Engineering Glycoprotein B of Bovine Herpesvirus 1 To Function as Transporter for Secreted Proteins: a New Protein Expression Approach

Günther M. Keil,* Constanze Höhle, Katrin Giesow, and Patricia König

Institute of Molecular Biology, Friedrich-Loeffler-Institutes, Federal Research Centre for Virus Diseases of Animals, Greifswald-Insel Riems, Germany

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Glycoprotein B (gB) of bovine herpesvirus 1 (BHV-1) is essential for BHV-1 replication and is required for membrane fusion processes leading to virus penetration into the target cell and direct spreading of BHV-1 from infected to adjacent noninfected cells. Like many of the herpesvirus gB homologs, BHV-1 gB is proteolytically processed by furin, an endoproteinase localized in the trans-Golgi network. Cleavage by furin is a common mechanism for the activation of a number of viral fusion (F) proteins. Among these, the F proteins of both human and bovine respiratory syncytial virus (RSV) have the so far unique feature that cleavage of the respective F protein precursors occurs at two furin recognition sites, resulting in the release of a 27-amino-acid intervening peptide which is secreted into the extracellular space. We showed recently that the intervening peptide of bovine RSV can replace by bovine interleukins which are secreted into the medium of cells infected with the respective bovine RSV recombinants (P. König, K. Giesow, K. Schuldt, U. J. Buchholz, and G. M. Keil, J. Gen. Virol. 85:1815–1824, 2004). To elucidate whether the approach to transport heterologous proteins as furin-excisable polypeptides functions in principle also in glycoproteins which are cleaved by furin only once, we inserted a second furin cleavage site into BHV-1 gB and integrated a 16-amino-acid peptide sequence, the 246-amino-acid green fluorescent protein (GFP), or the 167 amino acids for mature bovine alpha interferon (boIFN-α) as an intervening polypeptide. The resulting gB variants rescued gB-negative BHV-1 mutants, the resulting BHV-1 recombinants were fully infectious, and infected cells secreted biologically active GFP and boIFN-α, respectively. In contrast to the gB2Fu and gB2FuGFP precursor molecules, which were efficiently cleaved at both furin sites, the majority of pgB2FuIFN-α was not cleaved at the site between the amino-terminal (NH2) subunit and boIFN-α, whereas cleavage at the newly introduced site was normal. This resulted in virus particles that also contain the NH2-subunit/boIFN-α fusion protein within their envelopes. Our results demonstrate that BHV-1 gB can be used as a transporter for peptides and proteins which could be important for development of novel vaccines. In addition, the general principle might be useful for other applications, e.g., in gene therapy and also in nonviral systems.

Posttranslational processing of membrane and secreted proteins by endoproteolytic cleavage of the respective precursor molecules is a common pathway for, e.g., peptide hormones, serum albumin, cell surface receptors, and viral fusion proteins such as the hemagglutinin of influenza virus and the F proteins of respiratory syncytial virus (RSV), Sendai virus, and measles virus (12). Also, most of the herpesvirus glycoprotein B (gB) homologs are cleaved after the consensus sequence RX(K/R)R by furin, a subtilisin-like endoproteinase localized in the trans-Golgi network (TGN) (2, 12, 15). After cleavage, the amino-terminal subunit (NH2 subunit) and the carboxy-terminal subunit (COOH subunit) of gB remain covalently linked by disulfide bonds. There are, however, herpesviruses that express gB that is not cleaved by furin, among them being herpes simplex viruses types 1 and 2 and Epstein-Barr virus (5, 7, 9). In addition, it has been shown for the betaherpesvirus human cytomegalovirus and the alphaherpesviruses pseudorabiesvirus and bovine herpesvirus 1 (BHV-1) by mutagenesis of the furin recognition sequence that cleavage of gB is not essential for replication in cell culture (15, 23), indicating that this domain of gB might tolerate sequence modifications. Among the furin-cleaved viral fusion proteins, the F proteins of bovine RSV (BRSV) and human RSV share a so-far unique feature. The respective precursor molecules are cleaved at two furin recognition sites, resulting in release of a 27-amino-acid intervening peptide that is glycosylated (27, 28). For BRSV it has been shown that the intervening peptide is secreted as a bioactive peptide of the tachykinin family after further modification (29). We demonstrated recently that the intervening peptide of the BRSV F protein can be replaced by bovine interleukin 2 (boIL-2) and boIL-4, which are cleaved out of the precursor molecules and are secreted into the culture medium of cells infected with the respective BRSV recombinants (14).

We report here the introduction of a second furin cleavage site and intervening polypeptides into gB of BHV-1 and the isolation of viable BHV-1 recombinants that express the green fluorescent protein (GFP) and bovine alpha interferon (boIFN-α) as furin-excisable proteins which are secreted as biologically active proteins from infected cells.

