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Inhibition of Borna disease virus replication by an endogenous bornavirus-like element in the ground squirrel genome

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Animal genomes contain endogenous viral sequences, such as endogenous retroviruses and retrotransposons. Recently, we and others discovered that non-retroviral viruses also have been endogenized in many vertebrate genomes. Bornaviruses belong to the *Mononegavirales* and have left endogenous fragments, called endogenous bornavirus-like elements (EBLs), in the genomes of many mammals. The striking features of EBLs are that they contain relatively long open reading frames (ORFs) which have high sequence homology to the extant bornavirus proteins. Furthermore, some EBLs derived from bornavirus nucleoprotein (EBLNs) have been shown to be transcribed as mRNA and probably are translated into proteins. These features lead us to speculate that EBLs may function as cellular co-opted genes. An EBLN element in the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) genome, itEBLN, encodes an ORF with 77% amino acid sequence identity to the current bornavirus N. In this study, we cloned itEBLN from the ground squirrel genome and investigated its involvement in Borna disease virus (BDV) replication. Interestingly, itEBLN, but not a human EBLN, co-localized with the viral factory in the nucleus and appeared to affect BDV polymerase activity by being incorporated into the viral ribonucleoprotein. Our data show that, as with certain endogenous retroviruses, itEBLN potentially may inhibit infection by related exogenous viruses in vivo.

KEYWORDS; Endogenous non-retroviral viruses, Bornavirus, Anti-viral immunity

Significance statement

Sequences derived from ancient viruses have been shown to make up a substantial part of animal genomes. Bornaviruses, a genus of non-segmented, negative-sense RNA virus, have also left their DNA copies in the genomes of quite a number of vertebrate lineages. Recent studies have demonstrated that some endogenous bornavirus elements (EBLs) may have acquired functions in their hosts as a result of exaptation. In this study, we show that protein encoded by an EBL in the thirteen-lined ground squirrel genome efficiently blocks infection and replication of extant bornavirus. This is the first report showing that endogenous elements derived from non-retroviral RNA viruses may have been co-opted for a new function of anti-viral defense in hosts.

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Endogenous retroviruses have accumulated in the genomes of many organisms over evolutionary time and occupy about 8% of the human genome (1). Although almost all endogenous retroviruses in animal genomes appear to lack intact open reading frames (ORFs) and are not expressed in somatic tissues, some retain the potential to express mRNA and even produce the proteins they encode (2, 3). There is mounting evidence that expression of human endogenous retroviruses (HERVs) is associated with some autoimmune diseases, cancers and schizophrenia, as well as viral infections (4-6), suggesting that the induction of HERVs may be linked to immunological aberrations in the host. In addition, it has been reported that endogenous retroviruses have the ability to recombine with exogenous or other endogenous retroviruses to produce intact infectious viruses (7, 8). These observations indicate that the expression of endogenous retroviruses may cause deleterious consequences directly to the hosts.

On the other hand, recent studies have revealed clearly that endogenous retroviruses have been co-opted to play new and beneficial roles in their hosts (9, 10). For instance, in mammals, envelope genes from endogenous retroviruses are involved in the formation of the placenta during the fusion of syncytiotrophoblast cells (9, 11). In addition, a retroviral-like aspartic protease, skin aspartic protease, SASPase, is known to play a key role in determining the texture of skin by modulating the degree of hydration in mammals (12, 13). Furthermore, it has been known for decades that endogenous retrovirus-derived elements, such as Friend virus susceptibility 1 (Fv1) gene and endogenous fragments from Jaagsiekte sheep retrovirus (enJSRV), protect host cells from infection by exogenous retroviruses (10, 14, 15). These

lines of evidence suggest that evolution has favored persistence of endogenous retroviral elements that have the potential to protect their hosts against related viruses.

Recent advances in the availability of genomic sequences from many animal species, as well as the development of tools for sequence comparisons, have revealed that non-retroviral viruses also have endogenized in many mammalian species (16-18). Bornavirus, an enveloped, nonsegmented, negative-strand RNA virus in the order *Mononegavirales*, is unique among animal RNA viruses, not only because it replicates in the cell nucleus but also because it readily establishes a long-lasting persistent infection in the absence of overt cytopathogenesis (19). Although bornavirus does not integrate into the host genome during its replication cycle, interestingly, we and others found recently that DNA sequences derived from ancient bornaviruses are endogenized in the genomes of many vertebrate species, including humans (16, 17). The endogenous fragments of bornavirus in mammalian genomes originate predominantly from the region encoding the viral nucleoprotein (N), which encapsidates viral genomic RNA to form nucleocapsids, and we therefore designated them endogenous bornavirus-like nucleoproteins (EBLNs) (16).

In a previous study, we showed that EBLNs from the human and thirteen-lined ground squirrel (TLS) (*Ictidomys tridecemlineatus*) genomes, named hsEBLN and itEBLN, respectively, have significant sequence similarity to the N ORFs of extant mammalian bornaviruses, Borna disease virus (BDV). The elements, hsEBLN-1 and -2, which are located in chromosomes 10 and 3 of the human genome, respectively, encode ORFs with an overall 41% amino acid sequence identity to BDV N (16). itEBLN also has an intact ORF and shows approximately 77% amino acid sequence identity to N. Furthermore, it has been shown that

some hsEBLNs, including hsEBLN-1 and -2, are expressed as RNA and that a predicted mRNA transcript of itEBLN is provided by NCBI RefSeq (XM_005342477). Interestingly, we have found that long terminal repeats (LTR-1C and LTR-21B) from endogenous retroviruses exist in close proximity and upstream of itEBLN ORF (20). In fact, we could detect the predicted transcripts of itEBLN in several tissue samples from both breeding and wild-captured TLSs by RT-PCR (Supplementary Fig. S1). From these observations, it is tempting to speculate that EBLNs encode functional proteins as a consequence of exaptation or co-option by their hosts.

