

A novel Borna disease virus vector system that stably expresses foreign proteins from an intergenic noncoding region

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Running Title: Establishment of a novel BDV vector

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Abstract

Borna disease virus (BDV), a nonsegmented, negative-strand RNA virus, infects a wide variety of mammalian species and readily establishes a long-lasting, persistent infection in brain cells. Therefore, this virus could be a promising candidate as a novel RNA virus vector enabling stable gene expression in the central nervous system (CNS). Previous studies demonstrated that the 5' untranslated region of the genome is the only site for insertion and expression unit of a foreign gene. In this study, we established a novel BDV vector, in which an additional transcription cassette has been inserted into an intercistronic noncoding region between the viral phosphoprotein (P) and matrix (M) genes. The recombinant BDV (rBDV) carrying green fluorescent protein (GFP) between the P and M genes, rBDV P/M-GFP, expressed GFP efficiently in cultured cells and rodent brains for a long-period of time without attenuation. Furthermore, we generated a non-propagating rBDV, Δ GLLP/M, which lacks the envelope glycoprotein (G) and a splicing intron within the polymerase gene (L), by the trans-complementation system with either transient or stable expression of the G. Interestingly, rBDV Δ GLLP/M established a persistent infection in cultured cells with stable expression of GFP in the absence of the expression of G. Using persistently infected rBDV Δ GLLP/M-infected cells, we determined the amino acid region in the cytoplasmic tail (CT) of BDV G important for the release of infectious rBDV particles and also demonstrated that the CT region may be critical for the generation of pseudotyped rBDV having vesicular stomatitis virus G protein. Our results revealed that the newly established BDV vector constitutes an alternative tool, not only for stable expression of foreign genes in the CNS but also for understanding the mechanism of the release of enveloped virions.

Introduction

Borna disease virus (BDV) belongs to the *Bornaviridae* family within the non-segmented, negative-strand RNA viruses and is characterized by highly neurotropic and noncytopathic infection (18, 31). Previous studies revealed that BDV infects a wide variety of mammalian species, suggesting that its host range probably includes all warm-blooded animals. The most striking feature of BDV is that it readily establishes a long-lasting persistent infection in the cell nucleus (9, 31). BDV establishes a stable infection without causing apparent cell damage, even in brain cells (8), making this the only animal RNA virus capable of intranuclear parasitism. These features of BDV suggest that this virus could be a promising candidate for efficient and stable gene delivery system to the central nervous system (CNS).

Recent developments of the reverse genetics system of BDV revealed that the virus has the capacity to express foreign genes from the 5' end of the genome (26). Recombinant BDV (rBDV) with green fluorescence protein (GFP) near the 5' end of the genome (rBDV 5'GFP) successfully infected, and was propagated in, cultured cells. This study suggested that the 5' end of the BDV genome might be the only site for insertion of the GFP expression cassette, because rBDV could not be rescued after introduction of the cassette into other regions of the genome (26). On the other hand, a recent study using the rBDV 5'GFP revealed that the termination signal upstream of the GFP gene is modified by insertion of additional A residues, resulting in downregulation of GFP expression within several weeks of infection of rat brains (2). This observation suggested that although the 5' end of the BDV genome is capable of the expression of a foreign gene, an additional transcriptional cassette in this region may affect the replication of BDV adversely, probably because of destruction of specific genome-end

structures, such as the inverted terminal repeat (ITR) and panhandle structure, both of which could be important for the transcription/replication processes of the viral polymerase (24, 25, 27). Furthermore, because there is a clear polarity of initiation of transcription from the 3' end to the 5' end of the genome in mononegaviruses (17, 23, 29), a transcription unit at the 5' end of the genome may be unsuitable for efficient expression of foreign genes. Considering the complexities of genome construction and the transcription mechanism of BDV, it may be conceivable that BDV has a lower capacity than other mononegaviruses for the insertion of an additional expression unit in the genome.

As well as the site of insertion of a foreign gene cassette in the genome, the flexibility of the virus genome to retain the capacity to replicate efficiently, as well as host cell tropism, also could be important in establishing a more effective and useful virus vector system. In many virus vectors, propagation-defective and pseudotyped viruses have been developed by modifying viral genomes and deletion or mutation of viral genes (4, 5, 13, 35). In BDV, some point mutations, including in the phosphoprotein (P), X and RNA polymerase (L) genes, have been shown to modify the efficiency of virus replication in cultured cells and animal brains (1, 21). For example, two amino acid substitutions, L1116R and N1398D, in the L gene are known to enhance significantly the polymerase activity, as well as the replication kinetics, of rBDV (1). However, there have been only few studies on the modification of the viral genome to develop the efficient and safe BDV vector system.

In this study, we established a novel BDV vector, BDV P/M, which harbors an extra transcription unit in the short intergenic noncoding region of the genome between the P and matrix (M) genes, P/M. BDV P/M was shown to stably express several reporter genes,

including GFP, DsRed and luciferase, in cultured cells without attenuation of their expression. Furthermore, we developed a non-propagating rBDV, Δ GLLP/M, which lacks an envelope glycoprotein (G) gene and a splicing intron within the L gene, by the trans-complementation of the G. Interestingly, rBDV Δ GLLP/M-GFP was able to establish persistent infection in a single cell without the expression of G. We also show that the cytoplasmic tail (CT) of BDV G may be essential for the release of infectious particles and the generation of pseudotyped BDV virions having vesicular stomatitis virus (VSV) G protein, by using the rBDV Δ GLLP/M virus system. Our results show that the BDV P/M vectors may constitute a new system, not only for efficient and safe gene delivery in vivo, but also for understanding the mechanism of envelope assembly and virion formation of BDV, which also could be important for improving the utility of BDV as a virus vector.

Materials and Methods

Cells. Vero cells and Vero cells stably expressing BDV G (Vero-BG) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS). The OL cell line, derived from a human oligodendroglioma, was cultured in DMEM-high glucose (4.5%) supplemented with 5% FBS. HEK 293T cells were cultured in DMEM supplemented with 10% FBS.

Plasmid constructions. The RNA polymerase II (Pol II)-driven BDV minigenome plasmid, pCAG-HRSV3, has been described previously (34). To generate a plasmid expressing the BDV full-length antigenome RNA, pFct-BDV, a cloned full-length cDNA of

BDV strain He/80 was inserted into the *Xho* I and *Not* I sites of pCAG-HRSV3. A plasmid containing an extra transcription cassette, pFct-BDV P/M, was generated by insertion of transcription initiation (S3) and termination (T2) signal sequences in the P/M region with *Bst*B I and *Pac* I sites (Fig. 1A). The plasmid harboring the expression cassette in the 5' end of the genome, pFct-BDV 5', also was constructed using the *Bst*B I and *Pac* I restriction enzymes. The L_{RD} mutation (1) was introduced into the pFct-BDV P/M plasmid by PCR-based mutagenesis. To generate foreign gene expression vectors, reporter genes, including GFP, DsRed, luciferase and LacZ were amplified from peGFP-N1 (Clontech), pDsRed-Monomer-C1 (Clontech), pGL3-Promoter Vector (Promega), and pcDNA 5/TO/lacZ (Invitrogen) and inserted into pFct-BDV P/M or pFct-BDV Δ GLLP/M vector with *Bst*B I and *Pac* I sites.