**MATERIALS AND METHODS**

*Cells and viruses.* Madin-Darby bovine kidney (MDBK) cell clone Bu100 (kindly provided by L. Bello and W. Lawrence, Philadelphia, Pa.) was grown in

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*Corresponding author. Mailing address: Friedrich-Loeffler-Institutes, Federal Research Centre for Virus Diseases of Animals, Boddenblick 5A, 17493 Greifswald-Insel Riems, Germany. Phone: 49-38351-7273. Fax: 49-38351-7275. E-mail: Guenther.M.Keil@rie.bfav.de.
Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum, 2.4 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Bovine pharyngeal cell line 244 (KOP-R) and bovine kidney cell line ml. Cell cultures were incubated at 37°C in a humidified atmosphere containing (GenBank accession number AJ004801). (C) Scheme of the gB ORF. Sequences encoding the signal peptide (sig), the amino-terminal subunit, the carboxy-terminal subunit which contains the membrane anchor domain, and the furin cleavage site (FCS1) are indicated. The double-headed arrow indicates the sequence deleted in gB subunit, the carboxy-terminal subunit which contains the membrane anchor domain, and the furin cleavage site (FCS1) are indicated. Arrows indicate the isomerization of the U₄ segments are indicated, as are the internal and terminal repeat sequences (IR and TR, respectively). The fragment between the Apal cleavage sites (underlined) which encodes the codons for FCS1 (boldface) is expanded, and the nucleotide sequence is given. The amino acid sequence for FCS1 is indicated in three-letter code. (D) Nucleotide sequence introduced after the codon for Arg₅₀₄ within gB2Fu. The deduced amino acid sequence is given in three-letter code, and the position of the second furin cleavage site (FCS2) is marked. (E) Amino acid changes within mutants gB2FuGFP and gB2FuIFN-α. Additional amino acids are in boldface.

**FIG. 1.** Construction of the gB mutants. (A) Schematic representation of the BHV-1 genome. Unique long (U₄) and unique short (U₃) segments are indicated, as are the internal and terminal repeat sequences (IR and TR, respectively). Arrows indicate the isomerization of the U₄ during replication. (B) Position of BamHI fragments (3) and localization of the gB gene (hatched area) according to the complete genome sequence (GenBank accession number AJ004801). (C) Scheme of the gB ORF. Sequences encoding the signal peptide (sig), the amino-terminal subunit, the carboxy-terminal subunit which contains the membrane anchor domain, and the furin cleavage site (FCS1) are indicated. The double-headed arrow indicates the sequence deleted in gB subunit, the carboxy-terminal subunit which contains the membrane anchor domain, and the furin cleavage site (FCS1) are indicated. (D) Nucleotide sequence introduced after the codon for Arg₅₀₄ within gB2Fu. The deduced amino acid sequence is given in three-letter code, and the position of the second furin cleavage site (FCS2) is marked. (E) Amino acid changes within mutants gB2FuGFP and gB2FuIFN-α. Additional amino acids are in boldface.
counted after 2 days. The plaque count of untreated cultures was set as 100%
citrate buffer for 2 min. Cells were washed twice with cell culture medium and
adsorption. Cultures were then shifted to 37°C, and extracellular virions were
further incubated at 4°C for 2 h after addition of about 200 PFU to allow
a microscope by using a graduated ocular.

Diameters of 100 randomly selected plaques were determined under
cellulose. Plaques were counted after 2 days.

Cells, and cultures were incubated under semisolid medium containing methyl-
low-pH citrate buffer before harvest. Serial dilutions were titrated on MDBK
culture medium and incubated until the times indicated, when supernatants and
[pH 3.0] to inactivate extracellular virions (8). Cells were washed twice with cell
medium and then chased with normal cell culture medium for the times
which is also found in purified virions (17, 24) The 130-kDa gB
is then cleaved in the trans-Golgi network by furin at furin cleavage site 1 (FCS1) after Arg$^{506}$ (18, 26) into a 72-kDa NH$_2$
subunit and a 55-kDa COOH subunit, which remain covalently
associated by disulfide bonds (Fig. 1). To test whether intro-
duction of a second furin cleavage site (FCS2) 20 amino acids
downstream from FCS1 is compatible with gB function and results in release of the
intervening peptide, we constructed a modified ORF encoding gB2Fu (Fig. 1). The sequence of the
intervening peptide was derived from the A27L gene-encoded
fusion protein of vaccinia virus strain MVA and encompasses the linear epitope recognized by MAb 5B4 (6, 10); the with vaccinia virus-infected cells but failed to bind gB2Fu after
transient expression and also after cell-free synthesis, we
assumed that the MAb 5B4 epitope is not correctly presented in the
gB2Fu context. To demonstrate that BHV-1/gB2Fu con-
tained the modified gB ORF in which an Xbal cleavage was
introduced for diagnostic purposes (Fig. 1), purified viral DNA from wild-type BHV-1 and the recombinant
was cleaved with BamHI or with BamHI and XbaI. Restriction fragment pat-
terns after ethidium bromide staining and hybridization to a
MAb immunoglobulin G (Dianova).