In this study, we cloned hsEBLN-1 and itEBLN sequences from the human and TLS genomes, respectively, to determine whether these EBLNs encode potentially functional proteins. We found that the protein encoded by itEBLN, but not hsEBLN-1, colocalizes with the viral factory in the nucleus and markedly decreases infection and the replication of exogenous BDV. Furthermore, the protein encoded by itEBLN appeared to be incorporated into the viral ribonucleoproteins (RNPs) in infected cells. These results suggest that, like some endogenous retroviruses, itEBLN has the potential as a co-opted gene in the host to inhibit infection by genetically related viruses.

RESULTS

Expression and reconstruction of EBLN elements from the human and ground squirrel genomes. To investigate the potential roles of the EBLN elements in the human and TLS genomes, we first amplified EBLN sequences from the genomic DNAs and cloned the

118 products into expression vectors. Although the expression of hsEBLN-1 and itEBLN has been
119 predicted in previous studies (16, 18), the structure and transcription initiation sites of the
120 EBLN RNAs have not been characterized. In this study, therefore, we cloned only the
121 homologous regions to BDV N. To detect the expressed EBLN products, an HA-tag was
122 fused to the N-terminus of the coding sequences. At first, we transfected the plasmids into
123 human OL cells and investigated the expression of the reconstituted proteins by western
124 blotting at 24 h posttransfection. As shown in Fig. 1A, expression of the recombinant proteins
125 was detected as approximately 35 to 40 kDa products, indicating that the EBLNs may have
126 the ability to stably express the proteins in mammalian cells. In addition, we carried out
127 immunofluorescence assays to examine the subcellular localization of the proteins. As shown
128 in Fig. 1B, while BDV N clearly localizes in the nucleus, itEBLN and hsEBLN-1 appear to be
129 distributed predominantly in the cytoplasm of the transfected cells, probably because these
130 proteins lack the nuclear localization signal (NLS), which is present in the N-terminus of
131 BDV N (see Fig. 5).

132 Next, we transfected the EBLN plasmids into OL cells persistently infected with BDV.
133 In a previous study, we demonstrated that BDV generates dot-like structures, called vSPOT
134 (viral speckles of transcripts), in the nucleus (21). The vSPOT is the viral factory, in which
135 essential events in the BDV replication cycle take place and could be the same structured
136 nuclear bodies having the same function, as Joest-Degen inclusion bodies in vivo (22-24).
137 Despite the lack of a nuclear localization activity, itEBLN was strongly redistributed to
138 vSPOTs in the nucleus, as does N (Fig. 1C, arrowheads). On the other hand, hsEBLN-1
139 seemed to be not relocated in the infected cells. This observation suggests that itEBLN, but

not hsEBLN-1, may have the potential to interact with the viral components in the infected cells, leading to colocalization with vSPOT in the nucleus.

itEBLN inhibits BDV replication in persistently infected cells. In a previous study, Geib et al. (2003) revealed that transient expression of BDV P, but not N, inhibits viral replication (25) in Vero cells persistently infected with BDV. Therefore, we next investigated whether expression of hsEBLN-1 and itEBLN affects BDV replication in persistently infected cells. To evaluate viral transcription/replication, we measured the level of viral RNAs in cells transfected with the EBLN plasmids. Consistent with previous reports, transient expression of N in persistently infected OL cells did not exert any effect on BDV replication (Fig. 1D and 1E). In contrast, intriguingly, expression of itEBLN, but not hsEBLN-1, significantly reduced the levels of both viral genomic and mRNA at 48 h posttransfection (Fig. 1D and 1E). This result suggested that, unlike N, itEBLN could inhibit both the transcription and replication of BDV.

Expression of itEBLN confers resistance to exogenous BDV infection. Previous studies revealed that expression of BDV N protects cells from subsequent infection by BDV (25). Although the detailed mechanism of this resistance to superinfection has not been elucidated, it is assumed that an early step of BDV infection, such as nuclear transport of viral RNP, intranuclear dissemination or initiation of viral replication, may be interfered with by the overexpression of the viral nucleocapsid component (25). To determine whether itEBLN can also affect BDV infection, we established OL cell lines stably expressing itEBLN or

hsEBLN-1. In this experiment, we also generated a plasmid, pNL-itEBLN, in which itEBLN is fused with the NLS of BDV N at the N-terminus (Fig. 2A), in order to investigate the effect of nuclear localization of the protein. As for N, the product of pNL-itEBLN clearly localized in the nuclei of transiently transfected cells (Fig. 2B). We then infected the cell lines with cell-free virions of recombinant BDV (rBDV) expressing GFP, rBDV P/M-GFP, with an M.O.I. of 0.1 and monitored the propagation of GFP for at least 3 weeks. As shown in Fig. 2C and 2D, the expression of itEBLN and NL-itEBLN, but not hsEBLN-1, nearly completely protects the cells against BDV infection for the observation period, as does N, suggesting that itEBLN protects against BDV infection in the nucleus