The pFct-BDV Δ GP/M-GFP, in which two ATG codons (nt 2236 and 2248) in the G ORF were changed to ACG, was constructed from pFct-BDV P/M GFP by PCR mutagenesis (Fig. 3A). The intron II-deleted, L ORF-linearized BDV vector, pFct-BDV Δ GLLP/M GFP, was developed by deleting the intron II sequence (nt 2410 to 3703) from pFct-BDV Δ GP/M GFP using a PCR amplification method (Fig. 3A).

The expression vector, pBDV-G, was generated by subcloning the G ORF of strain He/80 into the *Kpn* I and *Sma* I sites of plasmid pCXN2. For efficient expression of BDV G in transfected cells, silent mutations were introduced at the splicing donor (SD) and acceptor (SA) sequences in the G gene. The cDNA of VSV G was amplified from pVPack-VSV-G (Stratagene) and subcloned into the *Eco*R I and *Eco*R V sites of pCXN2 vector to yield pVSV-G. The rabies virus (RaV) G expression plasmid, pRaV-G, was constructed by

subcloning of the Nishigahara strain G, which was kindly gifted by Naoto Ito (Gifu University, Japan), into the *Kpn* I and *EcoR* V sites of pCXN2 vector. Expression plasmids of chimeric G proteins of VSV and RaV, in which the CT regions were exchanged with that of BDV G, were constructed by PCR mutagenesis. Detailed information about the primers and PCR procedures used to generate these plasmids is available from the authors. Nucleotide sequences of the recombinant plasmids were confirmed by DNA sequencing.

Virus rescue. 293T cells were seeded in 35 mm dishes and transfected with 2 µg of BDV vector plasmid, 0.25 µg of pCA-N (34), 0.025 µg of pCXN2-P (34) and 0.25 µg of pCA-L (34) using LipofectamineTM2000 (Invitrogen). To rescue rBDV ΔGP/M GFP and ΔGLLP/M GFP, 0.1 µg of pBDV-G was transfected together with the helper plasmids expressing BDV N, P and L into 293T cells. Three days posttransfection, the cells were detached using trypsin and seeded onto 10 cm dishes. One day after the first passage, approximately 1×10^5 Vero or Vero-BG cells were cocultured with the transfected 293T cells. The cultures were split every 3 days and the rescue efficacy of recombinant viruses was evaluated by fluorescence microscopy using an anti-BDV N monoclonal antibody (mAb) or by monitoring the expression of GFP. The non-propagating rBDVs were rescued by transient transfection with expression plasmids encoding BDV, VSV and RaV G and chimeric G protein between these viruses. Vero cells stably infected with rBDV ΔGLLP/M-GFP in 35 mm dishes were transfected with 3 µg of the G expression construct using LipofectamineTM2000 (Invitrogen) and the virus particles were collected from the culture supernatant or from sonicated cells at 48 h posttransfection.

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156 **Virus infection.** Vero or Vero-BG cells were infected with the amounts of rBDV stock
157 indicated at 37°C. After absorption for 1 h, the cells were washed with PBS (-) and passaged
158 with the appropriate interval of a few days. Virus propagation was detected by indirect
159 immunofluorescence or GFP assay.

160 C57BL/6J mice and Lewis rats (Oriental Yeast Co. Ltd., Shiga, Japan) were inoculated
161 intracranially with 1×10^3 focus forming units (FFU) of rBDV stock per animal within 24 h
162 of birth. The infected animals were sacrificed at the appropriate month after inoculation and
163 the brains were collected for analysis. To assess GFP expression in the brain, the whole or
164 cross sections of the brains were rinsed with PBS (-) and examined under a Leica WILD M10
165 fluorescence stereomicroscope (Leica Microsystems, Wetzlar, Germany). Brain samples were
166 then fixed in 4% paraformaldehyde, cryopreserved in 25% sucrose, and frozen in Tissue-Tek
167 O.C.T. compound. Cryostat sections (4 μ m) were examined under a fluorescence microscope.
168 Sections also were examined by immunohistochemical analysis after staining with anti-BDV
169 N mAb. All animal experimentation conformed to the guide for the care and use of laboratory
170 animals of the Research Institute for Microbial Diseases, Osaka University.

171

172 **Histological analysis.** Frozen sections were incubated with trypsin solution (0.1% trypsin,
173 0.1% CaCl_2 , and 0.05 M Tris-HCl, pH 7.5) for 5 min at 37°C. The sections were then
174 permeabilized by PBS (-) with 0.05% Triton-X and blocked with 10% normal goat serum in
175 PBS (-) for 30 min at room temperature. The sections were incubated with primary antibody
176 with 5% normal goat serum in PBS (-) overnight at 4°C and then with the secondary

antibodies with PBS (-) for 1 hour at 37°C. The cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI).

Indirect immunofluorescence assay (IFA). Cells were fixed with 4% paraformaldehyde and permeabilized by PBS (-) with 0.4% Triton-X. After permeabilization, the cells were incubated with the primary antibody, anti-BDV N mAb, for 1 h at 37°C. Subsequently, the cells were washed three times with PBS (-) and incubated with secondary antibody, goat anti-mouse IgG antibody conjugated with Cy3 (Jackson Immuno Research) or goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (Invitrogen) for 1 h at 37°C. The cells were counterstained with DAPI. After further washing with PBS (-), the cells were examined with an inverted microscope Ti-E with a confocal laser scanning system C1 (Nikon).

Luciferase and LacZ reporter assays. Vero cells were infected with rBDV P/M-luciferase in 16 mm culture dishes. At 24, 48 and 72 h postinfection, cells were lysed and subjected to luciferase assay with Luciferase Cell Culture Lysis Reagent (Promega), according to the manufacturer's recommendations. The activity of β -Galactosidase from the rBDV Δ GLLP/M-LacZ infected Vero-BG cells was analyzed by β -Galactosidase Staining Kit (PanVera), according to the manufacture's recommendations.

