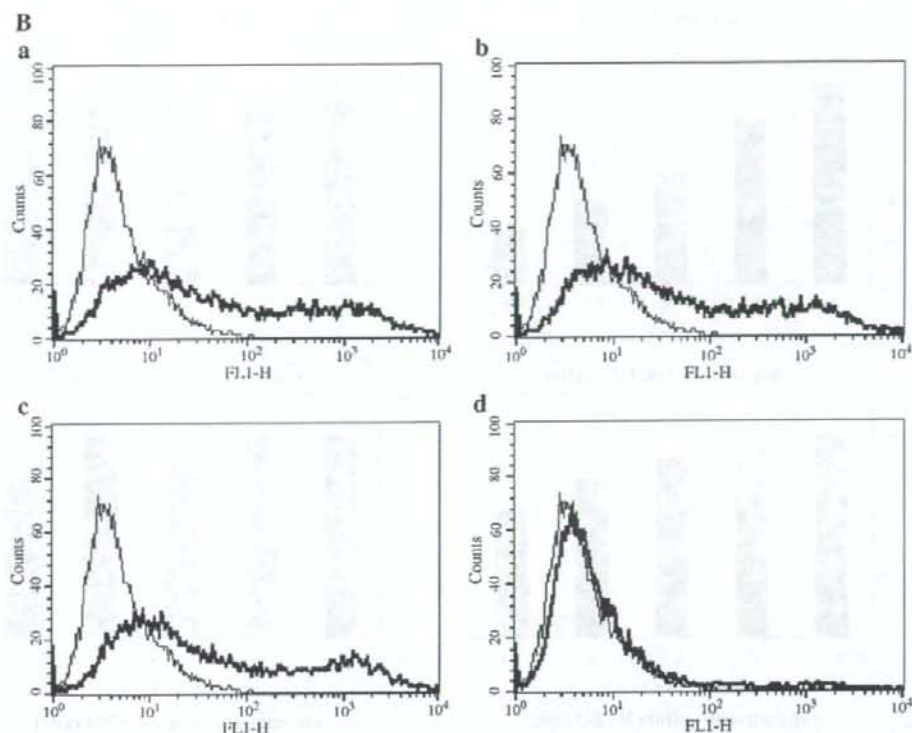
B

Fig. 2 (continued).

experiment, infection of B95a cells with rCDV-EGFP was not significantly inhibited by heparin, however, the inhibitory effect of heparin was pronounced when the B95a cells were treated with an anti-SLAM antibody to inhibit the SLAM-dependent infection. These results support the idea that the infection of B95a cells was mainly mediated by the high-affinity receptor SLAM, and the contribution of heparin-like molecules might be low. However, if the cells do not express high-affinity receptor, the virus manages to infect those cells via other pathway in which heparin-like molecules are involved. Increasing evidences suggest that, besides SLAM and CD46, there are yet unidentified receptor for MV since many cells are infected with MV even when these molecules are absent or blocked (Andres et al., 2003; Hashimoto et al., 2002; Ishida et al., 2004; Shingai et al., 2003; Takeuchi et al., 2003). Further studies on roles of heparin-like molecules in MV infection are now under investigation.

In this study, treatment of rCDV-EGFP with heparin or other GAGs did not completely block the infection with the rCDV-EGFP. These results suggest that other molecules than heparin-like molecules are also involved in the attachment process. It is thought that most of the heparan sulfate-binding proteins use heparan sulfate as a regulator of ligand-dependent activation of primary signaling receptors at the cell surface (Carey, 1997). Thus, it is possible that cell surface GAGs concentrate the virion

on the two-dimensional surface of the plasma membrane and the binding to heparin-like molecules increases the ability to bind to the low affinity receptor present in most cell species. Therefore, the binding to heparin-like molecules itself might not cause viral entry, and the function of these molecules may support the binding of virus to the low affinity or quantity receptor. Interestingly, it was recently reported that DC-SIGN expressed on dendritic cells is an attachment receptor for MV (de Witte et al., 2006). DC-SIGN itself does not support virus entry, however, it enhances CD46- and SLAM-mediated infection. It is possible that similar phenomenon occurs between heparin-like molecules with SLAM or other unknown receptor(s).

The reason why chondroitin sulfate B inhibited the infection with the rCDV-EGFP to a greater extent compared to chondroitin sulfate A or C (Fig. 1) remains to be elucidated. However, similar data have been obtained for other heparin-binding viruses (Byrnes and Griffin, 1998; Jackson et al., 1996; Summerford and Samulski, 1998). One possible explanation is the similarity of chondroitin sulfate B to heparan sulfate and heparin. Chondroitin sulfate B (dermatan sulfate) is the only chondroitin sulfate that contains the IdoA monosaccharides that are found in heparan sulfate and heparin (Rostand and Esko, 1997). The requirement of IdoA in glycosaminoglycans for infection with respiratory syncytial virus has been reported (Hallak et al., 2000), and it
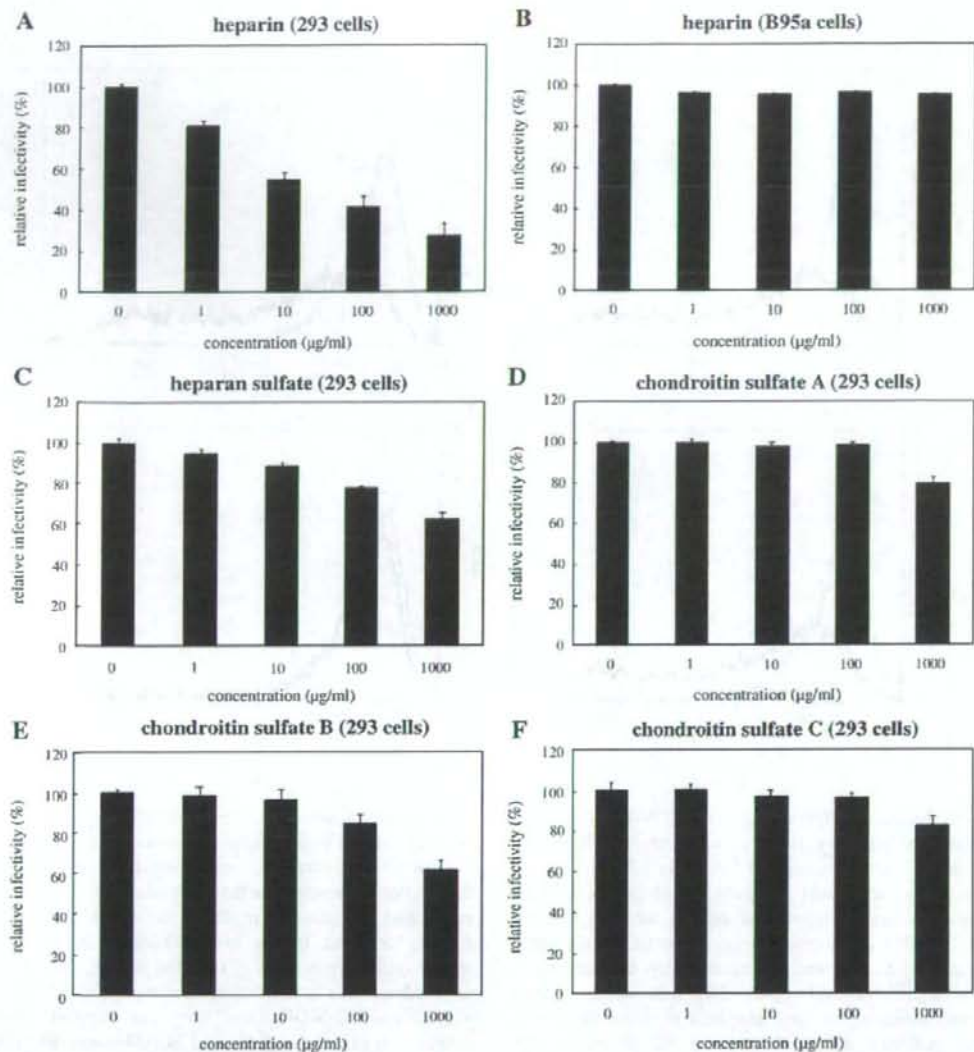
Fig. 3. Inhibition of rCDV-EGFP infection by soluble GAGs. rCDV-EGFP was treated with heparin (A), heparan sulfate (C), chondroitin sulfate A (D), chondroitin sulfate B (E) or chondroitin sulfate C (F) at different concentrations for 1 h and inoculated to 293 cells. The virus was also treated with heparin and inoculated to B95a cells in the same manner (B). The infectivities were measured by flow cytometry. Relative infectivities to mock-treated samples are shown.

is possible that the attachment of CDV requires the presence of IdoA units in GAGs.