Analyses of cell culture characteristics. For single-step growth curves, MDBK
cultures were infected with 10 PFU per cell. At 2 h p.i., cells were incubated for
2 min with low-pH citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl
[pH 3.0]) to inactivate extracellular virions (8). Cells were washed twice with cell
culture medium and incubated until the times indicated, when supernatants and
cells were harvested and stored at −70°C. Cells were incubated for 2 min with
low-pH citrate buffer before harvest. Serial dilutions were titrated on MDBK
cells, and cultures were incubated under semisolid medium containing methyl-
cellulose. Plaques were counted after 2 days. Plaques containing untreated cultures were set as 100%
penetration.

RESULTS

Insertion of a second furin cleavage site and an intervening peptide into gB. After transport into the endoplasmic reticu-
lum and removal of the signal peptide, the primary gB trans-
lation product is modified by the addition of mannose-rich N-glycans and the gB precursor molecules with an apparent
molecular mass of 117 kDa (24) are transported to the Golgi
apparatus, where the N-glycans are converted to the complex
form and O-glycans are added, resulting in the 130-kDa gB,
Secretion of proteins using gB as a transporter. To determine whether large polypeptides also can be expressed as furin-excisable secreted proteins, the ORF encoding GFP was integrated between the two furin cleavage sites (Fig. 1), and plasmid pSPgB2FuGFP was cotransfected with purified DNA of gB-lacZ into BHV-1 into MDBK cells. The transfected-cell supernatant was titrated on MDBK cells after development of a complete cytopathic effect. Virions from plaques which all showed autofluorescence (Fig. 3) were purified until homogeneity as described above, and isolate BHV-1/gB2FuGFP was analyzed further. Insertion of GFP into gB did not apparently influence the function of gB for cell-to-cell spread, since the sizes of plaques induced by BHV-1/gB2FuGFP and BHV-1/gBrev were comparable (Fig. 3). In addition, analysis of single-step growth and determination of the rate of entry into cells also did not reveal significant differences between BHV-1/gB2FuGFP and BHV-1/gBrev (data not shown). Intracellular transport of gB2FuGFP, however, was impaired. The pulse-chase experiment shown in Fig. 4 revealed that in comparison to that of wild-type gB, transport of GFP-excisable gB precursor molecules into the TGN is less efficient, which is reflected by the apparently unchanged intensity of the fusion protein precursor molecules throughout the chase period and the reduced accumulation of uncleaved gB2FuGFP in the TGN in presence of the furin inhibitor dec-RVKR-CMK (Fig. 4, lanes 90+). The delayed transport of pgB2FuGFP is probably responsible for the later appearance and relatively low abundance of the NH2 and COOH subunits, which migrate indistinguishably from the corresponding wild-type gB subunits, indicating that cleavage by furin is not affected by the incorporation of GFP.

The fate of GFP after transport of gB2FuGFP to the TGN and cleavage was analyzed by Western blotting of cell-associated and secreted proteins with anti-GFP MAb. KOP/R cells were infected with BHV-1/gBrev and BHV-1/gB2FuGFP. Cells and culture media were harvested 20 h later. Among BHV-1/gB2FuGFP-infected cell proteins, the anti-GFP MAb reacted with the ca. 150-kDa gB2FuGFP precursor, the comigrating uncleaved gB2FuGFP, and a protein of about 26 kDa, which corresponds to the size of GFP (Fig. 5A). Of the proteins in the culture medium of cells infected with BHV-1/gB2FuGFP, the anti-GFP MABs bound only to the 26-kDa GFP, and no proteins were detected by the MABs in cells and culture medium after infection with BHV-1/gBrev. To quantitate cell-associated and secreted GFP activity, the relative fluorescence activity in cells and culture medium was determined (Fig. 5B). 2-D evaluation of the recorded fluorescence images revealed that approximately 20-fold more GFP activity was found in the supernats after infection with BHV-1/gB2FuGFP than in the infected cells. Only background fluorescence levels were observed after infection with BHV-1/gBrev. These results demonstrated that GFP is efficiently transported and secreted in an active form into the culture medium after excision from the gB2FuGFP precursor molecules.