We next performed cocultivation experiments using the EBLN-expressing OL cell lines and Vero cells persistently infected with rBDV P/M-GFP, to investigate whether itEBLN can also inhibit the cell-to-cell spread of the infection, which is presumably the main route of BDV transmission. Repeated experiments revealed that, despite incomplete protection of the cells expressing N, itEBLN constructs were almost completely resistant to virus infection (Fig. 3A and 3B). This indicated that itEBLN could have a strong inhibitory effect on BDV infection, even following cell-to-cell transmission. To exclude the possibility that the NLS region (amino acids 1 to 38) of BDV N in the constructs has an effect on BDV replication, we also conducted the experiments using two different plasmids, pNLsv-itEBLN and pNL-DsRed, in which itEBLN and DsRed were fused with SV40 and BDV N NLS at the N-terminus, respectively (Supplementary Fig. S2). The results in Supplementary Fig. S3 show that the NLS region of N is not involved in the inhibitory effect of NL-itEBLN on BDV replication.

Incorporation of itEBLN into BDV RNP. The results shown above suggest the possibility that the itEBLN protein may be directly incorporated into BDV RNP, resulting in inhibition of viral replication. To test this possibility, we performed an immunoprecipitation analysis using BDV-infected cells transfected with the EBLN plasmids. At 24 h posttransfection, the cell extracts were immunoprecipitated with anti-HA antibody and the viral RNP components were detected by western blotting and RT-PCR. As shown in Fig. 4A, BDV genomic RNA, as well as BDV P, was clearly precipitated with itEBLN and N, whereas hsEBLN-1 seemed not to interact with BDV RNP in the cells. This observation revealed that itEBLN may be efficiently incorporated into the viral RNP in infected cells.

itEBLN inhibits BDV polymerase activity in a minireplicon system. We next determined whether itEBLNs can directly affect the polymerase activity of BDV. To this end, we used a minireplicon system of BDV, which synthesizes recombinant BDV nucleocapsids containing an artificial, minigenome reporter RNA, following transfection of expression plasmids encoding BDV N, P, L, and the minigenome (26). We carried out the minireplicon assay in the presence or absence of plasmids expressing itEBLN. Consistent with previous observation (26), the viral polymerase activity was strongly inhibited when BDV X was cotransfected with the minireplicon constructs (Fig. 4B). Interestingly, despite that BDV N could not inhibit the polymerase activity of the minireplicon, all of the itEBLN constructs, including itEBLN-M2, which is translated from the AUG codon at amino acid position 132 in the itEBLN sequence (see Fig. 5), efficiently decreased the polymerase activity in the system

(Fig. 4B). Altogether, these results suggest that itEBLN acts as a dominant negative inhibitor of N by being incorporated into the viral RNP.

The putative tetramer and RNA interaction domains of BDV N are conserved in itEBLN. Previous studies revealed that BDV N contains several signal sequences, including the NLS and nuclear export signal (NES), and putative P binding sites (27-29). Furthermore, structural studies of BDV N determined the amino acids essential for homotetramer formation and interaction with viral RNA (30, 31). To predict the function of itEBLN as a dominant negative inhibitor of N, we aligned the amino acid sequences of BDV N and itEBLN. As shown in Fig. 5, BDV N contains an NLS in the N-terminal region, whereas itEBLN lacks a homologous sequence in that region. On the other hand, putative binding sites for P (PBS-1 and -2) seem to be highly conserved between itEBLN and BDV N (Fig. 5). In addition, the sequences predicted to be involved in tetramerization of BDV N also seem to be conserved in the sequence of itEBLN, with the exception of the sequence in the N-terminus of BDV N (Fig. 5, asterisks). Furthermore, we also found that the corresponding residues essential for interaction with the viral RNA (K164, R165, K242 and R297) (Fig. 5, arrowheads) are well conserved in itEBLN. A structural model based on BDV N also revealed that the regions surrounding the RNA interaction sites are also conserved in itEBLN (Supplementary Fig. S4), suggesting that itEBLN may retain the ability to form nucleocapsids with BDV N.

DISCUSSION

In this study, we showed that the protein expressed from an endogenous bornavirus fragment from the TLS genome, itEBLN, is incorporated into BDV RNPs and inhibits BDV replication. This conclusion is supported by the following observations: first, we found that the itEBLN protein was colocalized with the viral factory of BDV in the nucleus. Second, the expression of this protein markedly reduced the replication level of BDV in persistently infected cells. Furthermore, the cells stably expressing the itEBLN protein were completely resistant to infection by exogenous BDV through both the cell-free and cell-to-cell routes. Third, we showed that the itEBLN protein can precipitate BDV RNP components, including viral genomic RNA, in infected cells. Finally, co-expression of itEBLN reduced BDV polymerase activity using the minireplicon system. To our knowledge, this is the first report that an endogenous, non-retroviral virus efficiently inhibits infection by the related exogenous virus.