We also demonstrated that both of the F and H proteins of CDV bind to heparin. It has been reported that human respiratory syncytial virus can bind to heparan sulfate with glycoprotein G and glycoprotein F (Feldman et al., 2000; Karger et al., 2001). We did not find the linear heparin-binding motif in the CDV H gene by sequence analysis (data not shown). In the F protein, there is a basic amino acid-rich region near the cleavage site, and it is possible that the F protein binds

to heparin via this region. It has been generally considered that, between the glycoproteins of the morbilliviruses, only the H protein is involved in virus attachment to cells. The results obtained in the present study indicate that not only the H but also the F protein may be involved in the virus attachment of morbilliviruses.

In foot-and-mouth disease virus, Sindbis virus and tick-borne encephalitis virus, it has been reported that the acquisition of the ability to bind to heparan sulfate correlates to the reduced pathogenicity in vivo (Klimstra et al., 1998; Mandl et al., 2001;
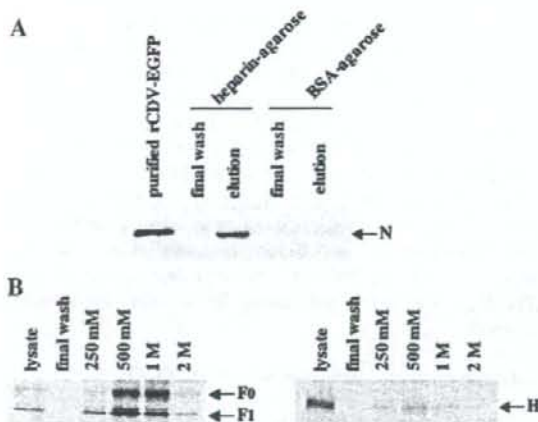
A

B

Fig. 4. Heparin affinity chromatography of rCDV-EGFP and its glycoproteins. (A) Heparin affinity chromatography of rCDV-EGFP. Lane 1: positive control (purified rCDV-EGFP), lane 2: final wash fraction from heparin-agarose, lane 3: elution fraction from heparin-agarose, lane 4: final wash fraction from BSA-agarose, lane 5: elution fraction from BSA-agarose. The fractions were analyzed on SDS-PAGE, and the presence of CDV was detected by western blotting using a monoclonal antibody against N protein of CDV. (B) Heparin affinity chromatography using cell extract of 293 cells transfected with the expression plasmids encoding either F (left panel) or H gene (right panel) of CDV. Proteins were eluted by increasing concentrations of NaCl in a stepwise manner. Individual fractions were immunoprecipitated with monoclonal antibodies against F or H protein of CDV. Lane 1: cell lysate of 293 cells transfected with the expressing plasmid of corresponding gene of CDV, lane 2: final wash fraction, lanes 3–6: elution fractions. NaCl concentrations used for elution are indicated on the top of the lanes.

Sa-Carvalho et al., 1997). The acquisition of heparan sulfate-binding activity is advantageous for virus in cell culture, but the acquisition of the ability to bind to a new receptor can also ameliorate or even abrogate disease by sequestering the virus to sites that are not favorable for replication. It has been reported that different strains in the same virus have a different affinity to heparin, and the adaptation of virus in cell culture selects for the use of heparan sulfate as an attachment receptor (Klimstra et al., 1998; Sa-Carvalho et al., 1997). In measles virus (MV), it has been shown that MV propagated in Vero cells lost its pathogenicity, while MV propagated in B95a cells maintained its virulence *in vivo* (Kobune et al., 1990). In addition, it was also reported that field isolates of MV passaged in B cell lines use different receptors for viral entry than a vaccine strain passaged in Vero cells (Bartz et al., 1998). The Yanaka strain of CDV was isolated and passaged in B95a cells, but this strain is apathogenic to dogs. It is not known whether the adaptation of CDV to a marmoset cell lines correlates with the loss of pathogenicity. Establishment of suitable canine cell lines is awaited. During the preparation of this manuscript, Baron published a paper reporting that cell culture-adapted strains of rinderpest virus, which is in the same genus as canine distemper virus, use heparan sulfate as a receptor (Baron, 2005). This study and ours reveal that morbilliviruses may have an ability to use or to be adapted to use heparin-like molecules in the process of viral entry.

Materials and methods

Virus and cells

Viruses were propagated in B95a cells (Kobune et al., 1990) as described previously. 293 cells were grown in Dulbecco's modified minimum essential medium (DMEM) containing 10% fetal calf serum (FCS) and antibiotics. B95a cells and 3132 cells (canine lymphoma cell line of B cell origin) were grown in RPMI containing 5% FCS. Primary-cultured cells from a dog embryo (DEB), MDCK (canine kidney), CRFK (feline kidney), Vero (monkey kidney), HeLa (human uterus), COS-7 (monkey kidney), 293 (human kidney), NIH-3T3 (mouse fibroblast), CPK (pig kidney), MDBK (cattle kidney), RK-13 (rabbit kidney), and BHK-21 (hamster kidney) cells were grown in DMEM containing 10% FCS and appropriate concentration of antibiotics. PBMC from a dog, a seal and a lion were isolated by density gradient centrifugation using Ficoll Plaque reagent (Pharmacia) and stimulated by 5 μg/ml of ConA in RPMI 1640 medium containing 10% FCS and 100 U/ml of recombinant human IL-2 for 3 days and then cultivated in RPMI 1640 medium containing 10% FCS and 100 U/ml of recombinant human IL-2.

DNA manipulations

The strategy for the construction of cDNA clones of CDV is summarized in Fig. 1A. At first, 3′ and 5′ non-coding sequences of the Yanaka strain were cloned into pMDB1 plasmid (generously provided by M. Baron). To facilitate the subsequent subcloning, nucleotides between 81 and 86 of the CDV genome (ACAAGG) were mutated to *Bsi*WI site (CGTACG), and nucleotides between 15,609 and 15,616 (CTGCTATTCA) were mutated to *Not*I site (GCGGCCGC). Primer pairs, 5′-GACGT ACGTCAGGGTTCAGACCTACCAGTATGGCTAGCCTT-CTTAAG-3′ and 5′-ACCTCGAGGCCGGCCTTAATTGA-GTAGCTCTCT-3′, 5′-GCCTCGAGGTGTTACATCAGT-CACCA-3′ and 5′-TATCTAGAGTTTAAACTTAAG-CATGTGTGATACT-3′, 5′-ACTCTAGATAATCTATTA-ACAGGTTCA-3′ and 5′-TAACGCGTTAGAGAATTTT-GAAAAG-3′, 5′-TAACGCGTTAGTTCATGAAC-TAAAACTC-3′ and 5′-GTGGTACCGCGATCGCTCAG-AGTGATCTTACATA-3′, 5′-GCGGTACCACGTCTTACCT-GATTGTTA-3′ and 5′-GAGTCGACTTAATTAACTATCA-AGGTTTTGAACG-3′, 5′-AAGTCGACTCTCAATTGAACT-TAAGGA-3′ and 5′-TGGCGGCCGCAGGGTTAGGATCCA-GACC-3′ were used to amplify the genes for N, P, M, F, H and L, respectively (unique restriction enzyme recognition sites underlined). Amplified products were sequentially cloned into the plasmid described above. Finally, sequences of *Bsi*WI and *Not*I recognition sites were mutated to the original sequences to produce the plasmid pCDV.

A derivative of pCDV (pCDV V(−)) which harbors mutations within editing site of V protein was made by site-directed mutagenesis using primers 5′-GAGTGTGGACCCAT-TAAGAAAGGCACAGGAGAGAG-3′ and 5′-CTCTCTC-CTGTGCCTTTCTTAATGGGTCCACACTC-3′.

Enhanced green fluorescent protein (EGFP) gene was amplified from pEGFP-N1 (Clontech) using the following primers, 5'-TAAGGCCGGCCAAACTCATTATAAAAAACT-TAGGGCTCAGGTAGTCCAACAATGGTGAGCAAGGGC-GAGGA-3' and 5'-TCGAGGCCGGCCTTACTTGTACA-GCTCGTCCA-3' (FseI site underlined). The PCR products were cloned into pCR2.1 vector (Invitrogen), and sequences were confirmed. Then, the plasmid was digested by endonuclease FseI and inserted into the FseI site of pCDV V(−) (downstream of the N gene). Firefly luciferase gene was amplified from pGL3-basic (Promega) using the following primers, 5'-GAATGCTTCTAAACTCATTATAAAAAACT-TAGGGCTCAGGTAGTCCAACAATGGAAGACGC-CAAAAACA-3' and 5'-GAATGCTTTACACGGCGAT-CTTTCCGC-3'. The PCR product was cloned into pCR2.1 vector (Invitrogen) and sequences were confirmed. The plasmid was digested by restriction enzyme BsmI, and the cutting ends were polished with T4 DNA polymerase (Takara). The fragments were ligated to FseI linker (5'-AGGGGC-CGGCCCCT-3'; FseI site underlined) which had been annealed and phosphorylated with T4 polynucleotide kinase (TOYOBO) and digested by FseI. The fragment was cloned into FseI site of pCDV V(−). The forward primers contain a transcription signal unit of CDV, that is transcription termination signal of the N gene, followed by intergenic trinucleotides, CTT, which are conservative except that in H–L junction and L–trailer junction and subsequently followed by transcription start signal of the H gene. This construct allows the additional gene to express in the form of an extra transcription unit. The resulting full-genome plasmids carrying EGFP gene or luciferase gene were designated as pCDV-EGFP or pCDV-Luc, respectively.

