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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.

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

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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 I (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 *Fse*I 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 *Fse*I 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

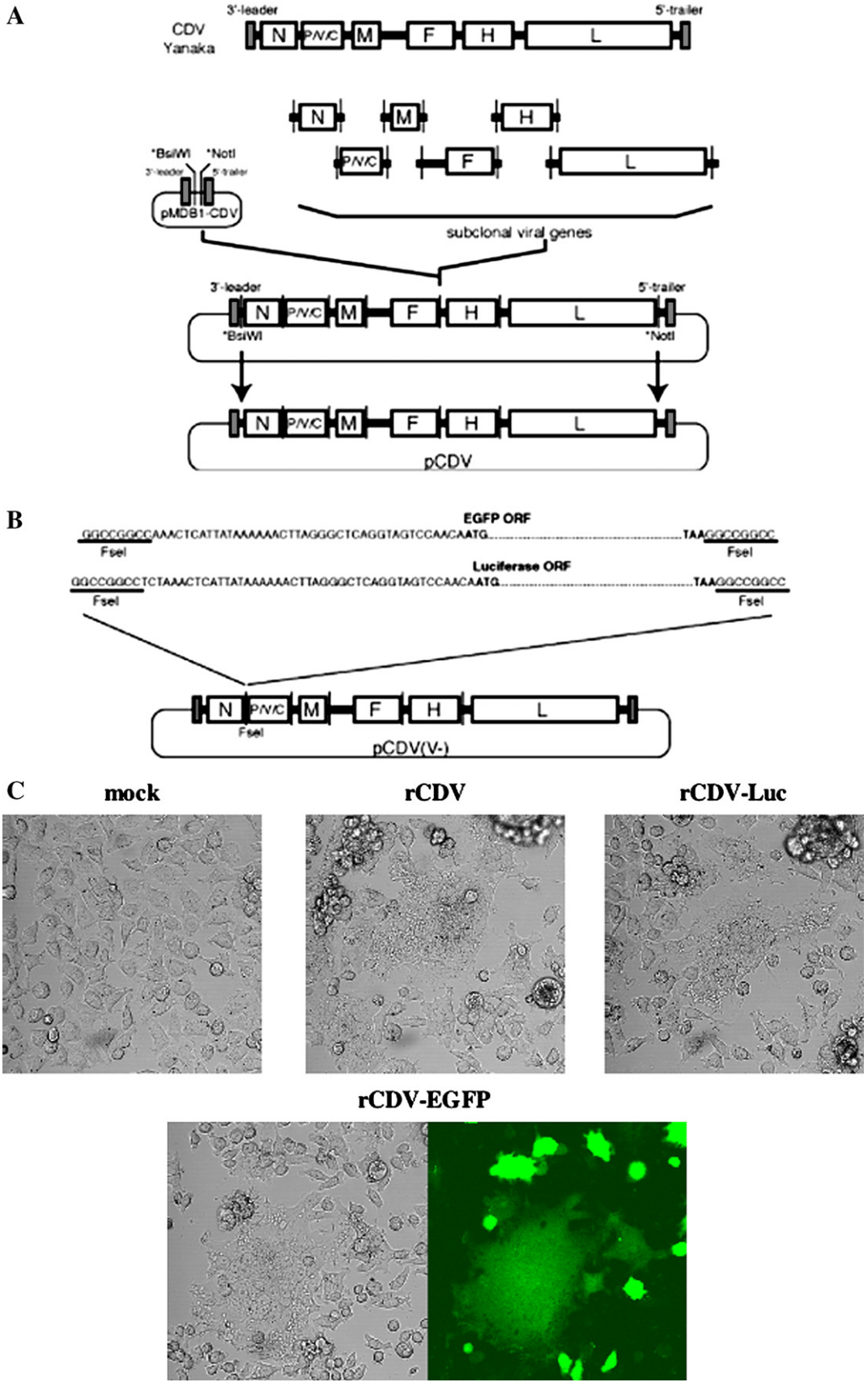
A
3'-leader
5'-trailer
CDV Yanaka
N
P/V/C
M
F
H
L
*BsiWI
*NotI
pMDB1-CDV
subclonal viral genes
pCDV
B
EGFP ORF
GGCCGGCCAAACTCATTATAAAAAACTTAGGGCTCAGGTAGTCCAACAATG
TAAGGCCGGCC
FseI
Luciferase ORF
GGCCGGCCTCTAAACTCATTATAAAAAACTTAGGGCTCAGGTAGTCCAACAATG
TAAGGCCGGCC
pCDV(V-)
C
mock
rCDV
rCDV-Luc
rCDV-EGFP