Western blot analysis. Vero cells infected with rBDV were lysed and the proteins separated with 12% SDS-PAGE, followed by transfer to Immobilon-P Transfer Membrane (Millipore). Membranes were then blocked with 5% skimmed milk in PBS (-) and incubated

with the primary antibodies (anti-BDV N and P mAb and anti-BDV M and G polyclonal antibodies [pAb]. After three washes with 0.05% Tween-20 in PBS (-), horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research) were applied for 1 h at 37°C. The membrane was washed three times and bound antibodies were detected using the ECL Western Blotting System (Amersham Pharmacia Biotech).

Northern blot analysis. Total RNA was extracted from cells infected with rBDV with TRIzol reagent (Invitrogen). Aliquots of 1.0 µg of total RNA were electrophoresed through a 1.0% agarose gel containing 2.2% formaldehyde and transferred onto Nylon Membranes (Roche) with 20 × SSC (1 × SSC = 150 mM NaCl plus 15 mM sodium citrate, pH 7.0). After UV cross-linking, the membrane was prehybridized in hybridization solution for 30 min at 65°C. This step was followed by hybridization overnight with a digoxigenin (DIG)-labeled RNA probe. The hybridized probe was detected with an alkaline phosphatase-conjugated anti-DIG Fab fragment (Roche)

Results

Efficient expression of foreign genes from an intergenic region between the P and M genes of BDV. A previous study by Schneider et al. (2007) showed that replication-competent rBDV was not able to be rescued when an extra transcription unit was inserted into the P/M region of the BDV genome (26). To examine further the availability of the P/M region for expression of a foreign gene, we inserted a GFP cassette into the site as indicated in Fig. 1A. The nucleotide sequences of the cassette could be slightly different from

those reported previously, at least in terms of the restriction enzyme sites for cloning of the cassette, whilst both cassettes use the same T2 and S3 sequences for additional transcription termination and initiation signals, respectively (Fig. 1A). We transfected the resultant plasmid, pFct-BDV P/M-GFP, which is driven by the Pol II promoter, into 293T cells along with helper plasmids (see Materials and Methods). A few weeks after cocultivation with Vero cells, rBDV harboring the GFP gene in the P/M region, termed rBDV P/M-GFP, was rescued successfully (Fig. 1B). Consistent with previous studies (26), the L1116R and N1398D mutations in the L gene (L_{RD}) enhanced the efficiency of rescue of the rBDV P/M-GFP virus (data not shown) and, therefore, we used the L_{RD} mutant plasmid in the backbone of BDV P/M vectors for further experiments.

At first, we infected Vero cells with cell-free rBDVs at a multiplicity of infection (MOI) of 0.01 and the growth kinetics of rBDV P/M-GFP were compared to wild-type (wt) recombinant virus and rBDV 5'GFP. As shown in Fig. 1C, the growth kinetics of both GFP-expressing viruses seemed to be slightly retarded compared to wt rBDV but they were propagated efficiently and reached over 90% infection by 3 weeks postinfection. We also performed IFA to investigate the expression of GFP in Vero cells. As shown in Fig. 1D, rBDV P/M-GFP induced an apparently stronger GFP signal than rBDV 5'GFP, while the expression of N is comparable among the various recombinant viruses. The western and northern blot analyses confirmed higher expression of GFP from the P/M region than the 5' end of the genome (Figs. 1E and F).

In addition to the GFP gene (720 bp), we could rescue rBDV P/M viruses harboring DsRed (678 bp) and luciferase (1653 bp) in the P/M region and verified the stable expressions

of these genes in infected cells. Both the rescue efficiency and growth kinetics of rBDV P/M-DsRed and -Luc were quite comparable to that of P/M-GFP virus (data not shown). Using rBDV P/M-Luc, we could monitor the transcription of BDV in infected cells at a very early stage of infection. The luciferase activity was clearly detected in Vero cells as early as 72 h postinfection (Fig. 1G), at which time BDV replication had not been demonstrated by other methods. Furthermore, efficient expression of DsRed was also demonstrated in Vero cells infected with the rBDV (Fig. 1H). The signal intensity of DsRed appeared to be overall equal to that of GFP, suggesting that the P/M region has the capacity to harbor various genes without attenuating viral replication.

Stable expression of GFP in rBDV P/M-GFP-infected rodent brains. To investigate whether rBDV P/M can efficiently and stably express a foreign gene in animal brains, we inoculated 1.0×10^3 FFU of rBDV P/M-GFP and 5'GFP viruses intracranially into neonatal Lewis rats and C57BL/6J mice and GFP expression was monitored by fluorescence stereomicroscopic and immunohistochemical analyses. As shown in Fig. 2A, a GFP signal was detected in the injected hemisphere of the rBDV P/M-GFP-infected rat brain and was much brighter than that of the 5'GFP at 2 weeks postinoculation. The expression of GFP was clearly visible in the neurons of the cerebrum cortex and cerebellum at 2 months after inoculation (Fig. 2B). Although the N-positive neurons were detected equally in the brain regions after infection by rBDV P/M-GFP and 5'GFP viruses, GFP fluorescence seemed to be much greater in the neurons infected with rBDV P/M-GFP than with 5'GFP virus (Fig. 2B). Furthermore, the stable expression of GFP from the P/M-GFP virus was demonstrated in

mouse brains (Fig. 2C). The neurons in the hippocampus region of infected mice showed a strong GFP fluorescence at 2-months postinoculation (Fig. 2C). Notably, the GFP signal in the brains of rBDV P/M-GFP-infected mice was observed and stable over an observation period of at least 8 months (Fig. 2D). These results indicated that rBDV P/M can establish a persistent infection, leading to the lasting expression of foreign gene in the brains.

Previous work using virus vectors derived from mononegaviruses revealed that insertion of an additional transcriptional unit into viral gene junctions causes mutations of the transcriptional initiation or termination sites that control the transcription of the inserted or neighboring genes (2, 33). Therefore, we next determined using RT-PCR whether the sequences of the newly introduced transcriptional cassette are stable during the replication of the P/M-GFP virus. No sequence alterations or deletions were found to have been introduced into the rBDV P/M-GFP genome at least 8 months after infection of mouse brains (data not shown), suggesting the additional transcriptional unit and its surrounding sequences are fairly stable during the multiplication of the P/M-GFP virus in the brains.