The expression plasmids for CDV F and H proteins were constructed as follows. The coding region of F gene was amplified using the following primers; 5'-CCGGAATTCT-TAGGGTCCAGGACATAGCA-3' and 5'-CCGGAATTC-GACTACCTGAGCCCTAAGTT-3'. The PCR product was phosphorylated by T4 polynucleotide kinase (Takara) and inserted into XhoI site of pCAGGS vector (a gift from Dr. J. Miyazaki, Osaka University, Osaka, Japan) (Niwa et al., 1991). The coding region of H gene was amplified using the following primers; 5'-CCGCTCGAGCAGGTAGTCCAACAATG-3' and 5'-CCGCTCGAGGTATCATCATACTATCA-3'. The PCR product was digested by EcoRI and inserted into EcoRI site of pCAGGS. The resulting plasmids were designated as pCAG-F and pCAG-H, respectively.

Recovery of recombinant CDVs

293 cells in 6-well culture dish were inoculated with recombinant vaccinia virus encoding T7 RNA polymerase (MVA-T7) for 1 h and then transfected with 1 μg of pCDV-EGFP or pCDV-Luc, 1 μg of pKSN1 and 1 μg of pKSP and 0.3 μg of pGEML per well, which express N, P and L protein of rinderpest virus (Baron and Barrett, 1997), respectively, under the control of T7 promoter, using Fugene 6 (Roche) as a transfection reagent. After 3 days of incubation, the cells were co-cultivated with B95a cells, a

marmoset B lymphoblast cell line (Kobune et al., 1990) in which CDV can replicate effectively (Kai et al., 1993), at a concentration of 2×10^6 cells per well and further incubated in RPMI (Sigma) containing 1.5% FCS until extensive cytopathic effects appeared. The recovered CDVs were designated as rCDV, rCDV-EGFP or rCDV-Luc from plasmids pCDV, pCDV-EGFP or pCDV-Luc, respectively. The cells were collected and lysed by 3 cycles of freezing and thawing. After sonicated, samples were centrifuged at 3000 rpm to remove cell debris and stored as a crude virus stock at −80 °C. The 50% tissue culture infective dose ($TCID_{50}$) was quantified using B95a cells by standard methods.

Infection of various cell types with rCDV-EGFP

All types of cells were inoculated with rCDV-EGFP or rCDV-Luc at an MOI of 2 $TCID_{50}$/cell (determined in B95a cells) and incubated for 40 h. The rCDV-EGFP-infected cells were suspended in PBS containing 0.02% EDTA and washed twice with sorter buffer (PBS containing 2% FCS and 0.1% NaN3). Then, the flow cytometric analyses were performed using FACScan (Becton Dickinson). The rCDV-Luc-infected cells were suspended in PBS containing 0.02% EDTA, washed twice with PBS and lysed in 50 μl of LCβ (Wako chemical). Luciferase activity was measured by luminometer (Berthold) using Picagene (Wako chemical) as a substrate.

Inhibition of recombinant CDV infection by antibodies

B95a and 293 cells (1×10^5 cells) in 24-well tissue plates were incubated with a monoclonal antibody specific for human SLAM (clone IPO-3) (KAMIYA industry) or human CD46 (M177; kindly provided by Dr. Seya, Department of Immunology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan) at a concentration of 5 μg/ml and 10 μg/ml at 37 °C for 1 h, respectively. The cells were inoculated with the rCDV-EGFP which was mock-treated or pretreated with 1 μg/ml of heparin at an MOI of 2 $TCID_{50}$/cell (determined in B95a cells) in the presence of each antibody at the same concentrations. After incubation for 1 h, the inoculum was removed and maintenance medium containing each antibody at the same concentration was added. The cells were further incubated for 40 h and analyzed by flow cytometry.

Inhibition of infection by soluble GAGs

For infection inhibition assay, the rCDV-EGFP (1×10^5 $TCID_{50}$) were incubated with heparin, heparan sulfate, chondroitin sulfate A, B or C (all purchased from Sigma) at various concentrations for 1 h at 37 °C and inoculated to 5×10^4 of B95a or 293 cells. After incubation for 1 h, the inoculum was removed and the cells were washed twice with RPMI containing 2% FCS or DMEM containing 5% FCS and then further incubated in the medium. Forty hours later, the cells were harvested and analyzed by flow cytometry.

Transfection and metabolic labeling of CDV glycoproteins

293 cells in 10 cm culture dishes were transfected with either pCAG-F or pCAG-H using Lipofectamine 2000 (Invitrogen) as the transfection reagent. The cells were metabolically labeled with ^{35}S-methionine and cysteine (Pharmacia) at 36 h after transfection for 14 h. Then, the cells were lysed in lysis buffer (20 mM sodium phosphate (pH 7.4) containing 150 mM NaCl and 1% Triton X-100).

Heparin affinity chromatography

For affinity chromatography of the rCDV-EGFP, the supernatant of rCDV-EGFP-infected B95a cells was concentrated with a size exclusion (100K) membrane filter (Amicon). After equilibration of heparin-agarose beads or BSA-agarose beads (purchased from Sigma) in PBS, concentrated virus supernatant was added to the beads and incubated for 30 min at 4 °C. Beads were washed 7 times in 1 ml of PBS followed by elution in PBS containing 2 M NaCl. The final wash and elution fractions were concentrated using a 100K membrane filter (VIVASPIN; Sartorius) and analyzed on SDS-PAGE followed by western blot analysis using a monoclonal antibody against CDV N protein (clone 3) (Masuda et al., in press). For affinity chromatography of CDV glycoproteins, heparin-agarose was equilibrated in lysis buffer, and cell lysates were mixed with the beads, followed by an incubation for 1 h at 4 °C. The beads were washed in the lysis buffer (7 times; 1 ml each), and bound materials were eluted with increasing concentrations of NaCl in a lysis buffer (stepwise gradient: 250 mM, 500 mM, 1 M and 2 M). The eluted fractions were immunoprecipitated with monoclonal antibodies against the F (clone a-8) (Hirayama et al., 1991) or H protein (clone d-7) (Hirayama et al., 1991) of CDV and analyzed on SDS-PAGE.

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Measles Virus N Protein Inhibits Host Translation by Binding to eIF3-p40[▽]

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The nonsegmented, negative-sense RNA genome of measles virus (MV) is encapsidated by the virus-encoded nucleocapsid protein (N). In this study, we searched for N-binding cellular proteins by using MV-N as bait and screening the human T-cell cDNA library by yeast two-hybrid assay and isolated the p40 subunit of eukaryotic initiation factor 3 (eIF3-p40) as a binding partner. The interaction between MV-N and eIF3-p40 in mammalian cells was confirmed by coimmunoprecipitation. Since eIF3-p40 is a translation initiation factor, we analyzed the potential inhibitory effect of MV-N on protein synthesis. Glutathione S-transferase (GST)-fused MV-N (GST-N) inhibited translation of reporter mRNAs in rabbit reticulocyte lysate translation system in a dose-dependent manner. Encephalomyocarditis virus internal ribosomal entry site-mediated translation, which requires canonical initiation factors to initiate translation, was also inhibited by GST-N. In contrast, a unique form of translation mediated by the intergenic region of *Plautia stali* intestine virus, which can assemble 80S ribosomes in the absence of canonical initiation factors, was scarcely affected by GST-N. In vivo expression of MV-N induced by the Cre/*lox*P switching system inhibited the synthesis of a transfected reporter protein, as well as overall protein synthesis. These results suggest that MV-N targets eIF3-p40 and may be involved in inhibiting MV-induced host translation.

Measles virus (MV) is a member of the genus *Morbillivirus* within the family *Paramyxoviridae* of the order *Mononegavirales*. This virus has a nonsegmented negative-sense single-stranded RNA genome that is encapsidated by multiple copies of the nucleocapsid protein (N) to form a helical ribonucleoprotein complex known as the nucleocapsid. Associated with the nucleocapsid are the two components of the viral RNA-dependent RNA polymerase: the phosphoprotein (P) and large protein (L). Together, these constitute the holonucleocapsid, which is packaged within a lipid envelope, bears the hemagglutinin and fusion glycoproteins (H and F), and is lined internally by matrix protein (M).