In this study, we employed an overexpression system of recombinant itEBLN with human cell culture systems. Thus, in order to demonstrate the host-specific exaptation of itEBLN, it would be necessary to investigate whether TLSs actually exhibit resistance to BDV infection. At present, however, we could neither find any available cultured cells of TLSs nor establish experimental infection using TLSs. On the other hand, we could detect the expression of predicted mRNA of itEBLN in tissue samples from both breeding and wild-captured TLSs by RT-PCR (Supplemental Figure S1A and B). The immunoblot analysis using a BDV N-specific polyclonal antibody showed only a faint band at the predicted size in the heart samples (Supplemental Figure S1C). Although the expression of itEBLN protein remained obscure due to the specificity of the antibody, given that the itEBLN mRNAs are efficiently translated into the protein in the squirrel cells, itEBLN may protect BDV infection

in vivo. We will continue to make an effort to establish infection systems of TLSs with BDV.

Our results showed that the expression of itEBLN not only reduces viral replication but also blocks de novo BDV infection. Immunofluorescence and immunoprecipitation assays indicated that the itEBLN protein interacted efficiently with BDV RNP in the infected cells. Among the RNP components, the most likely candidate is the N protein, because BDV N is known to tightly assemble as a homotetramer (30). BDV N forms the nucleocapsid, which serves as the template for RNA synthesis with the L and P proteins (31, 32), strongly suggesting that itEBLN participates in heteromultimerization with BDV N. It has been determined that the residues predicted to be important for tetramer formation by N are located in the N- and C-termini of the protein (Fig. 5) (30). An amino acid comparison between the itEBLN protein and BDV N revealed that the identity between BDV N and itEBLN may be sufficient to permit heteromultimerization. It is highly likely, therefore, that the itEBLN protein efficiently co-assembles with BDV N into viral nucleocapsids in infected cells. In a previous study, Geib et al. (25) demonstrated that transient expression of recombinant N in BDV persistently infected cells does not inhibit BDV replication, even though the transduced N was colocalized with the viral factory in the nucleus. This observation was also confirmed in our experiments shown in Fig. 1, suggesting that the itEBLN may act like a dominant negative mutant of N in the viral nucleocapsids and exert a deleterious effect on the viral replication. In fact, a numbers of substitutions, especially at the N-terminus, were found in the itEBLN sequence (Fig. 5). Such heterogeneity might destabilize tetramer formation or the interaction with viral RNAs, leading to inhibition of viral polymerase activity.

Alternatively, it may be possible that the itEBLN protein directly interacts with P and

inhibits the functions of P. In fact, the sequences corresponding to the P-binding sites in N have been shown to be well conserved in itEBLN. P plays important roles in viral replication, as a viral polymerase cofactor and in nucleocytoplasmic shuttling of the viral nucleocapsid (32-34). Although the intracellular distribution of P seems to be not altered by overexpression of itEBLN in the cells (Fig. 1C), the interaction between P and itEBLN may affect the dynamics of P in the infected cells. Furthermore, we previously demonstrated that expression of P regulates the translation efficiency of BDV X, which is a negative regulator of BDV polymerase (35). Thus, it may be also possible that their interaction affects the translation efficiency of X, resulting in the inhibition of polymerase activity of BDV. Together, it is conceivable that the effect of itEBLN on BDV replication may be multifaceted, via interaction with both N and P. Further experiments should be necessary to understand the mechanism of the inhibitory effect of itEBLN on BDV replication.

Our results may reveal an intriguing strategy for inhibition of exogenous virus infection by endogenous viral fragments. Previous studies clearly demonstrated that endogenous viral products are efficiently interacted into the capsids of incoming, genetically related viruses, resulting in inhibition of viral replication. A well-studied example is the Fv1 gene in mice. Fv1 originated from the *gag* gene of an ancient retrovirus, which was endogenized several million years ago into the genome of a common ancestor of mice and is known to restrict infection by specific strains of MLV (14, 36). Although the amino acid sequence of Fv1 is distant from the restricted MLVs, recent studies clearly indicate that direct interaction of Fv1 with the capsid protein of MLV induces the anti-MLV function of Fv1 (37). Another instance is the enJSRV. This endogenous virus is known to inhibit exogenous JSRV infection by two

different mechanisms (38). First, the envelope protein encoded by enJSRVs is expressed and binds to the cellular receptors used by JSRV (39). On the other hand, the Gag protein expressed by enJSRVs is also known to have deleterious effects on the replication of exogenous JSRV (15). A misfolded Gag protein of enJSRVs co-assembles with JSRV Gag and forms chimeric viral capsids, which are degraded by the proteasome system of the infected cells (40). In addition, Monde et al. have recently demonstrated that the Gag proteins of an endogenous betaretrovirus co-assemble with a distinct retrovirus, HIV-1, Gag protein to modulate the late phase of HIV-1 replication (41). Together with our observation that itEBLN also has the ability to form nucleocapsids with BDV N, interaction of endogenous viral products into the capsids or nucleocapsids of incoming exogenous viruses may be an effective way to regulate virus infections.

Recent studies have demonstrated that the human genome contains many intrinsic factors that prevent viral infection (10, 42). Such factors could be acquired during evolution as consequences of the battle between viruses and their hosts. There is no doubt that the arms race between the host and virus has affected the evolution of both. In addition to the sophistication of the immune system, host organisms must have acquired many genes to overcome infection by pathogens over many generations of evolution. Exaptation is the co-opting of exogenous sequences as new genes, with functions distinct from their original purpose, into the genome (10, 43). The co-option of endogenous retroviruses as anti-viral factors is a good example of such exaptation. Until now, retroviruses were considered to be the only virus family that has been co-opted as new functional genes in the host genomes. Our results strongly suggest that exaptation by non-retroviral viral genes may have occurred

during the co-evolution of bornaviruses and their hosts.