Generation of propagation-defective rBDV P/M viruses lacking the translation initiation codons for the G gene and an intron region within the L gene. To improve the BDV P/M vector system, we next tried to generate propagation-defective rBDV P/M. First, we changed two ATG codons (nt 2236 and 2248) in the G ORF to ACG to abrogate initiation of translation of the G protein, Δ GP/M (Fig. 3A). Furthermore, we deleted an intron II sequence within the L gene (nt 2410 to 3703) to create a linearized, intronless L gene, Δ GLLP/M (Fig. 3A), with the idea that the deletion of the intron in L mRNA may not only lead to the efficient

production of L during the replication but also increase the capacity for insertion of foreign genes into the genome. 293T cells were transfected with the resultant plasmid, pFct-BDV Δ GP/M-GFP or pFct-BDV Δ GLLP/M-GFP, together with three helper plasmids (N, P and L) and the G expression plasmid, and then cocultured with Vero cells stably expressing BDV G, Vero-BG. At first, the rescue efficiency was compared between P/M-GFP, Δ GP/M-GFP and Δ GLLP/M-GFP viruses at 12 days after the cocultivation. As shown in Fig. 3B, the cells transfected with pFct-BDV Δ GLLP/M-GFP yielded a much higher level of recombinant virus than those with pFct-BDV Δ GP/M-GFP. Furthermore, although the numbers of transfected plasmids, as well as the cells used for the rescue of recombinant viruses, were different between Δ GLLP/M-GFP and P/M-GFP viruses, the rescue efficiency of L linearized construct was comparable to or more efficient than that of wt P/M-GFP vector, suggesting that linearized, intronless L gene enables efficient replication of rBDV.

To examine the cell-free infectivity and stability of the Δ GLLP/M virus, the cell-free virus of rBDV Δ GLLP/M-GFP-infected Vero-BG cells were inoculated into either Vero-BG or parental Vero cells at an MOI of 0.01, and the growth kinetics of the viruses were monitored by IFA. Although the infection rate of Δ GLLP/M-GFP virus gradually increased in Vero-BG cells, the parental Vero cells did not support the propagation of the Δ GLLP/M-GFP virus (Fig. 4A). Interestingly, however, the infection by rBDV Δ GLLP/M-GFP was stably maintained during the observation period of 24 days (Figs. 4A and 4B). The Vero cells persistently infected with Δ GLLP/M-GFP virus exhibited the nuclear dot structure specific for BDV replication. (Fig. 4B, insets). Western blotting confirmed the lack of G expression in rBDV Δ GLLP/M-GFP-infected Vero cells (Fig. 4C). The persistent infection of Δ GLLP/M-GFP

virus without propagation was also observed in human-derived oligodendrogloma OL cells (Fig. 4D). On the other hand, we investigated whether the lack of G expression and the deletion of an intron within L gene increase the capacity of Δ GLLP/M vector to insert foreign gene into the P/M region. As shown in Fig. 4E, the rBDV Δ GLLP/M harboring LacZ gene (3060 bp) was successfully rescued and established persistent infection in Vero-BG cells. All these observations demonstrated that G is essential for virus propagation but not for the maintenance of persistent infection of BDV and suggested that rBDV Δ GLLP/M is a non-propagating virus, which may provide safe, stable and high-capacity foreign gene delivery in vivo.

The Δ GLLP/M vector shows that the carboxyl-terminal region in BDV G is essential for release of infectious particles.

In many virus vector systems, pseudotyping of the virus envelope protein may be an effective strategy to expand the host cell tropism. Furthermore, improvement of the efficacy of virus particle release from producer cells could be necessary to generate a high titer virus stock. Therefore, understanding the mechanisms of the release of BDV infectious particles, as well as the envelopment by BDV G, may be crucial for the development of further applications of the BDV P/M vector system. As a first step towards these goals, therefore, we tried to determine the region of BDV G essential for the release of infectious particles, using the Δ GLLP/M-GFP system. Previous studies clearly revealed that the CT region of the mononegavirus G glycoprotein may play a key role in assembly of infectious virus particles in concert with M (11, 12, 28). We thus generated a series of alanine substitution mutants in the CT region of BDV G (Fig. 5B) and transfected the expression

331 plasmids into a Δ GLLP/M-GFP virus persistently infected Vero cell line, Vero- Δ GLLGFP,
332 which was obtained by limiting dilution of Vero cells infected with Δ GLLP/M-GFP virus. At
333 48 h after transfection, the virus titers in the culture supernatants were evaluated in Vero cells.
334 As shown in Fig. 5B, none of the mutants, except for the QE^{502/503}A mutant, in which two
335 residues at the end of the C terminus are substituted by alanine, could rescue the infectious
336 viruses. This result indicated that at least the amino acids at positions 494 to 501 of BDV G
337 are required for efficient release or infectious particle formation of BDV in the cells.

338 We finally investigated whether the CT region of BDV G is required for the generation
339 of pseudotyped rBDV harboring other virus envelope proteins. To this end, we at first
340 transfected G expression plasmids of BDV, VSV, and RaV into the Vero- Δ GLLGFP cells, and
341 at 48 h after transfection the virus titers in the culture supernatants were evaluated. As shown
342 in Fig. 5D, although the cells transfected with BDV G plasmid yielded the rBDV in the
343 supernatant as shown above, the infectivity was not detected in the supernatants of both VSV
344 and RaV G-transfected cells. Interestingly, however, the chimeric VSV G, but not RaV G, of
345 which the CT region is exchanged with that of BDV G (Fig. 5C), was shown to produce
346 infectious particles in the culture supernatant of the transfected Vero- Δ GLLGFP cells (Fig.
347 5E). These results suggested that the CT region of BDV G may enhance the efficiency of
348 release or formation of pseudotyped rBDV using VSV G. All these data demonstrated that the
349 Δ GLLP/M virus system may provide a useful tool not only for investigating the mechanisms
350 of BDV particle formation and virion budding but also for generating the pseudotyped rBDV
351 particles.

Discussion

In this study, we showed that foreign genes can be stably expressed from an intercistronic region of the BDV genome in both cultured and animal brain cells. Together with the previous report by Schneider et al (26), this suggests strongly that BDV may be a good vector platform for gene delivery to the CNS. In this study, it was very surprising that our system expressing several foreign genes from the P/M region enabled successful rescue of the rBDV, despite the fact that a vector with the same genome construction did not yield recombinant virus in a previous study (26). Although the factors accounting for this difference are not obvious, it is highly likely that only a single nucleotide difference exerts the adverse effects on the rescue of rBDV in the reverse genetics system. It will be of interest to compare the sequence differences between these vectors to understand the genomic feature necessary for BDV replication or propagation.