Recently, several reports showed that MV-N can induce systemic immunosuppression. The C-terminal part of MV-N binds to the Fc receptor on B cells and dendritic cells and induces immunosuppression by inhibiting antibody production (20), impairing dendritic cell function (14), preventing interleukin-12 production (15), and suppressing hypersensitivity responses (14, 15). Interestingly, a recent study showed that MV-N also activates signal cascades of innate immunity by phosphorylating IRF-3 (28). The C-terminal domain of MV-N associates with IRF-3, as well as the virus-activated kinase. These findings strongly imply that MV-N is involved in mediating the immune response, as well as inducing immunological abnormalities and pathogenicity during the virus life cycle.

In the present study, we searched for MV-N-binding cellular

proteins by using a yeast two-hybrid screening system, and identified the p40 subunit of eukaryotic translation initiation factor 3 (eIF3-p40; eIF3γ) as a specific binding partner. We previously showed that MV shuts off host cell gene expression during virus infection (unpublished data). Therefore, we analyzed the implication of MV-N in the shutoff via binding with eIF3-p40 in the present study. We showed that MV-N inhibits in vitro translation of reporter mRNAs and in vivo protein synthesis in a dose-dependent manner. Based on these data, we proposed that MV-N shuts off host translation in MV-infected cells through protein-protein interactions with eIF3.

MATERIALS AND METHODS

Construction of yeast two-hybrid vectors. To construct yeast two-hybrid bait vectors encoding the full-length MV-N gene of the wild-type HL strain (pGBKT-N) and two deletion clones (pGBKT-NΔ1 and pGBKT-NΔ6), cDNAs were amplified by PCR from the full-length MV-HL strain clone (unpublished data) using specific primer pairs (data not shown) and LA-Taq DNA polymerase (TaKaRa). PCR products were subcloned into pGEM-T Easy vector (Promega) and digested with NdeI and EcoRI. The resulting cDNA fragments were inserted separately into pGBKT7 bait vector (Clontech). To construct four yeast two-hybrid bait vectors encoding MV-N deletion clones (pGBKT-NΔ2, -NΔ3, -NΔ4, and -NΔ5), the pGEM-T Easy vector containing the full-length MV-N cDNA was PCR amplified using the specific primer pairs (data not shown) and *Pfu* Turbo DNA polymerase (Stratagene). The PCR fragments were phosphorylated with T4 polynucleotide kinase (TOYOBO) and self-ligated. The plasmids obtained were digested with NdeI and EcoRI, and the resulting cDNA fragments were inserted separately into pGBKT7 bait vector.

Two-hybrid screening and interaction assays. Yeast AH109 cells were transformed with pGBKT7 bait plasmid containing full-length MV-N and the human T-cell cDNA library (10^7 clones) by using the lithium acetate method described in the Clontech manual. Transformed cells were plated on minimal selective synthetic dropout (SD) media (SD/−Ade/−His/−Leu/−Trp/X-α-Gal) containing 2.5 mM 3-aminotriazole (3-AT), and colonies were picked and replica plated after 5 to 7 days of incubation at 30°C. Plasmid DNA from positive clones was

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▽ Published ahead of print on 8 August 2007.

extracted by using the YeastMaker yeast plasmid isolation kit (Clontech) and electroporated into *Escherichia coli* ElectroMax DH10B competent cells (Invitrogen). The resulting plasmid recovered from *E. coli*, encoding eIF3-p40 cDNA, was retransformed into yeast with pGBKT7 vectors that encoded a series of MV-N deletion mutants (pGBKT-NΔ1 to -NΔ6). Positive clones were selected on SD medium in the absence of two nutrients (Leu and Trp), and the obtained colonies were spotted onto SD/−His/−Leu/−Trp plates in the presence of 0.5 mM 3-AT. Positive interactions were defined as the ability of transformed cells to grow on this medium.

Construction of eukaryotic expression plasmids. To create a plasmid expressing hemagglutinin (HA)-tagged eIF3-p40 (pCMV-HA-eIF3-p40), total RNA isolated from human embryonic kidney 293 cells by ISOGEN (Nippon gene) was reverse transcribed by using a random primer (9-mer) and SuperScript II reverse transcriptase (Gibco-BRL), followed by PCR amplification with a specific primer pair corresponding to eIF3-p40 cDNA (5′-GAATTCGGATGGCGTCCCGCA AGGAAGG-3′ and 5′-CTCGAGATTAGTTGTTGTATTCTTGAAGAGCCT G-3′; restriction sites are underlined). This PCR product was inserted into the EcoRI/XhoI site of pCMV-HA (Clontech). To create plasmids expressing myc-tagged MV-N (pCMV-Myc-N) and two N deletions (pCMV-Myc-NΔ1 and NΔ2), cDNAs were PCR amplified from pGBKT7N, pGBKT7-NΔ1, and pGBKT7-NΔ2, respectively, using specific primer pairs (data not shown), and the resulting cDNA fragments were inserted into the EcoRI/NotI site of pCMV-Myc (Clontech). To construct three expression vectors encoding myc-tagged deletions of MV-N (pCMV-Myc-NΔ2a, -NΔ2b, and -NΔ2c), pCMV-Myc-N was PCR amplified using specific primer pairs (data not shown) and *Pfu* Turbo DNA polymerase. PCR products were phosphorylated with T4 polynucleotide kinase and self-ligated.

Transfection and immunoprecipitation assay. Cos-7 cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 100 U of penicillin G per ml, 100 μg of streptomycin per ml (Gibco-BRL), and 10% fetal bovine serum (Sigma). Cos-7 cells in 3.5-cm-diameter dishes were transfected with 1 μg of pCMV-HA-eIF3-p40 and pCMV-Myc-N or its deletion mutants using FuGENE6 transfection reagent (Roche). At 24 h posttransfection, the medium was replaced with 1.5 ml of DMEM containing one-tenth the normal amount of methionine, 10% fetal bovine serum, and 150 μCi of [^{35}S] EasyTag Express protein labeling mix (Perkin-Elmer). At 16 h postlabeling, cells were lysed with lysis buffer (10 mM Tris-HCl [pH 7.5], 130 mM NaCl, 0.5% Triton X-100, 0.5 mM EDTA, 10 mM NaF, 1 mM Na$_3$VO$_4$) containing 2% (vol/vol) of a protease inhibitor cocktail (BD Bioscience) and clarified by centrifugation at 16,000 × g for 10 min. Cell lysates were incubated with a 1:200 dilution of anti-myc-tag monoclonal antibody (Clontech) or a 1:200 dilution of anti-HA-tag rabbit polyclonal antibody (Clontech), each containing 20 μl of protein A-Sepharose bead suspension, and rocked at 4°C overnight. The protein A-Sepharose beads were washed three times with phosphate-buffered saline (PBS), denatured at 100°C in sodium dodecyl sulfate (SDS) sample buffer, and subjected to SDS-10% polyacrylamide gel electrophoresis (PAGE). Immunoprecipitates were visualized by autoradiography.

MV infection and Western blotting. COBL-a cells (a human lymphoid cell line) (13) were cultured in RPMI medium supplemented with 100 U of penicillin G per ml, 100 μg of streptomycin per ml, and 10% of fetal bovine serum. COBL-a cells in a 10-cm-diameter dish were infected with MV-HL (23) at a multiplicity of infection (MOI) of 0.001. After 48 h of infection, cells were harvested and then cross-linked with 1% fonnaldehyde in PBS for 10 min at room temperature. Cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. Cells were washed with PBS, lysed with the lysis buffer described above, and then subjected to a 30-s sonication with a Sonifier 450 (Branson). Cell lysate was clarified by centrifugation at 16,000 × g for 10 min and subjected to an immunoprecipitation assay using a 1:500 dilution of anti-N monoclonal antibody 8G (16), as described above. For cross-link reversal, the immunoprecipitates were boiled in SDS sample buffer for 10 min and then resolved on SDS-10% PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were incubated with a 1:1,000 dilution of anti-N rabbit polyclonal antibody or a 1:100 dilution of anti-eIF3-p40 goat polyclonal antibody (Santa Cruz Biotechnology) at 4°C overnight. The membranes were washed three times with PBS and then incubated with a 1:2,000 dilution of horseradish peroxidase-conjugated rabbit anti-goat, or goat anti-rabbit, immunoglobulin G (Dako) at room temperature for 1 h. Proteins that bound antibodies were detected by ECL Plus Western blotting detection reagents (Amersham).