At present, some EBLNs from human and non-human primate genomes, including hsEBLN-1 and hsEBLN-2, have been shown to express RNAs that potentially encode proteins (16, 44). Although we could not demonstrate an inhibitory effect of the hsEBLN-1 protein on the BDV replication, the possibilities that hsEBLN-1 plays roles in the cellular environment or acts at the level of RNA remain to be elucidated. On the other hand, hsEBLN-2 has been shown to express protein in human cells (44). A recent study also revealed that hsEBLN-2 is a candidate gene of the recurrent 3p12-p14 loss in cervical cancer and could be a novel tumour suppressor in cervical cancer (45). The human EBLNs are considered to have been generated 40 to 45 million years ago. Nevertheless, relatively long ORFs are conserved in these elements, especially hsEBLN-1 and -2, and these have a high level of amino acid identity with the N protein of current bornaviruses (16). These observations suggest the intriguing possibility that hsEBLN-1 and -2 have been adapted during evolution with new functions in the host cells. We are currently working on understanding the co-opted roles of the EBLNs, as well as other endogenous non-retroviral elements, in mammalian genomes. These studies could provide new insights into the co-evolution and between viruses and their hosts and the co-option of new genes in hosts following infection by exogenous viruses.

Materials and Methods

Cells. OL (human oligodendroglioma) and Vero cell lines were cultured in Dulbecco's

modified Eagle's medium (DMEM)-high glucose (4.5%) supplemented with 5% fetal bovine serum (FBS) and 4 mM glutamine. HEK293T cells were cultured in DMEM-low glucose (1.0%) supplemented with 10% FBS. OL cells persistently infected with BDV strain huP2br (OL/BDV) were cultured using the same conditions as the parental cell line. The cell lines stably expressing hsEBLN-1, itEBLN or BDV N were established by the limiting dilution and maintained in culture medium with Zeocin (Invitrogen) or G418 (Invitrogen).

Virus Infection. OL cell lines stably expressing the EBLN constructs were infected with cell-free rBDV P/M-GFP (46) virions at an M.O.I of 0.1. After absorption for 1 h, the cells were washed with phosphate-buffered saline (PBS) and passaged within 2 or 3 days. In addition, the OL cell lines were cocultured with Vero cells persistently infected with rBDV P/M-GFP. Three days after the co-cultivation, the cells were treated by Zeocin or G418 to eliminate the Vero cells. The infection rates of the cells were determined by measuring GFP expression using a FACSCalibur flow cytometer (BD Biosciences) or Tali Image-Based Cytometer (Invitrogen).

Minireplicon Assay. Minireplicon assays were carried out according to Yanai et al. (26). Briefly, HEK293T cells were seeded in 12-well plates and transfected with expression plasmids of BDV N (0.1 μg), P (0.01 μg), RNA-dependent RNA polymerase (L) (0.1 μg) and Pol II-driven minigenome plasmids (0.1 μg), with or without EBLN and BDV X expression plasmids (0.5 μg), using Lipofectamine 2000 (Invitrogen). 48 h later, the cells were lysed and cell lysates were prepared for chloramphenicol acetyltransferase (CAT) assay.

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Figure legends

Fig. 1. Expression of itEBLN inhibits BDV replication in transfected human cells. (A) Expression of recombinant EBLN proteins in transiently transfected OL cells. The HA-fused itEBLN, hsEBLN-1 and BDV N constructs were transfected into OL cells and the expressed proteins were detected by western blotting using anti-HA antibody. (B and C) Subcellular localization of recombinant EBLNs in transfected cells. The expression plasmids were transfected into uninfected (B) and OL cells persistently infected with BDV (C), and the distributions of EBLN proteins and BDV P were visualized by immunofluorescence assay with anti-HA (red) and anti-P (green) antibodies, respectively. Cells were counterstained with DAPI. Scale bar, 10 μm. Arrowheads in (C) indicate bornavirus viral factories, vSPOT. (D and E) Expression of itEBLN decreases the level of BDV RNAs in persistently infected cells. Forty-eight hour after transfection of the indicated constructs, the amounts of BDV genomic RNA (D) and mRNA (E) were quantified by real-time RT-PCR. Empty indicates the cells transfected with an empty vector. The values are presented as the mean ± SE of three independent experiments. Statistical significance was analyzed by the two-tailed t test. $*p < 0.05$, $**p < 0.01$.

Fig. 2. Expression of itEBLN in human cells inhibits BDV infection. (A) Schematic representations of the recombinant proteins of BDV N and itEBLN. An expression plasmid, pNL-itEBLN, was generated by fusing the NLS of BDV N (amino acids 1 to 38) to the N-terminus of itEBLN ORF. (B) Subcellular localization of NL-itEBLN in the transiently

transfected BDV/OL cells. Scale bar, 10 μm. (C) OL cells stably expressing pHA-N, pitEBLN, pNL-itEBLN and phsEBLN-1 were inoculated with cell-free rBDV expressing GFP at an M.O.I. of 0.1. GFP expression was monitored by fluorescence microscopy. The cells were photographed 4 days after infection. (D) The percentage of GFP-expressing cells was monitored over a period of 35 days.