Recently, Ackermann et al. reported that GFP expression from the rBDV 5'GFP construct is reduced markedly in mouse brains from two months postinfection, probably because of mutations in the termination/initiation signals upstream of inserted GFP gene (2). This observation suggests that expression of an additional gene from the 5' end of BDV genome may have deleterious effects on virus replication, leading to attenuation of transcription of the inserted gene. The additional transcription initiation signal downstream of the L gene may affect the efficiency of the termination of transcription of L. Alternatively, the additional sequences may disrupt structures at the end of the BDV genome, such as the ITR and panhandle structures, important for the initiation of RNA replication and formation of the viral ribonucleoprotein (RNP) (24, 25, 27). Furthermore, very recently, Martin et al. clearly

showed that specific sequences in close proximity to the 3' ends of the viral genome and antigenomic RNA are required for the elongation of the genomic RNA after the realignment of the 3' termini (20). As this process could be essential for the maintenance of the BDV-specific genome-end structure during replication, it may be possible that the long extra sequences in the 5' end of the genome disrupt the realignment of the 3' terminus of antigenomic RNA, resulting in reduced virus replication. On the other hand, we demonstrated that the P/M-GFP virus exhibited no attenuation of the extra transcription unit, even in mouse brains during our observation periods of at least eight months. Sequencing analysis revealed that no attenuating mutations are introduced in the sequences of the inserted transcriptional unit. Furthermore, the expression levels of both P and M appeared not to differ between rBDV P/M and wt viruses. In addition, we could demonstrate that the P/M region must have a considerable capacity for insertion of various length foreign genes, at least below 3060 bp in Δ GLLP/M vector. All these observations indicated that P/M region may be a more flexible, stable and suitable site for insertion of an extra transcription cassette in the BDV genome, suggesting that the BDV P/M system provides an efficient platform as a BDV vector.

The potential for pathogenesis is an important concern for many virus vectors and replication- and propagation-incompetent viral vectors have been created for safety reason. In this study, we tried to generate propagation-defective rBDV by deleting the G ORF, using the P/M vector system. The G ORF of BDV encodes an envelope glycoprotein, which overlaps with the upstream M ORF, and contains a long intron of a 1293 nt for expression of the downstream L gene (7, 31, 32). The G precursor protein, GP, plays a key role in BDV entry to susceptible cells and the GP1, the N-terminal subunit of GP, is known to be required for

receptor recognition and virus entry (14, 15, 16, 19, 22). Previous studies suggested that the expression and correct processing of BDV GP are necessary for BDV dissemination in primary cultures of neurons and that neutralizing antibodies against BDV GP completely inhibited virus spread (3). These observations suggested that the enveloped viral particles, not nonenveloped viral RNPs, are crucial for dissemination of BDV between cultured cells (3), as in the cases of other mononegaviruses, such as RaV (10). On the other hand, in an independent system, it was reported that cell-to-cell spread of BDV does not require the expression of the primary receptor, as well as the processing of BDV GP (6). These observations showed the complexity of the roles of G in BDV infectivity and propagation in neurons. In this study, we mutated the first two methionines and an intron (intron II) for the L gene in the G ORF of the P/M-GFP vector to generate rBDV-lacking the G envelope protein, Δ GLLP/M virus. The transiently enveloped-rBDV from G-expressing Vero cells showed cell-free infectivity and established persistent infection of Vero cells in the absence of G expression. Interestingly, the infection seemed to be maintained without spreading in the cultured cells, suggesting that BDV G is not required for the establishment of persistent infection in a single cell but rather for cell-to-cell spread. Our result revealed that persistent infection by the Δ GLLP/M-GFP virus can be maintained for at least more than 2 months without losing the expression of GFP (data not shown), demonstrating that the Δ GLLP/M system may provide a promising BDV vector for in vivo use with safe and persistent foreign gene expression in the infected cells.

The Δ GLLP/M virus provided an intriguing finding that intron II of the L gene may negatively regulate the expression of L polymerase, because deletion of intron II markedly

enhances the efficiency of rescue of the rBDV when compared with the Δ GP/M virus. In addition, the L-linearized construct seemed to be more efficient than the wt P/M-GFP vector for rescue of rBDV, despite the trans-complementation of G in the rescue system. This result also demonstrated that the linearization of L gene does not have any adversary effect on the replication of rBDV. Previous studies revealed that the splice donor 2 (SD2; nt 2410) can produce two introns, introns II and III, in the BDV genome using alternative splicing machinery, which generate the mature L mRNA and predicted small ORFs, respectively (30). Because the SD2 sequence was mutated in the Δ GLLP/M vector, alternative splicing using intron III could be also defective in the recombinant viruses. Although control of the alternative splicing between introns II and III during BDV replication has not been elucidated yet, skipping over the splicing reactions of L transcripts might be likely to increase the polymerase activity, leading to high efficiency of rBDV rescue. Along with the L_{RD} mutation, the regulation of L splicing may be a key to improve the recovery rate of rBDV in the reverse genetics system.

Our analysis using rBDV Δ GLLP/M-GFP-infected cells also provided an excellent model for studying the release of infectious BDV and the virion formation in infected cells. Previous studies using other mononegaviruses revealed that M and G proteins may be critical for the assembly or budding of infectious virions in concert (11, 12, 28). In this study, we demonstrated that the CT region of BDV G may be essential for the release of infectious particles, using the rBDV Δ GLLP/M-GFP persistently infected cells. Our result showed that an 8-amino acid stretch, ⁴⁹⁴RRRRLGRW⁵⁰¹, in the CT region may play a critical role in the production of infectious virions. Understanding the interaction between M and G of BDV

may provide valuable insights into, not only the improvement of the efficiency of infectious particle releases from BDV-infected cells, but also the generation of pseudotyped viruses using other virus envelope proteins. In fact, we could show that the chimeric VSV G protein harboring the CT region of BDV G facilitates the production of infectious particles in the supernatant of Vero- Δ GLLGFP cells (Fig. 5D). This result suggested that the function of the CT region of BDV G may be at least a necessary prerequisite, if not sufficient, to generate pseudotyped virions using the envelope proteins of other viruses. On the other hand, we could not obtain evidence that the chimeric RaV G having the BDV CT region produces infectious virions in the supernatant of the transfected cells. This may be due to the differences of the intracellular localization of the maturation and/or the budding of G proteins between BDV and RaV. Alternatively, in RaV infection the specific interaction of G with other viral components might be necessary for efficient virion assembly and release. The difference in efficiency of formation of pseudotyped virions by the source of the G protein may be a key to understand the life cycle of BDV, especially assembly and budding of virions.

In this study, we could not demonstrate the detailed characteristics of the infectious virions released from the Vero- Δ GLLGFP cells by the trans-complementation assays, because the amount of infectious particles released into the supernatants were still low even in the supernatant of cells transfected with wt BDV G. Therefore, further studies using more detailed recombinant constructs between VSV, RaV and BDV G will be required to confirm the release mechanism and the efficient production of the pseudotyped virions in the Δ GLLP/M system, and such experiments are now underway in our laboratory. The pseudotyped BDV may not only extend the application of BDV vectors to many cell types and tissues other than

the CNS but also contribute to the development of low- or non-pathological rBDV lacking the immunogenicity of the envelope protein.