Expression and purification of GST-fused proteins. The cDNAs encoding MV-N and MV-NΔ2c were amplified by PCR from pCMV-Myc-N or pCMV-Myc-NΔ2c, respectively, using a specific primer pair (5′-CATATGCATGGCC ACACTTTTGAGGAG-3′ and 5′-GAATTCCTAGTCTAGAAGATCTCTG-

3′), as well as LA-Taq DNA polymerase. cDNA encoding MV-P was amplified from the full-length MV-HL strain clone with a specific primer pair (5′-CATA TGCATGGCAGAAGAGCAGGCACG-3′ and 5′-GAATTCCTACTTCATTA TTATCTTC-3′). These PCR fragments were cloned into the NdeI/EcoRI site of the baculovirus homologous recombination vector, pAcGHLT-A (Clontech), and recombinant baculoviruses were rescued according to the manufacturer's protocol. Briefly, the vector and BaculoGold linearized baculovirus DNA (BD Bioscience) were cotransfected into Sf9 insect cells by using Lipofectin (Gibco-BRL). After 3 days, the supernatant was screened for recombinant baculoviruses. Rescued recombinant baculoviruses were inoculated into Sf9 cells at an MOI of 5. After 4 days, the cells were harvested, washed with PBS, pelleted, lysed with lysis buffer described above on ice for 45 min, and centrifuged at 14,000 × g for 10 min at 4°C. Glutathione S-transferase (GST)-fused proteins were affinity purified by using prepacked glutathione-Sepharose 4B (Pharmacia Biotech) according to the manufacturer's protocol and dialyzed against 10 mM Tris-HCl (pH 7.5) and 20% glycerol. The dialyzed proteins were concentrated by using Vivaspin 500 (5,000 molecular weight cutoff; Vivascience) and stored at −70°C.

In vitro translation inhibition assay. To synthesize capped luciferase mRNA (m^{7}G-luc RNA), pRL-CMV vector (Promega) was linearized with BamHI and transcribed with the Ribomax large-scale RNA production system-T7 (Promega) in the presence of a cap analog, 7mGpppG (Promega). To synthesize encephalomyocarditis virus (EMCV)-internal ribosomal entry site (IRES)-luc RNA, pIRES (Clontech) was digested with XhoI and XbaI, and the fragment containing the EMCV-IRES sequence was inserted into pTNT (Promega) (pTNT-IRES). Firefly luciferase cDNA was amplified by PCR from pGL3-Basic (Promega) with the specific primer pair flanking the SalI and NotI sites (5′-GCGG TCGACGCCATGGAAGACGCCAAAAACATAAAG-3′ and 5′-GCGCGGC CGCTACACGGCGATCTTTCCGCC-3′) and inserted into pTNT-IRES. The obtained plasmid was transcribed with the Ribomax large-scale RNA production system-T7 in the absence of cap analog. For the synthesis of IGR-IRES-luc RNA, a pT7CAT-IRES-ΔaugRluc plasmid (25), containing the *Plautia stali* intestine virus (PSIV) intergenic region (IGR) sequence in front of the *Renilla* luciferase gene lacking the first ATG codon, was kindly provided by N. Nakashima (National Institute of Agrobiological Sciences of Japan). pT7CAT-IRES-ΔaugRluc was PCR amplified using the sense primer corresponding to nucleotides 5375 to 5401 in the PSIV genome (accession number AB006531) (5′-AGCTTTATTATTGGTCAAAATCTCTCC-3′) and the antisense primer corresponding to the end of the *Renilla* luciferase open reading frame (5′-TTA TTGTTCATTTTTGAGAACTCGCTC-3′), as well as LA-Taq DNA polymerase, and the PCR product was ligated into pGEM-T Easy vector. After the direction of insertion was checked, the plasmid was digested with SpeI and transcribed with the Ribomax large-scale RNA production system-T7 in the absence of cap analog. Translation reactions were performed by using the Flexi rabbit reticulocyte lysate system (Promega) as recommended by the manufacturer. Briefly, 16.5 μl of rabbit reticulocyte lysate was preincubated with 2 μl of increasing concentrations of GST or GST-fused protein at 30°C for 90 min. Then, 250 ng of reporter RNA was added to 25-μl reactions containing preincubated rabbit reticulocyte lysate, 4 μCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham Bioscience), 20 μM amino acid solution minus methionine, 0.5 mM magnesium acetate, 100 mM KCl, and 40 U of RNase inhibitor (TOYOBO), followed by incubation for 90 min at 30°C. Alternatively, reporter RNA was preincubated with GST-fused MV-N at 30°C for 90 min and then added to rabbit reticulocyte lysate, followed by incubation for 90 min at 30°C. Translation products were resolved by SDS-12% PAGE and visualized by autoradiography. Luciferase bands were quantified by densitometric analysis. Translation inhibition experiments were performed a minimum of three times.

Conditional switching expression of MV-N in vivo. A cDNA clone encoding the MV-N gene was amplified by PCR using a specific primer pair (5′-GATCG AATTCGATATCCGAGATGGC-3′ and 5′-GATCGAATTCGGTCCTAGTT TTT-3′) and was inserted into the EcoRI site of the pCALNL5 conditional expression vector which contains the expression-switching reporter unit CALNL consisting of a CAG promoter, a Cre/loxP cassette, and a neomycin resistance gene (9). The plasmid was linearized with ScaI and transfected into 293 cells by using FuGENE6 reagent. Cells were selected with 0.5 mg of Geneticin/ml, and *neo*-resistant cell clones were designated 293-MVN cells. A recombinant adenovirus expressing Cre recombinase, AxCANCre (9), was prepared by using a standard procedure (19) and purified by ultracentrifugation (10). To obtain MV-N switching expression, 293-MVN cells were infected with AxCANCre in 24-well plates at an MOI of 10, 20, or 50. After 24 h, cells were lysed with SDS sample buffer and sonicated for 6 s. Samples were boiled for 5 min and subjected to Western blotting with a 1:1,000 dilution of anti-N rabbit polyclonal antibody and a 1:2,000 dilution of goat anti-rabbit IgG as described above.

In vivo reporter synthesis inhibition assay. Triplicate samples of 2×10^4 293-MVN and 293 cells were infected with AxCANCre in 24-well plates at an MOI of 10, 20, or 50. After 24 h, 50 ng of phRL-TK(Int-) vector encoding a *Renilla* luciferase reporter gene (Promega) was transfected into the cells using FuGENE6 reagent. After 24 h, the cells were harvested and washed with PBS. One aliquot of the cells was lysed with passive lysis buffer (Promega), and the luciferase activity was measured by using the *Renilla* luciferase assay system (Promega); another aliquot was treated with ISOGEN reagent, and the total RNA was subjected to real-time reverse transcription-PCR (RT-PCR) using the One-Step SYBR RT-PCR kit (TaKaRa) as recommended by the manufacturer's protocol. Briefly, total RNA was mixed with One-Step SYBR RT-PCR mixture containing *Renilla* luciferase sense primer (5'-CGTCCAGATTGTCCGCAACT A-3') and antisense primer (5'-CAATAGCGTTGGAAAAGAACCC-3') in a total volume of 50 μL. RT-PCR was carried out according to the manufacturer's instructions, and amplification data were analyzed by using the ABI Prism 7900HT sequence detection system.

In vivo protein synthesis inhibition assay. Triplicate samples of 2×10^4 293-MVN and 293 cells were infected with AxCANCre in 24-well plates at an MOI of 10, 20, or 50. After 24 h, the culture medium was replaced with 0.5 ml of DMEM containing one-tenth the normal amount of methionine and 50 μCi of [^{35}S]EasyTag Express protein labeling mix (Perkin-Elmer). At 24 h postlabeling, the cells were harvested and washed with PBS. An aliquot was lysed with lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 5 mM Tris-HCl [pH 8.0], 30 mM NaCl) containing 2% (vol/vol) of a protease inhibitor cocktail and centrifuged at $16,000 \times g$ for 10 min. Radioactivity incorporated into the cell lysate was measured by using a scintillation counter. An aliquot of the cells was treated with ISOGEN reagent, and total RNA was subjected to real-time RT-PCR with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) sense primer (5'-GCCT CAAGATCATCAGCAATG-3') and antisense primer (5'-GGTCATGAGTCC TTCCACGATA-3') using the One-Step SYBR RT-PCR kit as described above. Alternatively, 293-MVN and 293 cells infected with AxCANCre were lysed with SDS sample buffer and sonicated for 6 s. Samples were boiled for 5 min and subjected to Western blotting with a 1:1,000 dilution of anti-GAPDH monoclonal antibody (Chemicon) and a 1:2,000 dilution of rabbit anti-mouse immunoglobulin (Dako) as described above.