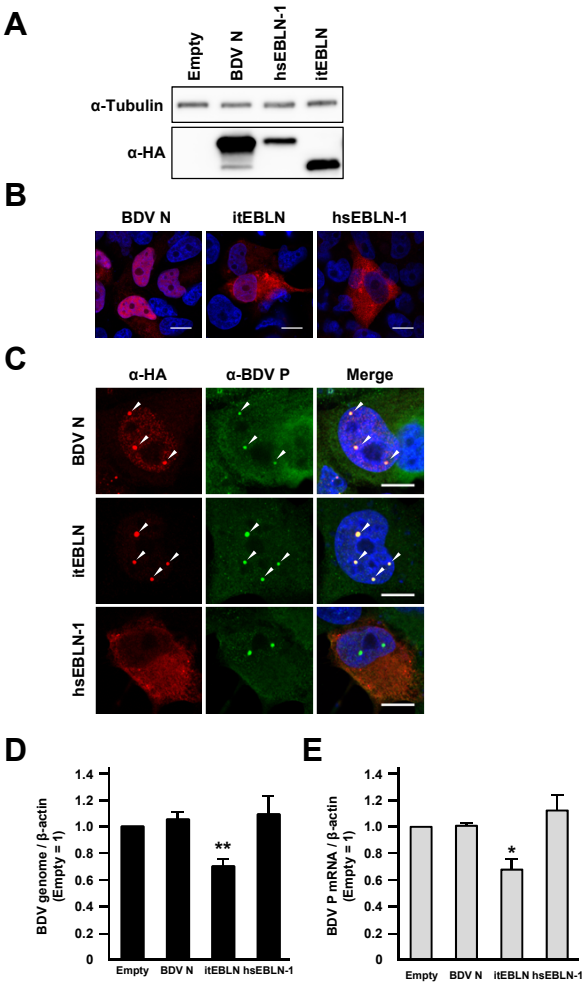
Fig. 3. Expression of itEBLN protects against cell-to-cell transmission of BDV. (A) OL cells stably expressing pHA-N, pitEBLN, pNL-itEBLN and phsEBLN-1 were co-cultured with Vero cells persistently infected with rBDV P/M-GFP. Three days after co-cultivation, the cells were treated with Zeocin or G418 to eliminate the Vero cells. The GFP expression was monitored by fluorescence microscopy. The cells were photographed 24 days after co-cultivation. (B) The percentage of GFP-expressing cells was monitored over a period of 24 days.

Fig. 4. itEBLN is incorporated into viral RNPs and affects BDV polymerase activity. (A) Immunoprecipitation analysis of itEBLN in OL/BDV cells. OL cells persistently infected with BDV were transfected with the indicated constructs, lysed twenty-four hour after transfection and immunoprecipitated with anti-HA antibody. Empty indicates empty vector (pcDNA3)-transfected cells. BDV P was detected by anti-BDV P antibody (arrow). The asterisk indicates non-specific bands detected in all cells. BDV genomic RNA was detected by RT-PCR using primers specific for the genome sense RNA within the BDV P region. (B) Expression of itEBLN inhibits BDV polymerase activity in a minireplicon assay. HEK293T

cells were co-transfected with a set of BDV minigenome plasmids and the expression constructs for itEBLN, hsEBLN-1, BDV N and BDV X. Forty-eight hours after transfection, the cells were lysed and subjected to CAT assays. The CAT activities are expressed as the ratio relative to empty vector-cotransfected cells (Empty). The values are the mean ± SE of three independent experiments. Statistical significance was analyzed by the two-tailed t test. ** $p < 0.01$

Fig. 5. Amino acid sequence alignment of BDV N and itEBLN. The identical amino acids in BDV N and itEBLN are shown by blue shading. The sequences of the NLS and NES and two P-binding sites (PBS-1/-2) are indicated. Arrowheads indicate the predicted amino acids residues essential for interaction with the viral RNA. The asterisks between the sequences indicate the residues predicted to be involved in the tetramerization of BDV N. Red and blue asterisks indicate residues interacting with the preceding and following crystallographic neighbors to form the tetramer, respectively. The initiation site of itEBLN-M2 is also indicated.