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 franked 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

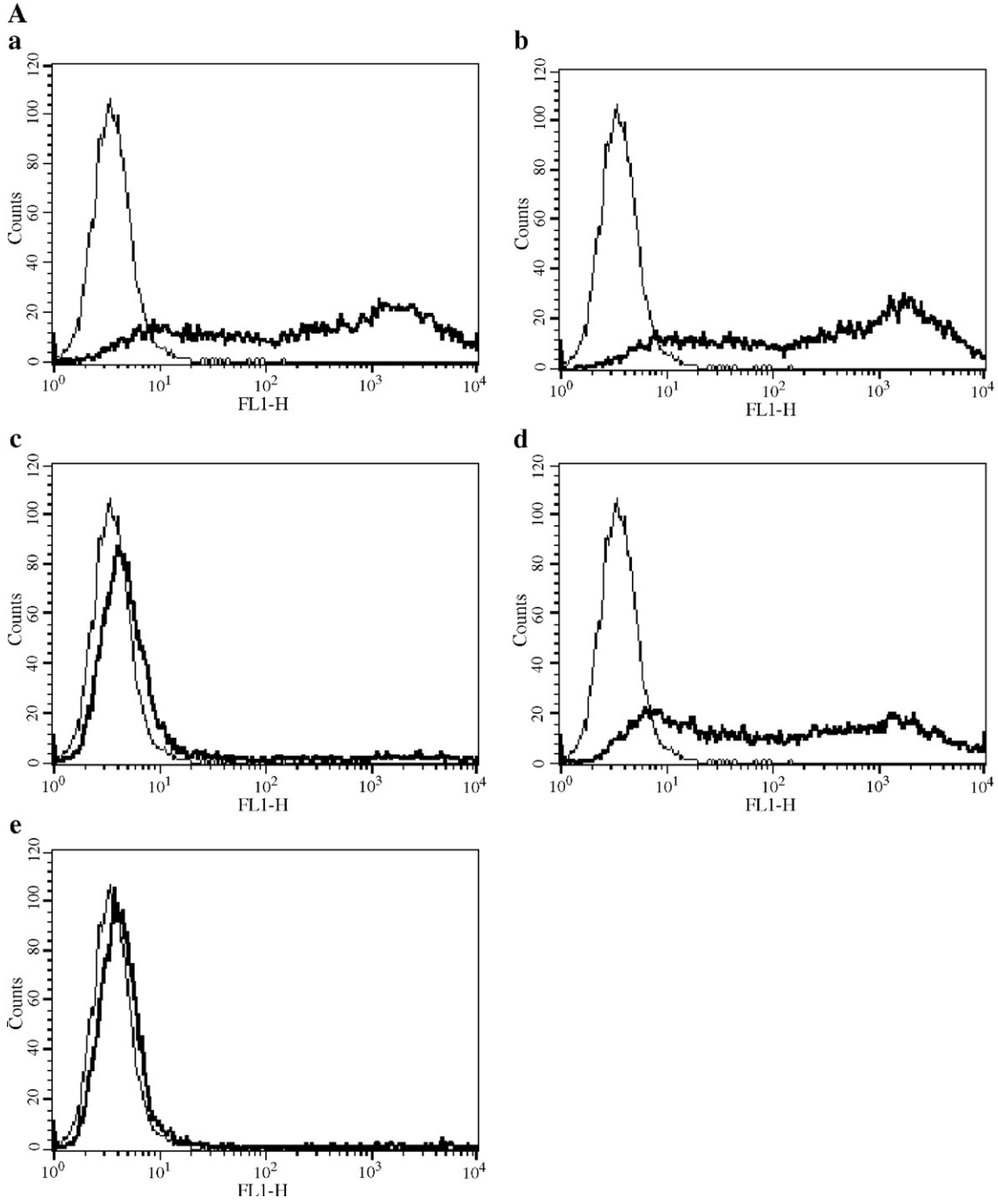

Fig. 2. Effect of monoclonal antibodies against CD46 or SLAM or heparin on rCDV-EGFP infection. Cells were treated with monoclonal antibodies against either CD46 or SLAM prior to infection with rCDV-EGFP. Meanwhile, rCDV-EGFP was treated with heparin prior to inoculation. (A) B95a cells. (B) 293 cells. 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.