RESULTS

eIF3-p40 interacts with MV-N in a yeast two-hybrid assay. To identify proteins that bind to MV-N, a yeast two-hybrid screen was performed using the N protein of wild-type MV (the HL strain) as bait to screen a human B-cell library. Two-hybrid screening of 10^7 cDNAs identified several independent cDNA clones that had potential to interact with MV-N. Sequence analysis identified a clone that encoded the p40 subunit of eIF3 (eIF3-p40). The specificity of the interaction between MV-N and eIF3-p40 was confirmed by cotransforming the yeast, AH109, with the bait and the eIF3-p40 construct. Growth was only observed when yeast were transfected with MV-N and eIF3-p40 (Fig. 1A). To confirm the interaction between MV-N and eIF3-p40 in mammalian cells, the entire eIF3-p40 cDNA was inserted into an HA-tagged mammalian expression vector, and MV-N cDNA was cloned into a myc-tagged mammalian expression vector. These vectors were simultaneously transfected into Cos-7 cells and immunoprecipitated. Anti-myc antibody against myc-tagged MV-N coprecipitated eIF3-p40 (Fig. 1B). The anti-HA antibody, in contrast, failed to coprecipitate MV-N (data not shown), suggesting that this antibody may interfere with the protein-protein interaction. To further confirm whether MV-N binds to eIF3-p40 during MV infection, COBL-a cells, which have a high sensitivity to wild-type MV (13), were infected with MV-HL and then cross-linked with formaldehyde, followed by an immunoprecipitation assay with anti-N antibody. Endogenous eIF3-p40 was detected in the immunoprecipitate obtained from the MV-
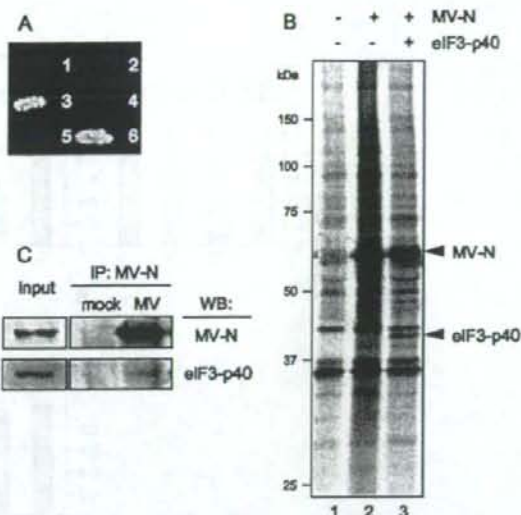

FIG. 1. Interaction between eIF3-p40 and MV-N. (A) Yeast strain AH109 was cotransfected with the following pairs of expression vectors and plated onto the selection medium without His, Leu, and Trp: (1) BD vector+AD-eIF3-p40, (2) BD-MV-N+AD-vector, (3) BD-MV-N+AD-eIF3-p40, (4) BD-lamin C+AD-eIF3-p40, (5) BD-MV-N+AD-SV40 large T antigen, and (6) BD-P53+AD-SV40 large T antigen. (B) Cos-7 cells were mock transfected (lane 1) or transfected with myc-tagged MV-N (lane 2) or myc-tagged MV-N and HA-tagged eIF3-p40 (lane 3). Immunoprecipitation was performed on total cell lysates using anti-myc antibody. Immunoprecipitates were detected by SDS-PAGE, followed by autoradiography. (C) COBL-a cells were infected with MV-HL at an MOI of 0.001. At 48 h postinfection, cells were cross-linked with formaldehyde, and the cell lysate was immunoprecipitated with anti-N monoclonal antibody. Immunoprecipitates were de-cross-linked by boiling and then detected by Western blotting with the indicated antibodies.

infected cells (Fig. 1C), suggesting that MV-N attaches, at least in part, to eIF3-p40 during MV infection.

MV-N protein inhibits translation of a reporter RNA in rabbit reticulocyte lysates. eIF3 is a 650-kDa complex formed by 11 or 12 different subunits and is one of the initiation factors required for protein synthesis in eukaryotes. Many viruses shut off host cell gene expression during virus infection using a variety of different strategies. Several RNA viruses inhibit the initiation of host mRNA translation by modulating various host translation initiation factors. Similarly, our preliminary data indicates that MV infection shuts off host protein synthesis without altering the level of cellular mRNAs (unpublished data), suggesting that inhibition occurs at the levels of host translation. To examine the functional consequences of the MV-N and eIF3-p40 interaction, we monitored the effect of MV-N on protein synthesis using an in vitro system. MV-N fused with GST (GST-N) was expressed by a recombinant baculovirus expression system and purified to homogeneity. GST and GST-fused MV-P protein (GST-P) were used as controls and prepared in a manner similar to that used for GST-N. Rabbit reticulocyte lysates were preincubated with increasing amounts of GST, GST-N, or GST-P, and the trans-
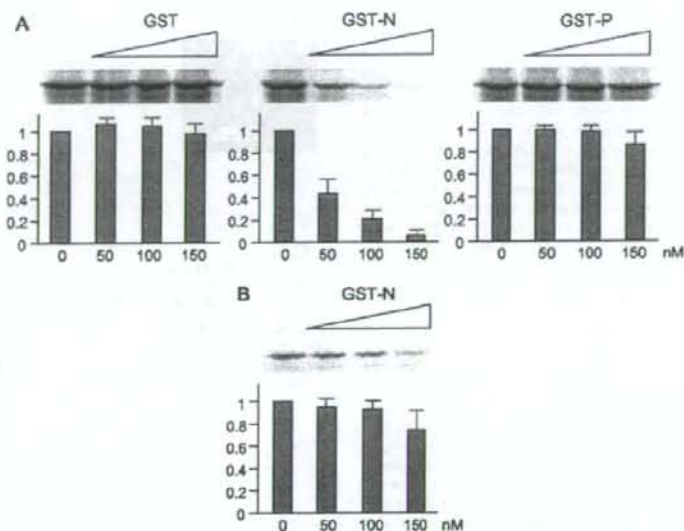
FIG. 2. Inhibition of in vitro translation by GST-fused MV-N. (A) Increasing amounts of recombinant GST, GST-N, or GST-P were incubated with rabbit reticulocyte lysates. A reporter RNA encoding the luciferase gene was added and translated in the presence of [^{35}S]methionine. Translated products were separated by SDS-PAGE and detected by autoradiography. The amount of luciferase synthesized was quantified by densitometric analysis. The results shown represent the means of three experiments. (B) A reporter RNA was preincubated with increasing amount of GST-N and then added to rabbit reticulocyte lysates. Translated products were quantified in a similar manner as panel A.

lation reaction was carried out using a capped reporter RNA. Translation products were detected by SDS-PAGE. As shown in Fig. 2, GST and GST-P did not interfere with the translation reaction, whereas GST-N markedly inhibited RNA translation in a dose-dependent manner (Fig. 2A), with up to 95% suppression observed at 150 nM GST-N. On the other hand, preincubation of reporter RNA with GST-N before addition to the rabbit reticulocyte lysate system had little effect on translation (Fig. 2B), indicating that the suppression by GST-N did not result from nonspecific binding between reporter RNA and GST-N. These results suggest that translation suppression is caused by interactions between MV-N and cap-dependent translation initiation factor(s) such as endogenous eIF3-p40.

Mapping of the eIF3-p40-interacting domain of MV-N. To map the domain of MV-N that is required for its interaction with eIF3-p40, six two-hybrid bait vectors with distinct deletions in MV-N (Fig. 3A) were constructed and cotransformed into yeast cells with the eIF3-p40 construct. Positive interactions were defined as the ability of transformed cells to grow on selection medium (SD/−His/−Leu/−Trp/3-AT). Two deletion clones (NΔ1 and NΔ2) failed to induce growth of the yeast cells (Fig. 3A). To confirm the region involved in binding, a mammalian expression vector containing the deletion clones NΔ1 or NΔ2 was coimmunoprecipitated with eIF3-p40. NΔ1 still bound eIF3-p40, whereas NΔ2 did not (Fig. 3B). These results indicate that the N-terminal residues, 81 to 192, of MV-N are required for binding to eIF3-p40. To identify the eIF3-p40-binding domain of MV-N more precisely, we generated three deletion clones between positions 81 and 192 (NΔ2a, -b, and -c) and performed coimmunoprecipitation in a similar manner. All of the deletion clones failed to bind eIF3-p40 (Fig. 3C),

indicating that the tertiary structure of the N-terminal region (residues 81 to 192) and/or residues 81 to 192 of MV-N is important for the interaction between MV-N and eIF3-p40.

Inhibition of in vitro translation by MV-N is mediated by its interaction with eIF3-p40. To examine the effect of eIF3-p40 binding to the N protein on MV-N-induced suppression of in vitro translation, the deletion clone, NΔ2c, which failed to interact with eIF3-p40, was fused with GST and added to the in vitro translation system. As expected, an equimolar amount of GST-NΔ2c showed a little ability to inhibit translation (Fig. 4). This result indicates that the inhibition of translation by MV-N is mediated by its specific interaction with eIF3-p40.