We investigated the potential of BDV as a virus vector using a novel BDV vector system. Our results showed that the newly established BDV P/M vector may provide a good system not only for efficient and safe gene delivery in vivo but also for basic research regarding BDV replication and virion formation. Further improvements of the BDV P/M vector system, including the reduction of possible pathogenesis and expansion of the capacity for insertion of foreign genes, are necessary to establish a useful virus vector and are now in progress.

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Figure legends

Fig. 1. Efficient expression of GFP from an intercistronic P/M region of a BDV vector.

(A) Schematic representation of a BDV vector construct harboring a GFP gene in an intercistronic region between the P and M genes. The genetic organization of BDV and the sequences surrounding the inserted transcription cassette are shown. The additional transcription start and termination signals (S3 and T2) and restriction enzyme sites (*Bst* BI and *Pac* I) are indicated. (B) Expression of GFP in rBDV P/M-GFP-infected Vero cells. The cell nuclei are stained by DPAI (blue). Bar, 10 μ m. (C) Growth kinetics of wt and GFP-expressing rBDVs in Vero cells. The viral growth rate was monitored by IFA using anti-N mAb. Symbols; rBDV wt (closed circle), rBDV P/M-GFP (closed square), rBDV 5'GFP (open triangle). (D) Comparison of the levels of GFP expression between rBDV P/M-GFP and rBDV 5'GFP in Vero cells. The rBDV-infected Vero cells were detected by anti-N mAb (red) and GFP fluorescence (green). Bar, 20 μ m. (E) and (F) The expression levels of GFP protein and mRNA were determined by western (E) and northern (F) blot analyses, respectively. The antibodies (E) and riboprobes (F) used in the analyses are indicated to the left. (G and H) The BDV-P/M vector has a great capacity for insertion of various foreign genes of different lengths. (G) The Vero cells were infected with cell-free rBDV P/M-Luc at an MOI of 1.0. The luciferase activity was detected in the infected cells as early as 72 h after infection. Symbols; rBDV P/M-Luc (closed diamond), rBDV P/M-GFP (open square). RLU, relative luciferase unit. (H) Stable expression of DsRed in the Vero cells infected with rBDV P/M-DsRed at 20 days postinoculation. Bar, 10 μ m.

Fig. 2. rBDV P/M stably expresses GFP in rodent brains. (A) The rat brains infected with rBDV P/M-GFP exhibited a brighter GFP signal than those with rBDV 5'GFP at 2 weeks postinoculation. The GFP expression was monitored by fluorescence stereomicroscopy. (B) The GFP fluorescence was compared between rats infected with rBDV P/M-GFP and 5'GFP in the cerebrum cortex and cerebellum at 2 months postinoculation. The cryosections of the brain samples were reacted with anti-N mAb. Merged images also are shown with nuclear DAPI staining (blue). Bar, 50 μ m. (C) A representative stereomicroscopic image of the cross section of the brain of 2-month-old C57BL/6J mice neonatally infected with rBDV P/M-GFP. Immunohistochemical staining of the cryostat sections was examined by fluorescence microscopy following staining with an antibody specific for BDV N. Bar, 100 μ m. (D) GFP signals in the brain of an 8-month-old mouse neonatally inoculated with rBDV P/M-GFP. The stereomicroscopic image of the sagittal section and the cryostat section of the brain are shown. Bar, 50 μ m.

Fig. 3. Construction and efficiency of rescue of the G-deficient BDV vectors. (A) Schematic representations of the Δ GP/M-GFP and Δ GLLP/M-GFP vectors. Arrows indicate the sites of the first two methionine codons that were mutated in the G-deficient vectors. The region of intron II (nt 2410 to 3703) also is shown. (B) Comparison of rBDV rescue efficiency between Δ GP/M-GFP, Δ GLLP/M-GFP and P/M-GFP vectors. The numbers of GFP-positive foci were counted by fluorescence microscopy at 12 days after cocultivation. The data of Δ GP/M-GFP and Δ GLLP/M-GFP viruses represent triplicate experiments.

624

625 **Fig. 4. The G-deficient rBDV establishes persistent infection in cultured cells.** (A)
626 Growth kinetics of Δ GLLP/M-GFP virus in Vero-BG and Vero cells. The cell-free virus was
627 inoculated onto the cells at an MOI of 0.01. Infected cells were split every 3 days and GFP
628 expression evaluated by fluorescent microscopy. The filled squares and open triangles
629 indicate the infection rates of rBDVs in Vero-BG (left vertical axis) and Vero (right vertical
630 axis) cells, respectively. (B) The fluorescence microscopic images of rBDV
631 Δ GLLP/M-GFP-infected Vero-BG (left panel) and Vero (right panel) cells at 12 days
632 postinfection. Cells were counterstained with DAPI. The cells were immunostained with
633 anti-N mAb (red; insets). Merged images are shown. Bar, 50 μ m (10 μ m in insets). (C)
634 Western blot analysis of Δ GLLP/M-GFP-infected Vero cells. The cloned Vero cells
635 persistently infected with rBDV Δ GLLP/M-GFP were analyzed by western blotting using
636 specific antibodies against BDV G (polyclonal) and N (monoclonal) and GFP (monoclonal).
637 (D) The fluorescence microscopic images of rBDV Δ GLLP/M-GFP persistently infected OL
638 cells. After the infection, the rBDV-infected cells were cloned by limiting dilution. The cells
639 were immunostained with anti-N mAb (red). Cells were counterstained with DAPI. Merged
640 images are shown. Bar, 20 μ m. (E) Stable expression of LacZ in the Vero-BG cells infected
641 with rBDV Δ GLLP/M-LacZ. Bar, 100 μ m.

642

643 **Fig. 5. Identification of the carboxyl-terminal region in the BDV G cytoplasmic tail**
644 **necessary for the release of infectious particles.** (A) Schematic representation of BDV G.
645 The amino acid number of BDV G is indicated. SP, signal peptide; TM, transmembrane

domain; CT, cytoplasmic tail. (B) Diagram of a series of alanine substitution mutants of CT region. The infectious titers in the supernatants of rBDV Δ GLLP/M-GFP-infected Vero cells transfected with each construct were monitored by focus-forming assay at 48 h after infection of the cells. The titers are shown to the right. N.D, not detected. (C) Schematic representation of VSV, RaV G, and chimeric G constructs. Bct, CT region of BDV G. (D and E) The infectious titers in the supernatants of the Vero- Δ GLLGFP cells transfected with G expression plasmids. The titers were monitored by focus-forming assay at 48 h after inoculation in Vero cells. ND, not detected. (E) VG-Bct and RV-Bct indicate chimeric VSV and RaV G constructs of which CT region are exchanged with that of BDV G, respectively. Data represent triplicate experiments.

