Dysfunction of Bovine Endogenous Retrovirus K2 Envelope Glycoprotein Is Related to Unsuccessful Intracellular Trafficking

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ABSTRACT
Endogenous retroviruses (ERVs) are the remnants of retroviral infection of ancestral germ cells. Mutations introduced into ERVs halt the production of infectious agents, but their effects on the function of retroviral proteins are not fully understood. Retroviral envelope glycoproteins (Envs) are utilized in membrane fusion during viral entry, and we recently identified intact coding sequences for bovine endogenous retrovirus K1 (BERV-K1) and BERV-K2 Envs. Amino acid sequences of BERV-K1 Env (also called Fematrin-1) and BERV-K2 Env are similar, and both viruses are classified in the genus Betaretrovirus. While Fematrin-1 plays an important role in cell-to-cell fusion in bovine placenta, the BERV-K2 envelope gene is marginally expressed in vivo, and its recombinant Env protein is defective in membrane fusion due to inefficient cleavage of surface (SU) and transmembrane subunits. Here, we conducted chimeric analyses of Fematrin-1 and BERV-K2 Envs and revealed that defective maturation of BERV-K2 Env contributed to failed intracellular trafficking. Fluorescence microscopy and flow cytometric analysis suggested that in contrast to Fematrin-1 Env, BERV-K2 Env could not be transported from the endoplasmic reticulum to the trans-Golgi network, where cellular proteases required for processing retroviral Envs are localized. We also identified that one of the responsive regions of this phenomenon resided within a 65-amino-acid region of BERV-K2 SU. This is the first report to identify that retroviral Env SU is involved in the regulation of intracellular trafficking, and it may help to elucidate the maturation process of Fematrin-1 and other related Envs.

IMPORTANCE
Retroviruses utilize envelope glycoproteins (Envs) to enter host target cells. Mature retroviral Env is a heterodimer, which consists of surface (SU) and transmembrane (TM) subunits that are generated by the cleavage of an Env precursor protein in the trans-Golgi network. SU and TM mediate the recognition of the entry receptor and virus-host membrane fusion, respectively. However, unexplained issues remain for the maturation process of retroviral Env. We previously reported that bovine endogenous retrovirus K2 (BERV-K2) Env lost fusogenicity due to a defect in the cleavage of SU and TM. In this study, we identified that mutations residing in BERV-K2 SU disturbed intracellular trafficking of BERV-K2 Env and resulted in inefficient cleavage. Because SU is not known to play an important role in this process, our study may provide novel insights into the maturation mechanism of retroviral Envs.

Eight to thirteen percent of mammalian genomes are occupied by endogenous retrovirus (ERV) sequences (1). ERVs are the remnants of exogenous retroviral infection of ancestral host germ cells. Although most ERVs are inactivated by mutations, deletions, and epigenetic modifications, some ERVs produce proteins that provide benefits for their hosts (2). Syncytins are derived from envelope glycoproteins (Env) of particular ERVs identified in humans, mice, carnivores, and rabbits. Moreover, they play critical roles in cell-to-cell fusion and immunosuppression during morphogenesis of placental syncytiotrophoblasts (3–8). Multiple copies of endogenous Jaagsiekte sheep retroviruses (enJSRVs) are also present in the sheep genome (9–12). The enJSRVs are reported to be involved in placental development and blockade and/or attenuation of exogenous JSRV infection (9–12).

Recently, we identified two novel bovine ERVs, named BERV-K1 and BERV-K2, which belong to the genus Betaretrovirus (13, 14). While both BERV-K1 and BERV-K2 harbor intact Env coding sequences (env), only BERV-K1 Env (here referred to as Fematrin-1) was preferentially expressed in bovine placental binucleate cells (BNC) throughout the gestation period and exhibited high fusogenic activity with bovine endometrial cells (13, 14). From these results, we concluded that BNCs utilize Fematrin-1 to generate fetomaternal hybrid cells called trinucleate cells in bovine placenta (14).