MV-N exhibits distinct effects on two types of IRES-mediated translation. To further evaluate the effect of the interaction between MV-N and eIF3 on translation, we investigated in vitro translation initiation mediated by two different IRESs. The mouse EMCV IRES utilizes canonical initiation factors, including eIF3, for translation initiation (18), and the RNA genome of PSIV possesses a unique IGR that functions as an IRES (22). The IGR-IRES can assemble 80S ribosomes in the absence of canonical initiation factors and can initiate translation in a methionine-independent manner (22, 29). We tested whether MV-N inhibited mRNA translation under the control of the EMCV-IRES or IGR-IRES. A reporter gene was added downstream of the EMCV-IRES or IGR-IRES, and these RNAs were translated in vitro with increasing amounts of GST-N. EMCV-IRES-mediated translation was inhibited by GST-N in a dose-dependent fashion (Fig. 5B). The inhibition rate was similar to that of cap-dependent translation, with up to 95% inhibition observed at 150 nM GST-N.
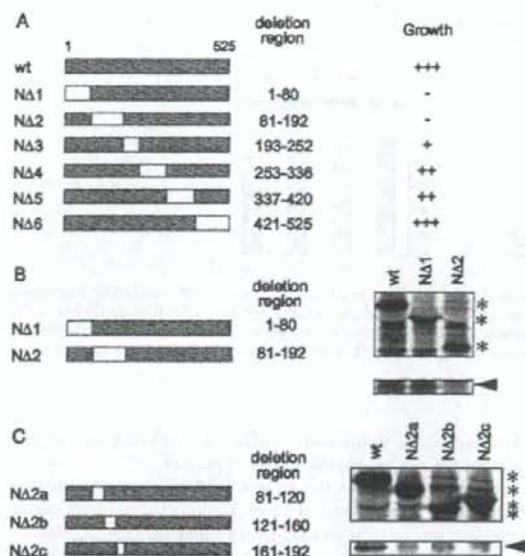
FIG. 3. Mapping the eIF3-p40-interacting domain of MV-N. (A) Parts of the N gene corresponding to amino acid residues 1 to 80, 81 to 192, 193 to 252, 253 to 336, 337 to 420, and 421 to 525 were deleted, and the resulting deletion clones were inserted into a BD vector for yeast two-hybrid analysis. These constructs were cotransformed with the AD-eIF3-p40 vector into yeast and plated onto selection medium lacking Leu and Trp (SD/−Leu/−Trp). The colonies obtained were spotted on SD/−His/−Leu/−Trp/3-AT plates and incubated at 30°C for 4 days. The apparent binding strength was assessed by the degree of growth and is scored as strong (+++), intermediate (++), weak (+), or absent (−). (B) Myc-tagged MV-N and its deletion clones, NΔ1 and NΔ2, were coexpressed with eIF3-p40 in Cos-7 cells and coimmunoprecipitated using anti-myc antibody. Asterisks indicate immunoprecipitated MV-N or its deletions. An arrowhead indicates the band of eIF3-p40 that was coprecipitated. (C) Parts of the NΔ2 deletion region corresponding to amino acid residues 81 to 120, 121 to 160, and 161 to 192 were further deleted, and the resulting deletion clones were coexpressed with eIF3-p40 in Cos-7 cells and coimmunoprecipitated. The asterisks indicate MV-N or its deletion clones precipitated using anti-myc antibody. Arrowhead indicates the band of eIF3-p40 coprecipitated.

On the other hand, inhibition of IGR-IRES-mediated translation was markedly reduced, with ca. 32% inhibition observed in the presence of equimolar amounts of GST-N (Fig. 5C). These results demonstrate that IGR-IRES-mediated translation is apparently insensitive to inhibition by GST-N compared to cap-dependent or EMCV-IRES-mediated translation and that MV-N inhibits host translation by directly interacting with eIF3.

MV-N inhibits translation in vivo. The physiological effect of MV-N-mediated translation inhibition was examined using whole cells. We examined the effects of MV-N on a cotransfected reporter gene by using a transient-transfection assay and observed that MV-N only slightly inhibited translation, probably because it was expressed in low levels (data not shown). As a result, we established a stable cell line that possessed a Cre/loxP expression-switching unit containing the MV-N gene (293-MVN cells). MV-N expression in 293-MVN cells was
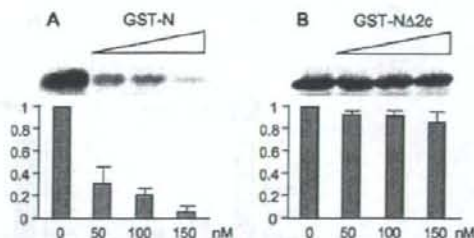

FIG. 4. MV-N lacking eIF3-p40-binding ability shows no inhibitory effect on translation. Increasing amounts of recombinant GST or GST-NΔ2c were incubated with rabbit reticulocyte lysates. A reporter RNA encoding the luciferase gene was added and translated in the presence of [³⁵S]methionine. Translated products were separated by SDS-PAGE and detected by autoradiography. The amount of luciferase synthesized was quantified by densitometric analysis. The results shown represent means of three experiments.

induced by infection with the recombinant adenovirus, AxCANCre, which expresses Cre recombinase. 293-MVN and parental 293 cells were infected with increasing amounts of AxCANCre, and MV-N expression was confirmed by Western blotting (Fig. 6A).

To examine the ability of MV-N to inhibit translation in whole cells, 293-MVN and parental 293 cells were infected with increasing doses of AxCANCre and MV-N was allowed to accumulate. The cells were transfected with a reporter gene encoding *Renilla* luciferase, and luciferase mRNA levels and activity were measured simultaneously. The luciferase levels and activity were comparable in 293-MVN and 293 cells (Fig. 6B). However, luciferase synthesis was progressively suppressed in 293-MVN cells as MV-N began to accumulate, resulting in 59% inhibition in the presence of AxCANCre used at an MOI of 50. To further investigate the effect of MV-N on the overall rate of cellular protein synthesis, AxCANCre-infected 293 and 293-MVN cells were pulse-labeled with [³⁵S]methionine in vivo, and the amount of incorporated radioactivity was determined. mRNA levels were also measured based on expression of the housekeeping gene, GAPDH (Fig. 6C). There was no difference in the rate of GAPDH mRNA transcription between 293 cells and 293-MVN cells, but overall protein synthesis in 293-MVN cells decreased up to 48% compared to 293 cells in the presence of AxCANCre used at an MOI of 50. In addition, Western blotting analysis demonstrated that the protein level of GAPDH was also decreased in 293-MVN cells (Fig. 6D). These results demonstrate that intracellular accumulation of MV-N results in a partial but substantial reduction in the overall rate of cellular protein synthesis.

DISCUSSION

In a previous study, it was demonstrated that although MV infection induces significant host shutoff, infected cells contain normal levels of host mRNA (unpublished data). This suggests that MV-induced shutoff occurs at the level of host translation. In most eukaryotic mRNAs, translation initiation commences with the recruitment of cap binding protein complex, eIF4F, composed of eIF4E (cap binding protein), eIF4A, and eIF4G, to the capped 5′ end (6). The 40S ribosomal subunit, which
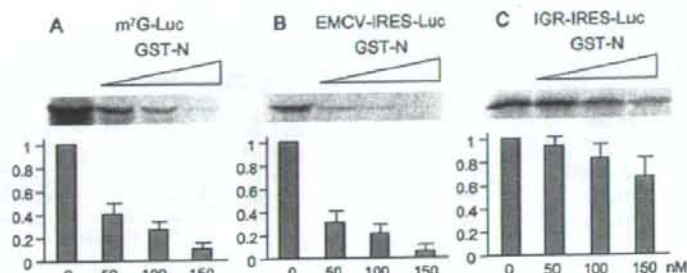
FIG. 5. MV-N selectively inhibits cap-dependent and EMCV-IRES-mediated translation but not IGR-IRES-mediated translation. Increasing amounts of GST-N were incubated with rabbit reticulocyte lysate. m^{7}G-luc RNA (A), EMCV-IRES-luc RNA (B), and IGR-IRES-luc RNA (C) were added and translated in the presence of [^{35}S]methionine. Translated products were separated by SDS-PAGE and detected by autoradiography. The amount of luciferase synthesized was quantified by densitometric analysis. The results shown represent the means of three experiments.