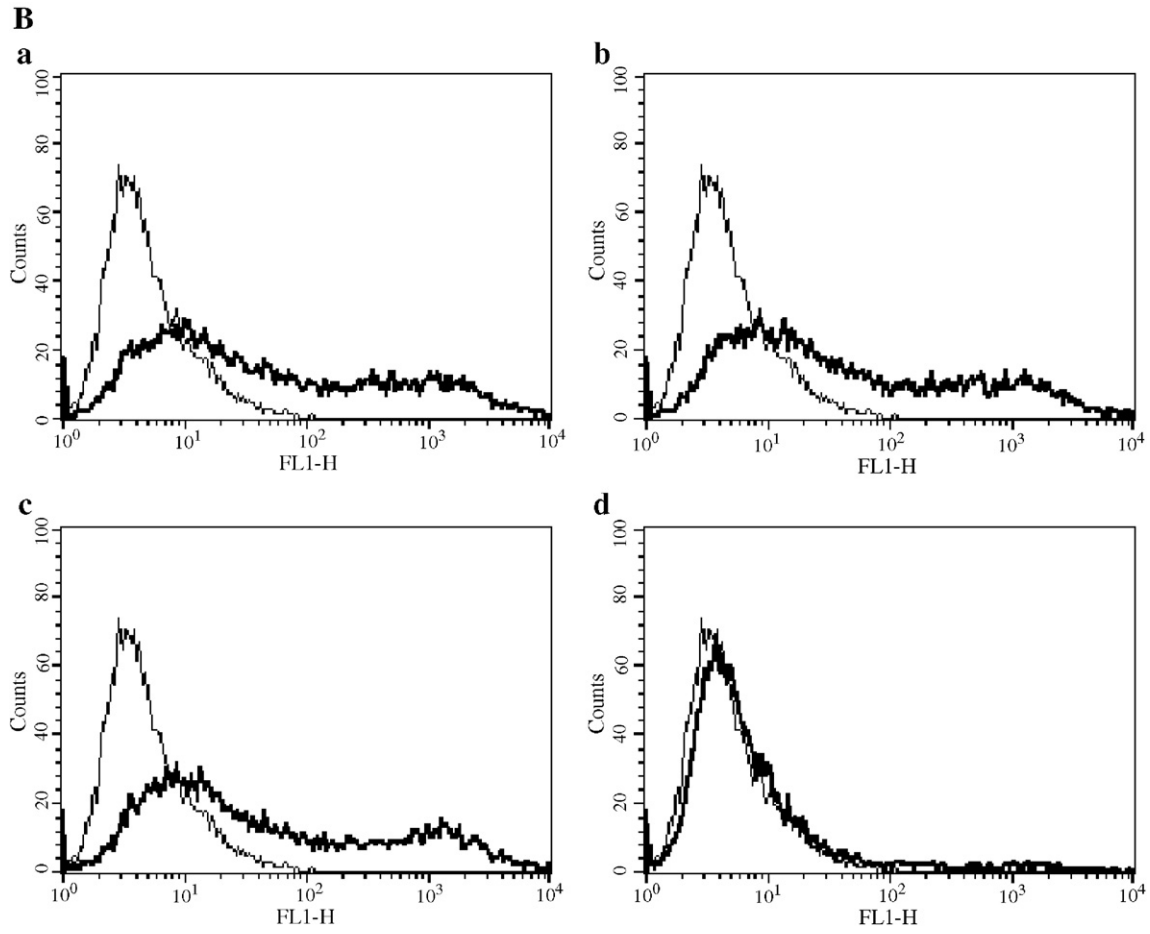

Fig. 2 (*continued*).