Although some ERVs retaining open reading frames (ORFs) can also produce viral proteins and/or particles, their function and/or infectivity is attenuated by mutations associated with endogenization (2, 15, 16, 17). For instance, Oliveira et al. reported that the infectivity of koala retrovirus (KoRV), which is endogenizing into koalas in Australia, was attenuated by amino acid substitutions in the late domain of Gag and receptor binding domain of Env (17). These substitutions were considered to be endogenization-associated adaptive mutations (17). However,
more studies are required to gain a complete view of the functional modification following endogenization.

Retroviral Env generally consists of signal peptide (SP), surface (SU), and transmembrane (TM) subunits. Translated Env is transported into the endoplasmic reticulum (ER) and then exported to the cellular surface membrane through the trans-Golgi network (TGN) (18). Envs of retroviruses including syncytins are cleaved and divided into SU and TM at an R-X-K/R-R amino acid motif by furin or related cellular proteases in TGN (19–22). SU and TM form heterodimers in a covalent or noncovalent manner before acquiring mature functions, such as receptor binding and membrane fusion (23, 24). However, unsolved issues remain in relation to the maturation mechanism of retroviral Envs. For example, it is unclear which domain is important for intracellular trafficking and whether this domain is different depending on the retroviral species.

Previously, we found that BERV-K2 Env was incapable of cleavage and cell-to-cell fusion despite the presence of a furin cleavage motif (R-T-R-R) and receptor-binding potency of the SU domain (see Fig. 1) (14). However, the precise mechanism remains to be fully elucidated. In this study, we investigated and characterized BERV-K2 Env maturation in detail and compared it to that of Fematin-1.

MATERIALS AND METHODS

Cell cultures. Cos-7 (ATCC CRL-1651) and HEK293T cells (CRL-11268) were cultured in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 5% CO₂ in air.

Construction of expression plasmids. C-terminal FLAG-tagged Fematin-1 SU (Fema1SUFLAG) and BERV-K2 SU (BK2SUFLAG) expression vectors, designated pCMV3Fema1SUFLAG and pCMV3BK2SUFLAG, respectively, were constructed as follows: both pSG5Fema1SUFLAG and pSG5BK2SUFLAG (14) were digested with EcoRI and BglII, and then each fragment was ligated into EcoRI/BglII sites of a phCMV3 vector (Genlantis, San Diego, CA). Stop codons at the 3′ end of the FLAG sequence excluded the hemagglutinin A epitope (HA) tag of the phCMV3 vector from the ORF.

pDisplayFema1SU and pDisplayBK2SU vectors (see Fig. 4B) were constructed using the In-Fusion HD cloning system (Clontech, Mountain View, CA) as follows; Fema1SU and BK2SU sequences were amplified by primer sets for Fema1SU (forward, 5′-GCCAGATCTCCCCGCTCCAGAAAGTGATTG-3′; reverse, 5′-GACCTGCAATTAACCCGGCCTTGGTTG-3′) and BK2SU (forward, 5′-GCCAGATCTCCCCGCTCCAGAAAGTGATTG-3′; reverse, 5′-GACCTGCAATTAACCCGGCCTTGGTTG-3′), respectively. Plasmid backbone sequences were also amplified using a common forward primer (5′-TATGCTGAGCGCTGACGAA CAAAAACTC-3′) and a specific reverse primer for Fema1SU (5′-AGCG GGAGATCTGGGCCCCGCTTGGGCCCCAGC-3′) or BK2SU (5′-AGTGGG AGATCTGGGCCCCGCTTGGGCCCCAGC-3′). Each fragment was treated with Cloning Enhancer (Clontech) and ligated by In-Fusion enzyme (Clontech). Chimeric Env expression plasmids were constructed using In-Fusion HD Cloning System. The detailed procedures for the construction of the plasmids are available upon request.

Transfection. Transfection was performed under various conditions as described below. For the experiment shown in Fig. 4, 2 × 10⁶ cells of Cos-7 cells and 5 × 10⁶ cells of HEK293T cells were subcultured in 6-well plates prior to transfection. Cells were transfected with 3 μg of pCMV3Fema1SUFLAG, pCMV3BK2SUFLAG, pDisplayFema1SU, or pDisplayBK2SU using 3 μl of Lipofectamine 2000 (Invitrogen) at 37°C for 3 h. Cells and culture supernatants were collected at 72 or 48 h for the experiment shown in Fig. 4A or C, respectively, and subjected to analysis.