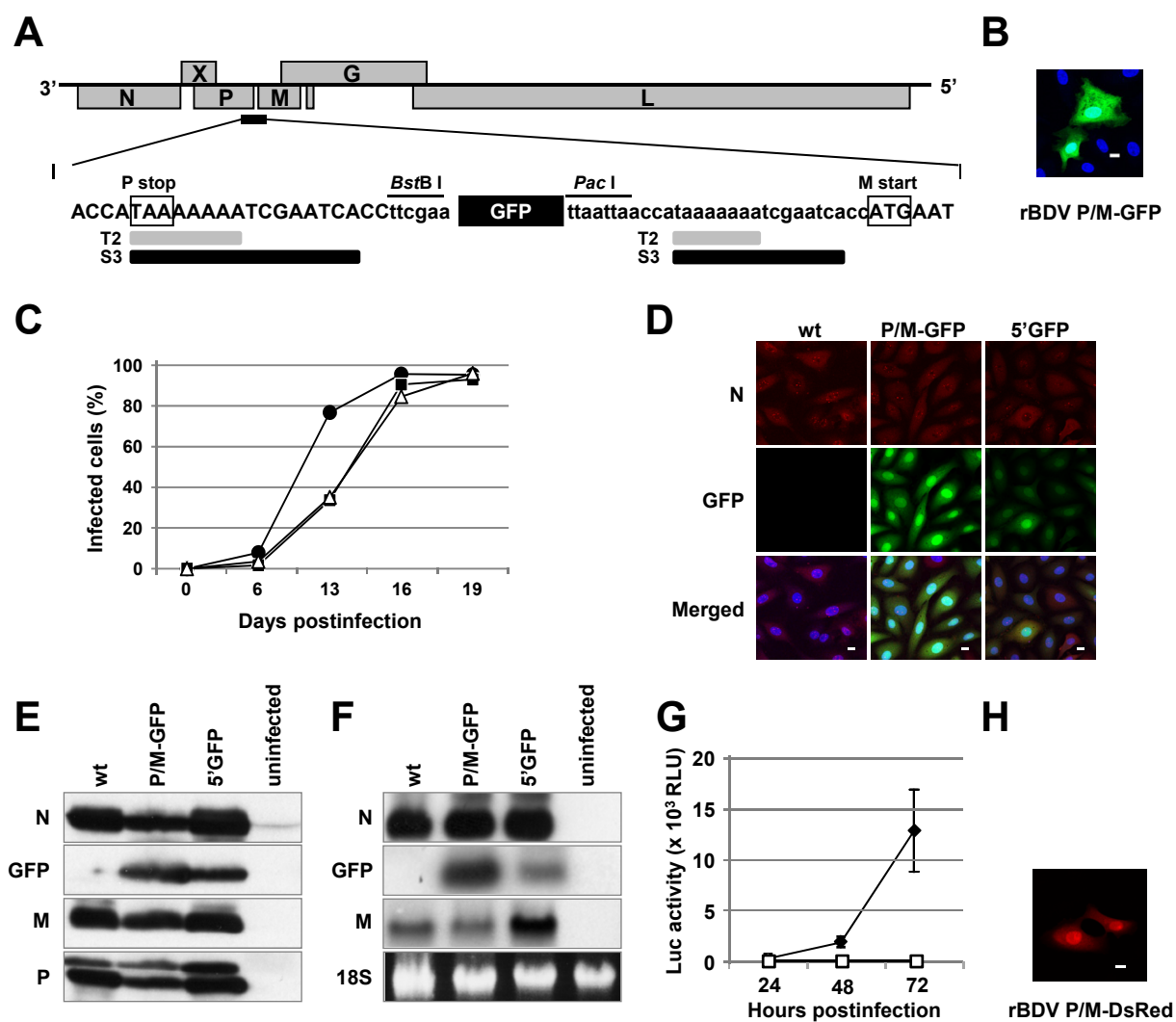


Fig. 1

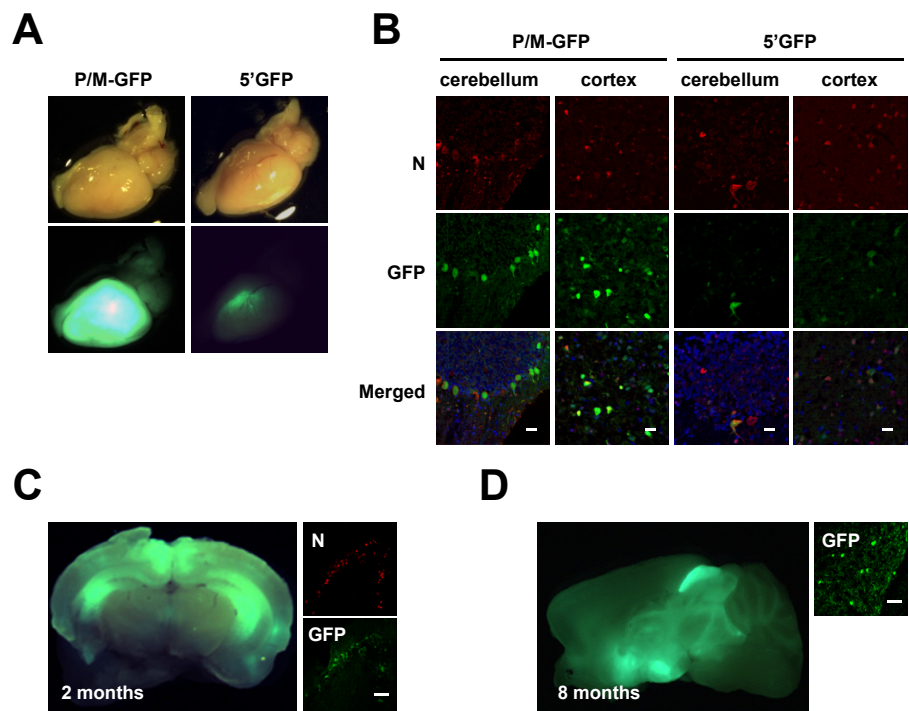


Fig. 2

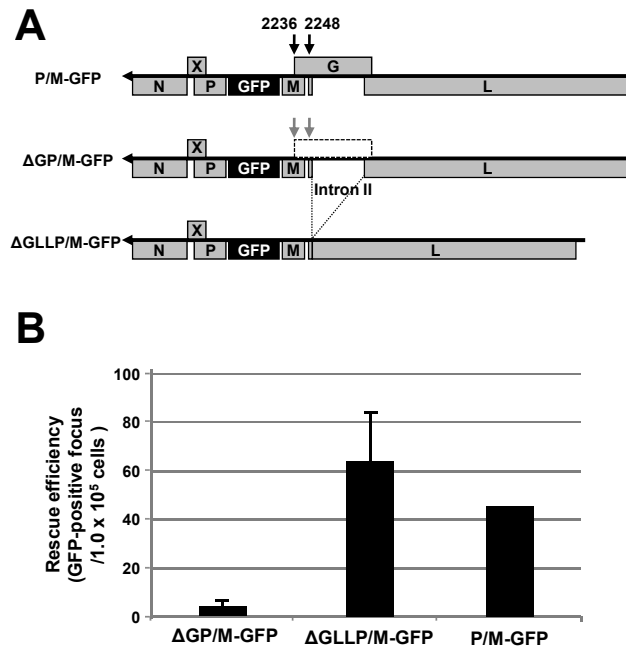