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

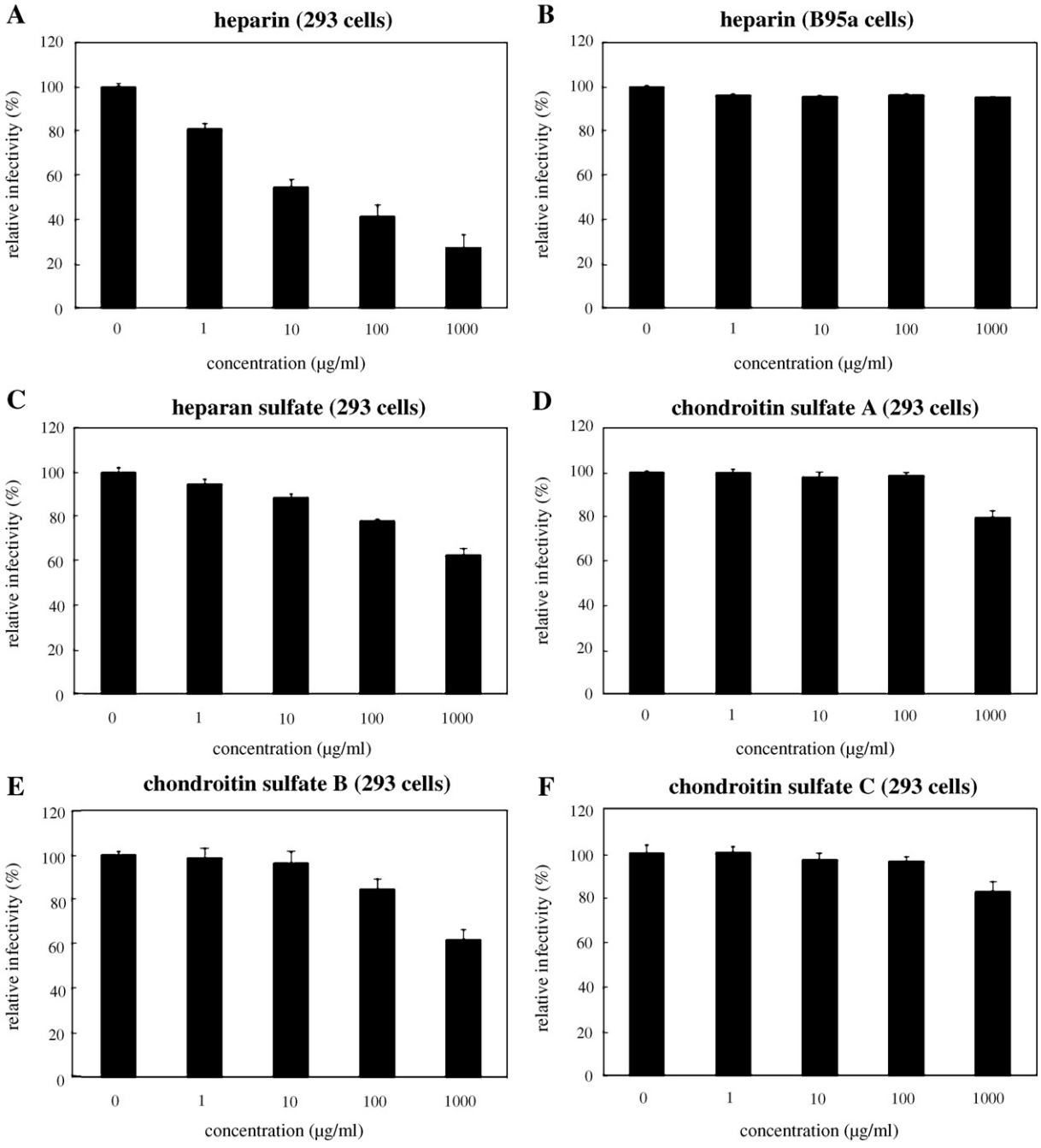

Fig. 3. Inhibition of rCDV-EGFP infection by soluble GAGs. rCDV-EGFP was treated with heparin (A), heparan sulfate (C), chondroitin sulfate A (D), chondroitin sulfate B (E) or chondroitin sulfate C (F) at different concentrations for 1 h and inoculated to 293 cells. The virus was also treated with heparin 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;