For the experiment shown in Fig. 5, 2 × 10⁶ wells of Cos-7 cells, which were subcultured in 6-well plates containing cover glasses, were cotransfected with 1 μg/well of pER-MAG1 (MBL, Nagoya, Japan) or pEYFP-FLAG (Clontech) and 1.5 μg/well of pSG5Fema1SUFLAG or pSG5BK2SUFLAG, using 3 μl/well of Lipofectamine 2000 at 37°C for 4 h. Cells were subjected to indirect immunofluorescence antibody staining (IFA) at 48 h posttransfection. pER-MAG1 contains a coding sequence for a green fluorescent protein, which was derived from stony coral, tagged with an N-terminal signal peptide and a C-terminal ER retention sequence (K-D-E-L). pEYFP-FLAG encodes a fusion protein of enhanced yellow fluorescent protein, and the N-terminal 81 amino acids of human β1,4-galactosyltransferase contains the membrane-anchoring signal peptide utilized in TGN localization.

For immunoblotting for Fig. 2, 3, and 6, 2 × 10⁵ cells of Cos-7 cells were subcultured in 6-well plates and transfected with 3 μg/well of each construct using 3 μl/well Lipofectamine 2000 as described above. Cells were collected at 48 h posttransfection and subjected to immunoblotting.

For fusion assays shown in Fig. 2, 3, and 6, 2 × 10⁵ cells of Cos-7 cells were subcultured in 6-well plates and divided into two transfection groups as follows. While one group was cotransfected with 3 μg/well pT7EMCVLuc (24), 3 ng/well pHRL-TK (Promega, Madison, WI), and 3 μg/well of each chimeric Env expression plasmid using 6 μl/well of Lipofectamine 2000, the other was transfected with 3 μg/well pCAT7pol (25) using 3 μl/well of Lipofectamine 2000. The procedure for the fusion assay is described below.

Purification of FLAG-tagged Envs. Supernatants of Cos-7 and HEK293T cells transfected with pCMV3 vector, pCMV3Fema1SUFLAG, or pCMV3BK2SUFLAG were harvested at 72 h posttransfection and incubated with ANTI-FLAG M2 affinity gel (Sigma) at 4°C for 16 h. Then, FLAG-tagged SUs were eluted using 150 ng/μl of 3×FLAG peptides. Purified proteins were subjected to immunoblot analysis.

Immunoblotting. Immunoblotting was conducted as described previously (14). Briefly, cell lysates were harvested with RIPA buffer (50 mM Tris·HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]), and culture supernatants were filtered with disc filters. Collected samples were denatured at 100°C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis. Electrophoresed samples were transferred onto polyvinylide fluoride membranes, followed by blocking with 10% (wt/wt) skim milk, and reacted with relevantly diluted antibodies described below, and the reacted proteins were visualized using the SuperSignal West Femto maximum-sensitivity substrate (Thermo Fisher Scientific Inc., Waltham, MA) and the ImageQuant LAS4000 mini instrument (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, England). In the antibody reactions, primary antibodies for FLAG tag (anti-FLAG M2 [Sigma]), HA tag (anti-HA [6E2] [Cell Signaling Technology Inc., Danvers, MA]), and tubulin (anti-alpha-tubulin [Sigma]) were diluted at 1:2,000, 1:1,000, and 1:10,000, respectively, followed by secondary antibody (anti-mouse IgG conjugated with horseradish peroxidase [Thermo Fisher Scientific Inc.] diluted at 1:1,000).