Fig. 3

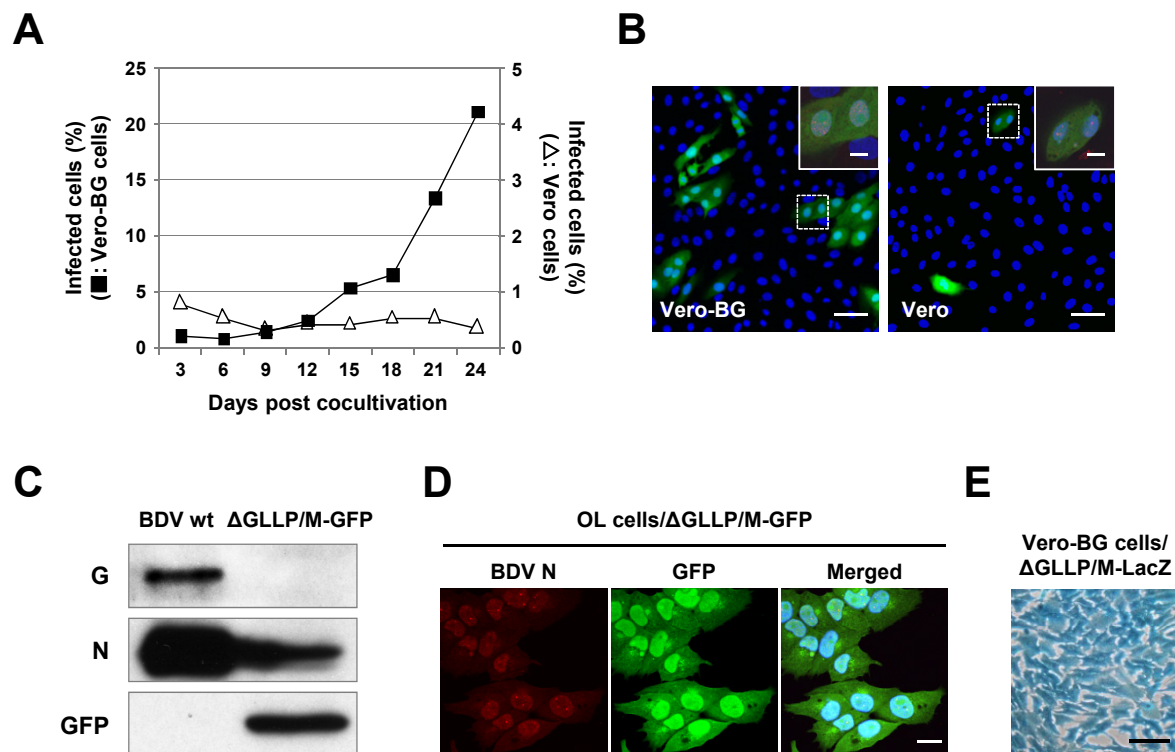


Fig. 4

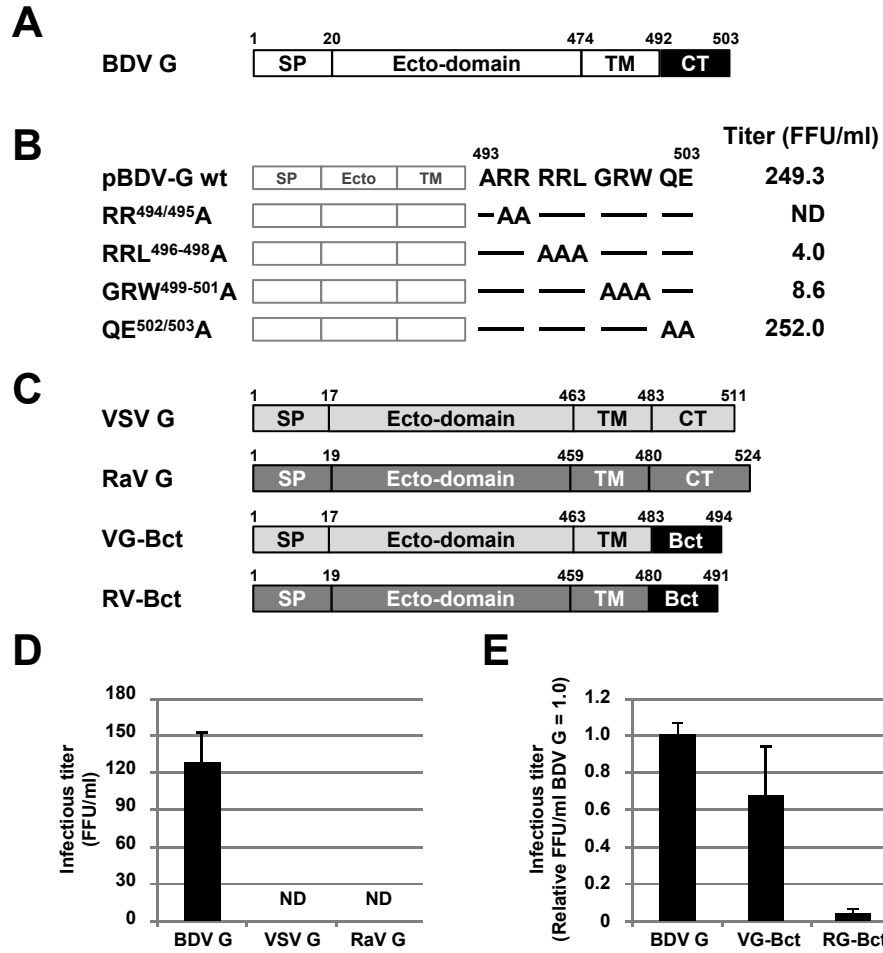


Fig. 5