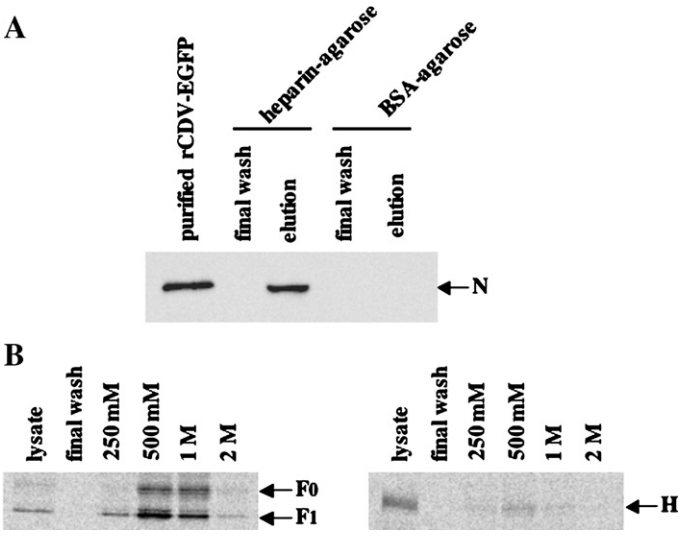

Fig. 4. Heparin affinity chromatography of rCDV-EGFP and its glycoproteins. (A) Heparin affinity chromatography of rCDV-EGFP. Lane 1: positive control (purified rCDV-EGFP), lane 2: final wash fraction from heparin-agarose, lane 3: elution fraction from heparin-agarose, lane 4: final wash fraction from 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′-GACGTACGTCAGGGTTCAGACCTACCAGTATGGCTAGCCTTCTTAAG-3′ and 5′-ACCTCGAGGCCGGCCTTAATTGAGTAGCTCTCT-3′, 5′-GCCTCGAGGTGTTACATCAGTCACCA-3′ and 5′-TATCTAGAGTTTAAACTTAAGCATGTGTGATACT-3′, 5′-ACTCTAGATAATCTATTAACAGGTTCA-3′ and 5′-TAACGCGTTAGAGAATTTTGAAAAG-3′, 5′-TAACGCGTTAGTTCATGAACTAAAACTC-3′ and 5′-GTGGTACCGCGATCGCTCAGAGTGATCTTACATA-3′, 5′-GCGGTACCACGTCTTACCTGATTGTTA-3′ and 5′-GAGTCGACTTAATTAACTATCAAGGTTTTGAACG-3′, 5′-AAGTCGACTCTCAATTGAACTTAAGGA-3′ and 5′-TGGCGGCCGCAGGGTTAGGATCCAGACC-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′-GAGTGTGGACCCATTAAGAAAGGCACAGGAGAGAG-3′ and 5′-CTCTCTCCTGTGCCTTTCTTAATGGGTCCACACTC-3′.

Enhanced green fluorescent protein (EGFP) gene was amplified from pEGFP-N1 (Clontech) using the following primers. 5′-TAAGGCCGGCCAAACTCATTATAAAAAACTTAGGGCTCAGGTAGTCCAACAATGGTGAGCAAGGGCGAGGA-3′ and 5′-TCGAGGCCGGCCTTACTTGTACAGCTCGTCCA-3′ (*Fse*I site underlined). The PCR products were cloned into pCR2.1 vector (Invitrogen), and sequences were confirmed. Then, the plasmid was digested by endonuclease *Fse*I and inserted into the *Fse*I site of pCDV V(−) (downstream of the N gene). Firefly luciferase gene was amplified from pGL3-basic (Promega) using the following primers. 5′-GAATGCTTCTAAACTCATTATAAAAAACTTAGGGCTCAGGTAGTCCAACAATGGAAGACGCCAAAAAACA-3′ and 5′-GAATGCTTTACACGGCGATCTTTCCGC-3′. The PCR product was cloned into pCR2.1 vector (Invitrogen) and sequences were confirmed. The plasmid was digested by restriction enzyme *Bsm*I, and the cutting ends were polished with T4 DNA polymerase (Takara). The fragments were ligated to *Fse*I linker (5′-AGGGGCCGGCCCCT-3′; *Fse*I site underlined) which had been annealed and phosphorylated with T4 polynucleotide kinase (TOYOBO) and digested by *Fse*I. The fragment was cloned into *Fse*I 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′-CCGGAATTCTTAGGGTCCAGGACATAGCA-3′ and 5′-CCGGAATTCGACTACCTGAGCCCTAAGTT-3′. The PCR product was phosphorylated by T4 polynucleotide kinase (Takara) and inserted into *Xho*I 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 *Eco*RI and inserted into *Eco*RI 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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