Flow cytometric analysis. Flow cytometric analysis was performed as described previously with slight modifications (14). Cells transfected with pDisplayFema1SU or pDisplayBK2SU and cells mock transfected with only transfection reagent were harvested 48 h after transfection and fixed with 4% paraformaldehyde (PFA) on ice for 30 min, followed by incubation in blocking buffer (phosphate-buffered saline [PBS], pH 7.4, containing 3% bovine serum albumin [BSA]) on ice for 1 h, incubation with anti-HA (6E2) diluted at 1:50 in blocking buffer on ice for 1 h, and incubation with anti-mouse IgG conjugated with Alexa Fluor 488 (Invitrogen) diluted at 1:250 in blocking buffer on ice for 30 min. Then, samples were analyzed by using a FACScalibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ). Assays were repeated as three independent experiments.
ments ($n = 3$) and the results were exhibited as relative mean fluorescence intensities (MFI).

IFA. IFA was applied as previously described, with slight modifications (26). Briefly, cells transfected with indicated plasmids were fixed with 4% PFA on ice for 30 min, followed by blocking in blocking buffer at room temperature (RT) for 1 h, reaction with anti-FLAG M2 diluted at 1:500 in antibody dilution buffer (PBS containing 1% BSA) at RT for 2 h, staining with anti-mouse IgG conjugated with Alexa Fluor 594 (Invitrogen) diluted at 1:1,500 in antibody dilution buffer at RT for 1 h, and staining with 1 ng/ml 4,6-diamidino-2-phenylindole (DAPI) solution at RT for 5 min. Cells were embedded in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA) and subjected to confocal fluorescence microscopy (Digital Eclipse C1si spectral imaging confocal laser microscope; Nikon Inc., Tokyo, Japan). Tiff images taken by a confocal microscope were divided into three channels (red, green, and blue) by the ImageJ software program. Red and green channels were merged and analyzed using a plug-in, named colocalization finder. For colocalization finder analysis, we adjusted the measuring points (yellow spots) using the correlation diagram and obtained the colocalization values. Values were exhibited as percent colocalization in a window of ImageJ. Measurements were conducted for 10 randomly selected cells per gene. Mean values ± standard errors are indicated in bar graphs of Fig. 5.

Fusion assay. Fusion assays were performed as previously described (14). Briefly, at 24 h posttransfection, $5 \times 10^4$/well of Cos-7 cells transfected with pT7EMCVluc, pRL-TK, and each chimeric Env expression vector were cocultured with $5 \times 10^4$/well of Cos-7 cells transfected with pCAGT7pol in 96-multiwell plates at 37°C for 24 h. Then, cells were lysed and subjected to a dual-luciferase reporter assay. Assays were conducted in triplicate for 3 independent experiments ($n = 9$), and the results were shown as relative luciferase activities.

Statistical analyses. Student’s t test was conducted for the all experiments, and significant differences were considered to be $P$ values of $<0.05$.

RESULTS

Chimeric analysis of Fematrin-1 and BERV-K2 Env. Because Fematrin-1 and BERV-K2 Env have more than 70% amino acid identity (Fig. 1), we succeeded in constructing chimeric mutants and investigated their cleavability and fusogenic activities by immunoblotting analysis and fusion assay, respectively (Fig. 2 and 3). Env C-terminal FLAG tags allowed the detection of Env precursor and cleaved TM by immunoblotting. Reciprocal replacement of each cleavage site (the mutants’ names are 1CS2 and 2CS1) did not affect each cleavability and fusogenic activity (com-

FIG 1 Sequence comparison of Fematrin-1 and BERV-K2 Env. Amino acid sequences of Fematrin-1 and BERV-K2 Env are aligned. Identical amino acids and gap sequences are shown as dots and hyphens, respectively. Each Env subunit is indicated as SP, SU, and TM, and putative cleavage motifs are boxed.
Failure of BERV-K2 Env Trafficking and Processing