Fig. 1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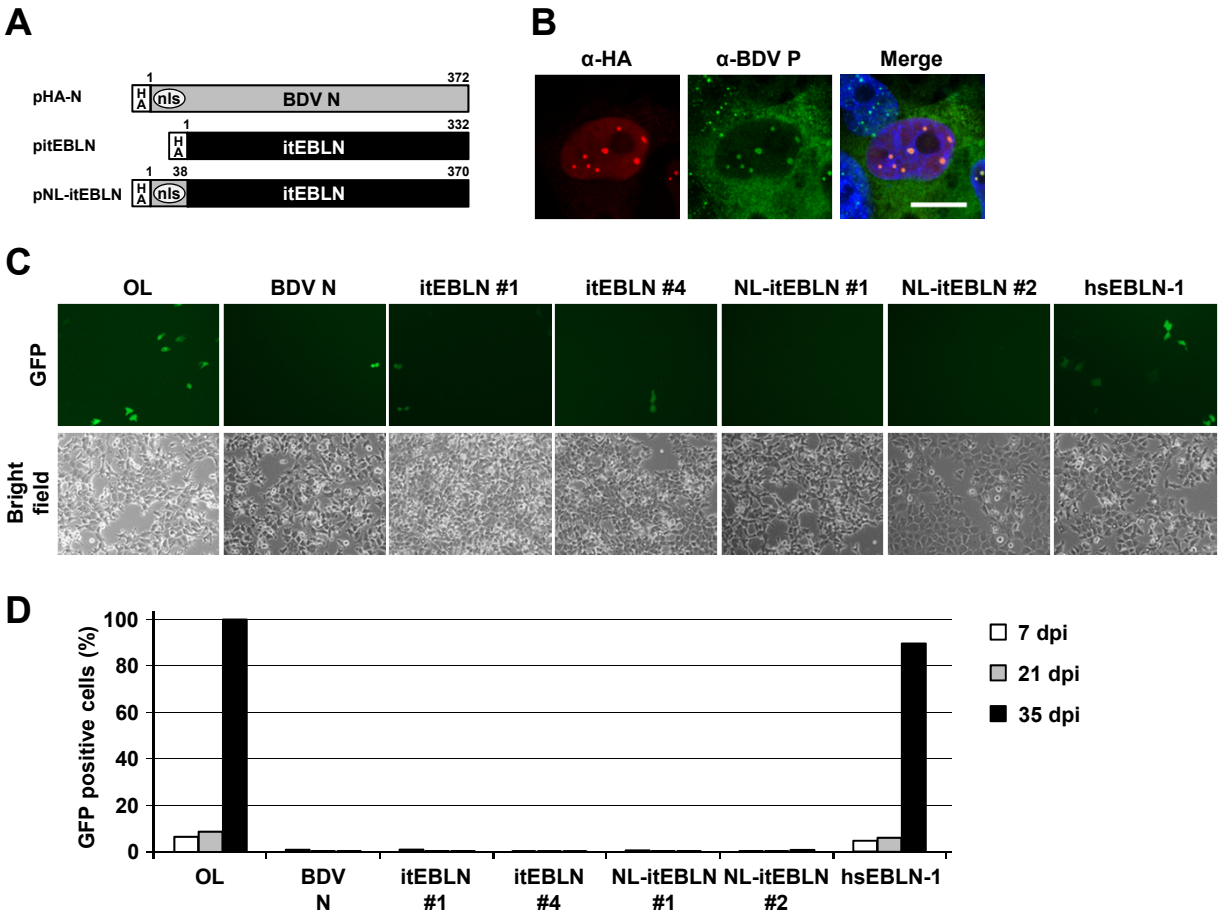
hsEBLN-1
itEBLN
α-Tubulin
α-HA
B
BDV N
itEBLN
hsEBLN-1
C
α-HA
α-BDV P
Merge
BDV N
itEBLN
hsEBLN-1
D
BDV genome / β-actin (Empty = 1)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
Empty
BDV N
itEBLN
hsEBLN-1
E
BDV P mRNA / β-actin (Empty = 1)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Empty
BDV N
itEBLN
hsEBLN-1

Fig. 2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HA
nls
BDV N
pitEBLN
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332
HA
itEBLN
pNL-itEBLN
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38
370
HA
nls
itEBLN
B
α-HA
α-BDV P
Merge
C
OL
BDV N
itEBLN #1
itEBLN #4
NL-itEBLN #1
NL-itEBLN #2
hsEBLN-1
GFP
Bright field
D
GFP positive cells (%)
100
80
60
40
20
0
OL
BDV N
itEBLN #1
itEBLN #4
NL-itEBLN #1
NL-itEBLN #2
hsEBLN-1
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21 dpi
35 dpi