carries eIF3 and the ternary initiator tRNA$_i^{Met}$-eIF2-GTP complex, is then recruited to the 5′ end of the mRNA through interactions between eIF3 and eIF4G (6). The 40S subunits scan the mRNA in a 5′-to-3′ direction until an appropriate start codon is encountered. At this point, the anticodon in initiator tRNA (tRNA$_i^{Met}$), positioned in the ribosomal P site, engages in base pairing with the start codon in the mRNA. The large ribosomal 60A subunit joins and protein synthesis commences (6). It is known that several RNA viruses disrupt host translation initiation as part of their selective translation strategies. These viruses primarily target the eIF4F complex and related auxiliary factors in order to inhibit host translation. For example, during picornavirus infection, eIF4G is cleaved by virus-encoded proteases (5, 11, 17). In cells infected with EMCV and poliovirus, an eIF4E-binding protein (4E-BP) is dephosphorylated, resulting in sequestration of eIF4E by 4E-BP (3). During coxsackievirus and poliovirus infection, the poly(A)-binding protein, which forms a closed-loop translation complex in conjunction with eIF4G, is proteolyzed (8, 12). In influenza virus-infected cells, eIF4E is partially inactivated by dephosphorylation (2). Our preliminary studies indicate that no modifications of the eIF4F complex or any related auxiliary factors are observed during MV infection (unpublished data). In the present study, however, we show that MV-N has the ability to bind to eIF3-p40 and suppress mRNA translation in vitro (Fig. 1 and 2). Binding was necessary for suppression since the MV-N deletion clone, which does not interact with eIF3-p40, scarcely inhibited translation reactions (Fig. 4). These results indicate that MV-N uniquely suppresses translation reactions by binding to eIF3-p40. Our preliminary studies revealed that 293 and Cos-7 cells, which overexpressed SLAM (a receptor for wild-type MV) by plasmid transfection, permitted wild-type MV infection and replication (unpublished data). This implies that the results observed in Cos-7 cells (Fig. 1 and 3) and 293-MVN cells (Fig. 6) reflect the phenomenon during MV infection. To further confirm the effect of MV-N on translation in MV-infected cells, we attempted to generate a recombinant MV that possesses mutant N protein lacking the binding site to eIF3-p40 using reverse genetics. However, the variant virus could not be rescued by several trials using conditions with which other recombinant MVs were well rescued (data not shown). This result implied that the amino acid residues

that participate in interaction with eIF3-p40 may also be important for the other function of N protein.

Mammalian eIF3 is the largest (650 kDa) of all initiation factors and is composed of 11 or 12 different subunits whose precise patterns of interaction with other factors and stoichiometry remain poorly understood. To analyze the binding manner of MV-N on eIF3 complex, we attempted to immunoprecipitation assay using antibodies against several subunits of eIF3, but they did not pull down the whole eIF3 complex effectively, and the exact interaction between MV-N and eIF3 complex could not be defined (data not shown). Recent reports have revealed that a viral protein and several host factors regulate host protein synthesis by modulating eIF3 function. For example, the virus- and interferon (IFN)-inducible human protein, p56, binds to the p48 subunit of eIF3 and inhibits in vitro translation and cellular protein synthesis (4). Mouse p56 also inhibits in vitro translation by binding to the p110 subunit of eIF3 (4). Cyclin-dependent kinase 11 interacts with the p47 subunit of eIF3 and suppresses translation by phosphorylating a specific serine residue in the p47 subunit (24). In Norwalk virus, the RNA genome-linked protein VPg binds to the p66 subunit of eIF3 and inhibits in vitro translation reactions (1), which are thought to initiate protein synthesis from viral RNA. Our present study reveals a new mode of mammalian protein synthesis regulation that involves the modulation of eIF3 function via the p40 subunit.

GST-N inhibited both cap-dependent and EMCV-IRES-mediated translation in a dose-dependent manner (Fig. 5). The EMCV-IRES requires canonical eIFs, including eIF3, in order to initiate translation (18). Stable binding of eIF3 to the IRES is essential for the attachment of the 40S subunit to the IRES (26). This suggests that the suppression of EMCV-IRES-mediated translation by MV-N may occur through binding of MV-N to eIF3-p40, as seen in cap-dependent translation. In contrast, IGR-IRES-mediated translation is apparently insensitive to the inhibitory effects of GST-N (Fig. 5). The IGR-IRES can assemble 80S ribosomes from purified 40S and 60S ribosomal subunits in the absence of eIF2, tRNA$_i^{Met}$, or GTP hydrolysis and without any known canonical eIFs (29). Thus, eIF3 is not involved in IGR-IRES/40S subunit assembly. These unique characteristics may explain why IGR-IRES-mediated translation is only modestly inhibited by GST-N. At high con-
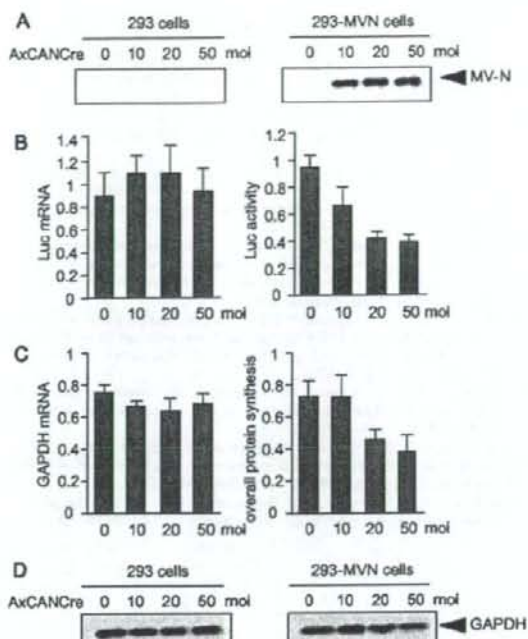
FIG. 6. Inhibition of protein synthesis by MV-N in vivo. (A to D) 293-MVN cells or 293 cells were plated in 24-well plates and infected with a recombinant adenovirus, AxCANCre, expressing Cre recombinase at an MOI of 10, 20, or 50. (A) At 24 h postinfection, switching expression of MV-N in 293-MVN cells was detected by Western blotting. (B) At 24 h postinfection, a reporter plasmid encoding the luciferase gene was transfected and incubated for 24 h. Cells were harvested, an aliquot was lysed, and luciferase activity was measured by using a luminometer. Another aliquot was harvested, and the total RNA was analyzed for luciferase transcripts using one-step real-time RT-PCR. The luciferase activity and mRNA levels in 293-MVN cells are expressed relative to those in 293 cells. The results shown represent means of three experiments. (C) At 24 h postinfection, cells were labeled with [^{35}S]methionine for 24 h. An aliquot of the cells was lysed, and the level of radioactivity incorporated in all proteins was measured by using a scintillation counter. An aliquot of the cells was harvested, and the total RNA was analyzed for transcripts of the housekeeping gene, GAPDH, using one-step real-time RT-PCR. The levels of radioactivity and GAPDH mRNA in 293-MVN cells are expressed relative to those in 293 cells. The results shown represent means of three experiments. (D) At 48 h postinfection, cells were lysed and endogenous GAPDH was detected by Western blotting.

centrations of GST-N, slight suppression of IGR-IRES-mediated translation is observed; however, this may result because endogenous initiation factors are still be present in the lysates, and binding of MV-N to endogenous eIF3 may be interfering with 40S subunit binding to IGR-IRES.

MV-N induced the suppression of protein synthesis at the level of translation both in vitro and in whole cells (Fig. 6). However, suppression in whole cells was partial, and suppression rates reached a plateau at ca. 50 to 60% inhibition. Our preliminary data indicate that MV-infected cells suppress more than 90% of host protein synthesis at 36 h postinfection (unpublished data), suggesting that one or more additional

pathways are involved in MV-mediated host shutoff. Previous reports on other viral infections show that type I IFN induced by infection activates multiple pathways to shutoff host function, including phosphorylation of eIF2α by double-stranded RNA-activated protein kinase (21), activation of RNase L and subsequent degradation of mRNA by 2',5'-oligoadenylate synthetase (27), and induction of virus-inducible protein P56 which binds to eIF3-p48 (4, 7). However, a previous report showed that the suppression of overall cellular protein synthesis by IFN is not complete but rather only ca. 50% inhibition (4). Indeed, we have shown that MV infection induces phosphorylation of eIF2α, but its participation for the inhibition of host protein synthesis in the overall host shutoff is partial and restricted at an early phase of infection (Inoue et al., unpublished). These data indicate that significant host shutoff induced by MV may occur through multiple cooperating pathways.

In addition to the significant suppression of host translation, we have confirmed that the selective translation of viral mRNA still occurs in MV-infected cells (unpublished data). Recently, our preliminary study identified a host factor that is implicated in the preferential translation of MV mRNAs in infected cells (unpublished data). From these data, it can be speculated that MV utilizes advanced strategies that control the host cells while evade MV itself from shutoff state in the infected cells.

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