FIG 2 Processing of Fematrin-1 and BERV-K2 chimeric mutants. (A to C) Schematic representation of expression constructs which were transfected into Cos-7 cells. All constructs contain FLAG epitope tags and 3’ long germinal repeats (LTR) downstream of the TM C termini. (D to F) Immunoblotting analyses of lysates of Cos-7 cells transfected with chimeric mutants. Mutants are indicated above the gels. The names of antibodies and detected proteins are at right. Molecular masses are displayed at left. (G to I) Fusion assay of chimeric mutants. Cos-7 cells cotransfected with indicated expression vectors for chimeric Envs and the pT7EMCVluc and pRL-TK vectors were cocultured with the other Cos-7 cells transfected with pCMVT7pol at 37°C for 24 h. Then, cocultured cells were subjected to a dual-luciferase reporter assay. Assays were performed in triplicate and repeated as three independent experiments (n = 9). Values are exhibited as means ± SE of relative luciferase activities. Significantly higher (P < 0.05) activities are marked with asterisks. Fematrin-1 is abbreviated as Fema-1. ShPep indicate the short peptides, under 20 kDa in mass.
We confirmed that the reciprocally replaced sequences functioned as cleavage sites because the alanine substitutions lost both cleavability and fusogenicity (compare Fema-1 with 1CSA and 1CS2 with 1CSA in Fig. 2A, D, and G). Although immunoblotting of BERV-K2 Env showed two major bands around 30 kDa (Fig. 2D to F and 3B), the disappearance of the upper band after alanine mutagenesis (Fig. 2D) revealed that it was cleaved TM (see 2CSA). We could not determine the significance of the lower band but suspect that it might be a partially processed product or ER-associated degradation fragment. Next, each SP and SU was reciprocally swapped, and the constructs were termed 2SS1T and 1SS2T (Fig. 2B). We found that both cleavability and fusogenicity were inverted by this recombination (compare Fema-1 with 2SS1T and K2 with 1SS2T in Fig. 2B, E, and H). These results indicate that amino acid differences residing in SPs and/or SUs but not cleavage sites might cause functional differences. We then investigated whether SPs and/or SUs are responsible for the functional differences by chimeric analysis. Exchanging each other’s SUs (compare Fema-1 with 1S2S1T and K2 with 2S1S2T in Fig. 3) but not SPs (compare Fema-1 with 2S1ST and K2 with 1S2ST in Fig. 2C, F, and H) changed maturation status and fusogenicity of each Env protein. These data suggested that the inefficient cleavage can be attributed to any mutations accumulated in BERV-K2 SU. In these subsequent experiments, we identified additional bands under 20 kDa by immunoblotting as shown in Fig. 2D to F and 3B. The number of short peptides seemed to inversely correlate with cleavability and fusogenicity. These peptides might consist of C-termini of Envs, as with other retroviral R peptides (27); however, the amount of R peptide is usually directly proportional to fusogenicity. Therefore, these fragments should not function as R peptide. It is possible that these peptides might be accessory proteins encoded by unidentified spliced mRNAs, because related betaretroviruses, including mouse mammary tumor virus and Jaagsiekte sheep retrovirus, encode unique accessory proteins in their genome (28, 29). In addition, these short peptides may be degradation products. Nonetheless, additional studies are required to figure out the generation mechanism and significance of these peptides.

**Solely expressed BERV-K2 SU is not secreted from the cells in vitro.** Because we identified that BERV-K2 SU is responsible for the deficiency of Env processing, we attempted to gain insights into characteristics of BERV-K2 SU by comparing them with those of Fematrin-1 SU. Retroviral SU has an SP and is shed from cells when it is expressed alone (26). We therefore examined whether BERV-K2 SU is normally exported into culture medium when it is expressed in Cos-7 and HEK293T cells (Fig. 4A). FLAG-tagged Fematrin-1 SU and BERV-K2 SU were equally detected in both cell lysates, but only Fematrin-1 SU was detected in culture supernatants in both cells. Immunoprecipitation of FLAG-tagged Fematrin-1 and BERV-K2 SU in the supernatants confirmed these results (data not shown) and suggested that BERV-K2 SU was not shed from the cells.
tag, Myc tag, and platelet-derived growth factor receptor transmembrane domain (PDGFR TM). The fusion protein from the plasmid is expressed on the cellular surface membrane.

pDisplayFema1SU or pDisplayBK2SU contains Fematrin-1 SU or BERV-K2 SU at the boundary region of the HA and Myc tags, respectively (Fig. 4B). These fusion proteins were expressed in Cos-7 cells, and then whole and cellular surface expression levels were confirmed by immunoblotting and flow cytometric analyses, respectively (Fig. 4C and D). While both of the fusion proteins were similarly expressed in Cos-7 cells (Fig. 4C), the surface expression level of PDGFR TM fused with BERV-K2 SU was significantly lower than that of PDGFR TM fused with Fematrin-1 SU (Fig. 4D). These data indicated that BERV-K2 SU impaired intracellular trafficking when fused with transmembrane proteins, including unrelated proteins.