Fig. 3

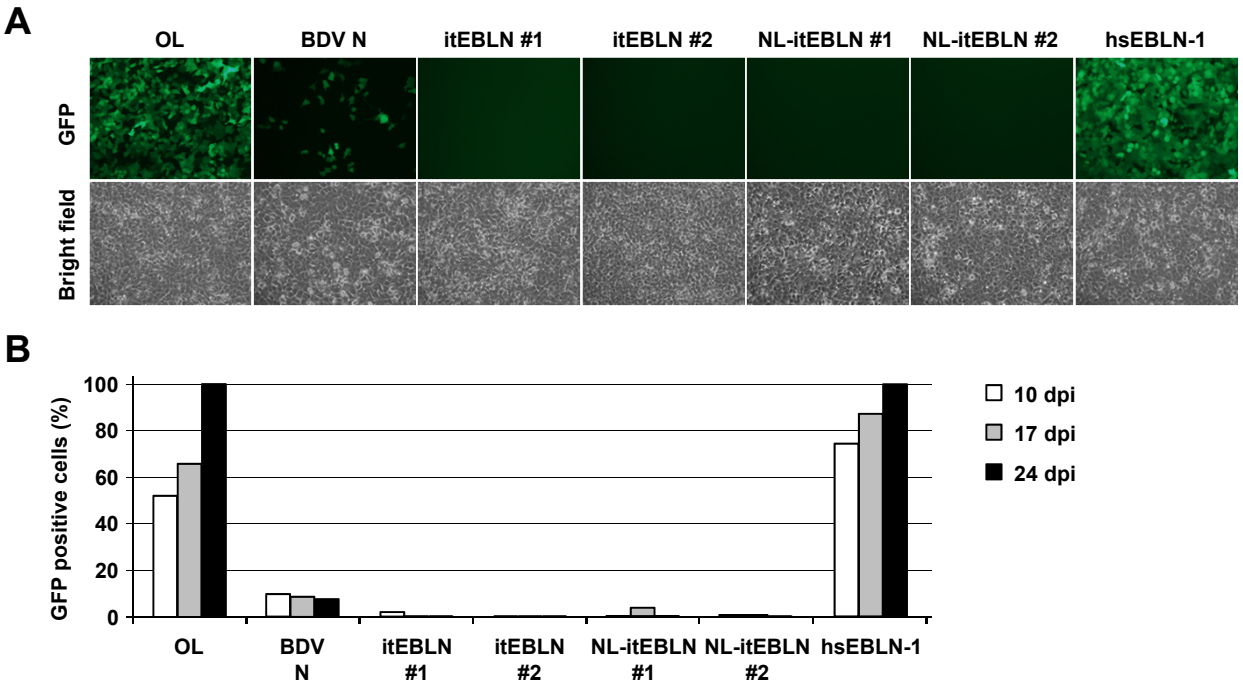
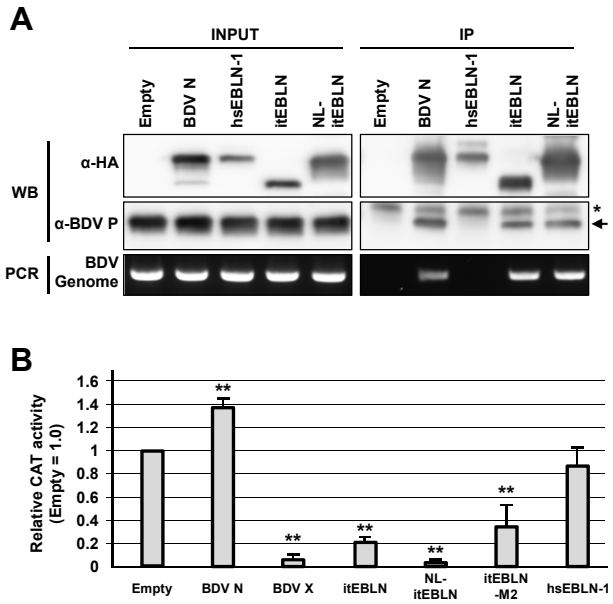

A
OL
BDV N
itEBLN #1
itEBLN #2
NL-itEBLN #1
NL-itEBLN #2
hsEBLN-1
GFP
Bright field
B
GFP positive cells (%)
100
80
60
40
20
0
OL
BDV N
itEBLN #1
itEBLN #2
NL-itEBLN #1
NL-itEBLN #2
hsEBLN-1
10 dpi
17 dpi
24 dpi

Fig. 4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BDV N
hsEBLN-1
itEBLN
NL-itEBLN
WB
α-HA
α-BDV P
PCR
BDV Genome
*
B
Relative CAT activity (Empty = 1.0)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
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Empty
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BDV X
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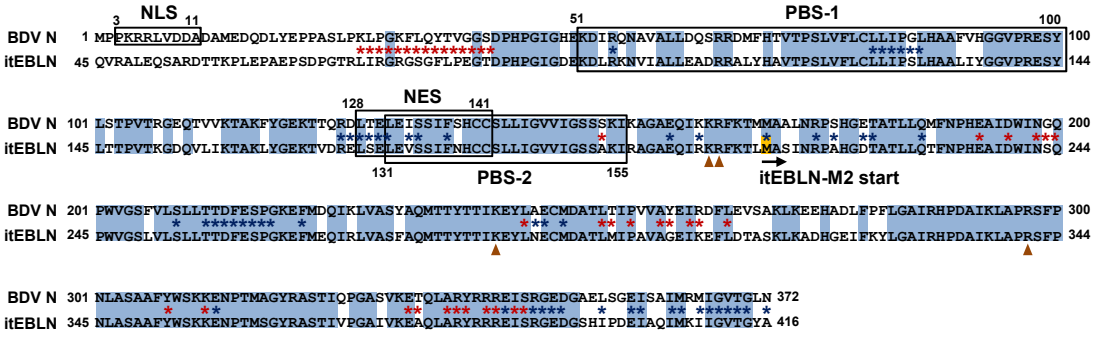
NL-itEBLN
itEBLN-M2
hsEBLN-1

Fig. 5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100
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itEBLN 45 QVRALEQSARDTTKPLEPAEPSDPGTRLIRGRGSGFLPEGTDPHPGIGDEKDLRKNVIALLEADRRALYHAVTPSLVFLCLLIPSLHAALIYGGVPRESY 144
NES
128
141
BDV N 101 LSTPVTRGEQTVVKTAKFYGEKTTQRDLTELEISSIFSHCCSLLIGVVIGSSSKIKAGAEQIKKRFKTMMAALNRPSHGETATLLQMFNPHEAIDWINGQ 200
itEBLN 145 LTTPVTKGDQVLIKTAKLYGEKTVDRELSELEVSSIFNHCCSLLIGVVIGSSAKIRAGAEQIRKRFKTLMASINRPAHGDTATLLQTFNPHEAIDWINSQ 244
131
PBS-2
155
itEBLN-M2 start
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