Both BERV-K2 Env and BERV-K2 SU do not localize in TGN.

FIG 4

Expression levels of FLAG-tagged Fematrin-1 SU and BERV-K2 SU in culture supernatants and those of fusion proteins of Fematrin-1 SU or BERV-K2 SU on the cellular surface. (A) Cell lysates and culture supernatants (Sup) of Cos-7 and HEK293T cells, which were transfected with each SU expression plasmid, were subjected to immunoblotting analysis using indicated antibodies (α-FLAG or α-Tubulin). Molecular masses are indicated at left. α-Myc, Myc, and PDGFR TM were inserted between HA and Myc tags. pDisplaySUs encode fusion proteins of MIKLS, HA, SU, Myc, and PDGFR TM. (B) Schematic representation of pDisplaySU plasmids. Each BERV-K SU was inserted between HA and Myc tags. pDisplaySUs encode fusion proteins of MIKLS, HA, SU, Myc, and PDGFR TM. (C) Cell lysates of Cos-7 cells transfected with each pDisplaySU plasmid were subjected to immunoblotting analysis using anti-FLAG signals shown in Fig. 5 reflected the localization of Env or TM, even in the presence of detected short peptides (under 20 kDa in immunoblotting of Fig. 2 and Fig. 3).

Altogether, these results suggest that BERV-K2 Env was inefficiently transported from the ER to the TGN, and the responsive region of the phenomenon resided in BERV-K2 SU.

Determination of the region responsible for cleavage deficiency of BERV-K2 Env. We investigated the region responsible for the low cleavability of BERV-K2 Env. We generated various expression plasmids harboring coding sequences for chimeric Env proteins, as shown in Fig. 6A, and expressed them in Cos-7 cells. Immunoblotting analysis revealed that cleaved Fematrin-1 TM was detected when the cells were transfected with the 1-298 and 1-187 constructs but not the 1-491, 1-465, 1-394, and 1-387 constructs (Fig. 6B). Fusion activities of the 1-298 and 1-187 constructs were also significantly higher than those of the other four chimeric constructs (Fig. 6C). Four additional chimeric Envs were subjected to immunoblot analysis and fusion assay (Fig. 6D to F).

Altogether, these results suggest that BERV-K2 Env was inefficiently transported from the ER to the TGN, and the responsive region of the phenomenon resided in BERV-K2 SU.

DISCUSSION

In this study, we investigated the root of inefficient BERV-K2 Env cleavage in cells from the standpoint of intracellular trafficking. We determined that inefficient cleavage of BERV-K2 SU and TM could be attributed to the SU subunit (Fig. 2 and 3). We also verified that BERV-K2 SU was not shed into the culture medium when expressed alone, and PDGFR TM fused with BERV-K2 SU was marginally expressed on the cellular surface (Fig. 4). Intracellular sublocalization of BERV-K2 Env was also similar to that of BERV-K2 SU (Fig. 5). These data suggested that BERV-K2 SU harbors mutations disturbing the transport of membrane proteins...
fused with BERV-K2 SU from the ER to the TGN. The retroviral
Env SU subunit is utilized for receptor recognition, and the con-
formation of SU and its receptor usage depend on retroviral spe-
cies (30, 31). Currently, it is unclear whether Fematrin-1 and
BERV-K2 Env recognize the same receptor; however, we previ-
ously reported that they both could bind Cos-7 cells at a similar
level (14). Therefore, differences in their fusogenicity would not
be attributed to their receptor usages. Although we could not have
examined the cellular surface expression level of BERV-K2 Env
because we do not have any antibodies to BERV-K2 Env, our
current results indicated that BERV-K2 Env was not successfully
transported to the cellular surface. Retroviral Env precursor pro-
teins are generally processed into SU and TM by cellular furin or
related proteases (19–22). Because these proteases are localized
predominantly in TGN, Envs have to be properly transported into
TGN for successful processing (18). We therefore considered that
unsuccessful processing of BERV-K2 Env might result from im-
proper trafficking.

Furthermore, we found that three chimeric mutants of Fematrin-1 with the region from position 298 to 362 replaced with
that of BERV-K2 Env exhibited attenuated processing efficiencies and fusion activities (Fig. 6). These results were also associated with defects in their trafficking (data not shown). We also con-
structed BERV-K2 Env with the corresponding region from 298 to
362 replaced with that of Fematrin-1 and performed immuno-
blotting analysis and fusion assays. However, its cleavage effi-
ciency and fusion activity were not significantly restored (data not
shown). This indicated that although the region from 298 to 362
of BERV-K2 Env is one of the critical domains, other regions of
BERV-K2 Env or the overall structure of BERV-K2 Env may con-
tribute to the defect. There are significant differences between
Fematrin-1 and BERV-K2 Env in the region from 298 to 362 of
BERV-K2 Env (26 amino acid differences in a 65-amino-acid re-
gion) (Fig. 1).

In silico analysis suggested that the replacement of
these different amino acids altered the two-dimensional structure of Fematrin-1. Two β-sheets (from position 297 to 303 and from

FIG 5 Intracellular sublocalization of FLAG-tagged Fematrin-1 and BERV-K2 Env in Cos-7 cells. (A and C) Cos-7 cells were cotransfected with pER-mAG1 (A)
or pEYFP-Golgi (C) and each Env expression plasmid, which was C-terminally tagged with FLAG, and were subjected to IFA 48 h posttransfection. Anti-FLAG
M2 and anti-mouse IgG Alexa Fluor 555 were used as the primary and secondary antibodies, respectively. Enlarged images are shown in small windows. Signals
of Envs and ER-mAG1/EYFP-Golgi are highlighted with red and green colors, respectively, and merged signals are shown in yellow. Nuclei were stained with
DAPI (blue). (B and D) The bar graphs indicate the relative numbers of merged spots of ER-mAG1 and Envs (B) or EYFP-Golgi and Envs (D). Fluorescent spots
were quantified using ImageJ software and colocalization finder. Values are represented as means ± SE for each 10 microscopic fields. Significant differences were
considered to be P values < 0.05 and are indicated with asterisks (*).
343 to 356) and an α-helix (319 to 340) of Fematrin-1 were lost or disrupted by these mutations (data not shown). ER export of secretory and cellular surface proteins is a very complex event (32). ER cargos are sorted into the coat protein complex II (COPII) vesicles at exit sites of the ER. Then, COPII vesicles are exported to compartments of pre-TGN and TGN in an anterograde direction. Sec24 is a coat protein of COPII vesicles and has an important role in incorporating cargo proteins into COPII (33, 34). Sec24 forms oligomers with Sec23 and Sar-1 and binds with cargo proteins (33, 34). Sec24 recognizes short amino acid motifs and/or conformational epitopes that reside in the cytosolic region of cargo proteins (34). Therefore, proteins lacking binding motifs for Sec24 or insufficiently folded proteins are retained within the ER and degraded (35). Considering the intracellular trafficking mechanisms mentioned above, BERV-K2Env but not Fematrin-1 might be retained in the ER due to inefficient incorporation into COPII vesicles.

**FIG 6** Determination of the region responsible for functional defects in BERV-K2Env. (A and D) Schematic representation of expression constructs which were transfected into Cos-7 cells. All constructs contain FLAG epitope tags and 3’ LTR downstream of the TM C termini. Plasmid names indicate ranges of amino acid sequences which originated with BERV-K2 Env. For example, “1-491” means that amino acids from 1 to 491 of BERV-K2 Env were recombined into the corresponding region of Fematrin-1. (B and E) Immunoblotting analysis of lysates of Cos-7 cells transfected with chimeric mutants. Mutants’ names are shown at the top. The names of used antibodies and detected proteins are indicated at right. Molecular masses are displayed at left. (C and F) Fusion assay of chimeric mutants. Cos-7 cells cotransfected with indicated expression vectors for chimeric Envs and pCMV7pol vectors were cocultured with other Cos-7 cells transfected with pCMV7pol at 37°C for 24 h. Then, cocultured cells were subjected to dual-luciferase reporter assay. Assays were performed in triplicate and repeated as three independent experiments (n = 9). Values are shown as means ± SE of relative luciferase activities. Significantly higher (P < 0.05) activities are marked with asterisks (*). Fematrin-1 is abbreviated as Fema-1. “ShPep” indicates the short peptides, under 20 kDa in mass.
vesicles, because BERV-K2 Env might lose a Sec24 binding motif and/or change its steric structure through endogenization.

A related study previously reported that human immunodeficiency virus type 1 (HIV-1) Env mutants with mutations in the membrane-spanning domain (MSD) of TM were inefficiently transported from the ER to the TGN and cleaved into SU and TM (36). Membrane proteins are subjected to quality control by checking their MSD, and then defective proteins are retained in the ER (37). Therefore, the mutated HIV-1 Env might be inefficiently transported and processing defective (36). Because BERV-K2 SU does not harbor any MSDs but had a functional defect in BERV-K2 Env due to substitutions in SU, there should be different mechanisms. However, because mechanisms of retroviral Env maturation, especially in intracellular trafficking, are complicated, it remains unclear how Env is incorporated into transport vesicles and which domains and motifs are required for successful transport. We consider that comparative studies of Femmatrin-1 and BERV-K2 Env might provide helpful information to resolve these issues.

We reported that BERV-K2 had integrated into the bovine genome much later than BERV-K1, and its coding sequences, including gag, pol, pro and env, seem to be conserved as intact ORFs (13, 14). However, BERV-K2 env was marginally expressed in all tissues examined (13, 14), and epigenetic modification was involved in the transcriptional inactivation (data not shown). Moreover, BERV-K2 Env was also inactivated by amino acid mutations. Thus, hosts would have protected themselves from BERV-K2 reactivation and reinfection by using various strategies. Recently, we also identified that although bERVE-A, which is an intact Env coding sequence of a bovine ERV, was specifically expressed in trophoblast cells (38), a recombinant bERVE-A protein was incapable of cell-to-cell fusion in vitro (K. Koshi, Y. Nakaya, K. Kizaki, T. Miyazawa, and K. Hashizume, unpublished data). This might also be the result of functional mutations that were introduced during endogenization. As with findings of the studies mentioned above, the ERVs might be subjected to posttranslational defects in various ways by their hosts’ defensive machineries.

A recent study revealed that a defective murine ERV, which is named Emv2 and is physiologically suppressed at a very low level, was reactivated and acquired full infectivity in mice with artificial knockout (KO) of specific Toll-like receptors (37, 38). The reactivated Env was generated by recombining with other replication-defective ERVs and induced tumors in mice (39, 40). In the study, normal mice but not KO mice were reactivated to generate neutralization antibodies to Emv2 under the homeostatic condition (39). This indicated that antigens of Emv2 are normally expressed at a leaky level and its host can recognize them to gain acquired immunity. In other words, marginally expressed proteins of ERVs could activate the hosts’ immune systems, and then the hosts would come to protect themselves against contingent reactivated ERV and its related exogenous retroviruses. To date, we do not know the significance of BERV-K2 and its related ERVs present in the bovine genome (14). BERV-K2 is defective in replication and is expressed marginally, like Emv2 (13, 14). Therefore, these studies might provide an insight into the physiological functions of BERV-K2 and its related ERVs.

Although more studies are required to determine the precise mechanism, this is the first report elucidating the relationship between mutations of retroviral SU and a failure of intracellular trafficking. This study may provide useful information for the maturation process of Femmatrin-1 and related EnvS, as well as the complex mechanism underlying retroviral endogenization.

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