The somewhat surprising result that integration of the 246 amino acids into the gB precursor is compatible with correct folding and oligomerization required for a biologically active gB and the observation that this modification had no detectable negative effects on BHV-1 replication in cell culture led us to incorporate the amino acids for mature boIFN-α as a furin-excisable protein into gB (Fig. 1) to test whether this approach raised against the carboxy terminus of gB. The precursor molecule of gB2Fu (pgB2Fu) is about 2 kDa larger than the 117-kDa wild-type gB precursor expressed by BHV-1/gBrev, which provides good evidence that the intervening peptide is contained within pgB2Fu. The gB precursor molecules become converted to slower-migrating forms of about 130 kDa which are subsequently cleaved into the respective NH2 and COOH subunits. Figure 2 shows that the kinetics of appearance of the uncleaved gB and the subunits and the mobilities of the 72- and 55-kDa subunits were identical for wild-type gB and gB2Fu, indicating that the intervening peptide was removed from pgB2Fu as envisioned and that its presence within pgB2Fu does not interfere with the transport from the endoplasmic reticulum to the TGN and cleavage by furin. This interpretation is supported by the result of a pulse-chase experiment performed in the presence of the furin inhibitor decanoyl-RVKR-chloromethylketon (dec-RVKR-CMK) (kindly provided by Wolfgang Garten, Marburg, Germany) (22), which shows a comparable conversion of the endoplasmic reticulum- associated precursors of wild-type gB and gB2Fu to their respective uncleaved forms when cleavage by furin is inhibited (Fig. 2). The absence of any other intermediates further demonstrates that the introduced FCS2 indeed requires active furin for cleavage. It should be noted that after prolonged exposure the NH2 and COOH subunits of both gBs also become visible and again migrate indistinguishably (not shown in Fig. 2).
is also applicable for transport and export of biologically active secreted glycoproteins. Generation and isolation of BHV-1/gB2FuIFN-α was done as described above with plasmid pSPgB2FuIFN-α and purified DNA of gB- BHV-1/GKD. BHV-1/GKD was chosen as the progenitor strain to provide the same genetic background for animal experiments as for the previously tested bovine cytokine-expressing BHV-1 recombi-

FIG. 3. Expression of GFP by BHV-1/gB2FuGFP. MDBK cells were infected with diluted stocks of BHV-1/gBrev and BHV-1/2FuGFP and incubated under methylcellulose-containing medium for 2 days. Cells were fixed and stained with gB-specific MAb 42/18/7 and tetramethyl rhodamine isocyanate-conjugated anti-mouse secondary antibody. GFP autofluorescence and indirect immunofluorescence were visualized by using appropriate filters and photographed with an Olympus digital camera.

FIG. 4. Maturation of gB2FuGFP. Pulse-chase analysis was performed with BHV-1/gBrev and BHV-1/gB2FuGFP as described in the legend to Fig. 2A, with the exception that the sample marked 90* was incubated in presence of 100 μM dec-RVKR-CMK during the labeling and chase period to inhibit furin cleavage.

FIG. 5. Secretion of GFP from BHV-1/gB2FuGFP-infected cells. (A) KOP/R cells were infected with BHV-1/gB2FuGFP (lanes 1) and BHV-1/gBrev (lanes 2). Proteins from cells (left panel) and culture media (right panel), harvested at 20 h p.i., were separated by SDS-12.5% PAGE and analyzed by Western blotting with anti-GFP MAb. Positions of marker proteins are indicated on the left. (B) Aliquots of the samples analyzed in panel A were transferred to 96-well cell culture plates, and autofluorescence was visualized with a Fuji FLA-3000 fluorescence scanner. Relative fluorescence intensities (RFI) were determined by using the AIDA 2D evaluation software.
FIG. 7. Incorporation of the gB-NH₂/IFN-α fusion protein into virions. KOP/R cells were infected with BHV-1/GKD (lanes 1 and 3) and BHV-1/gB2FuIFN-α (lanes 2 and 4) at 5 PFU/cell and incubated with [³⁵S]methionine-[³⁵S]cysteine from 4 to 40 h.p.i. Cell culture supernatants were clarified by low-speed centrifugation, and virions were pelleted by ultracentrifugation. Labeled proteins from virions were immunoprecipitated with anti-gb serum (lanes 1 and 2) or BHV-1 gD-specific MAb 21/3/3 (lanes 3 and 4) and analyzed by SDS–10% PAGE.

MDBK cells. Cultures were infected with 10 PFU per cell, nonpenetrated virions were inactivated by low-pH treatment at 2 h.p.i., and cells and culture supernatants were harvested at the times indicated in Fig. 8A. In contrast to cell-associated infectivity, which showed kinetics comparable to those for BHV-1/GKD, the start of release of infectious BHV-1/gB2FuIFN-α was delayed for about 4 h. In addition, it took roughly 22 h until extracellular infectivity surpassed the titer of intracellular virions, whereas the parental strain BHV-1/GKD needed only approximately 12 h to reach this point. In addition, the final titers reached by BHV-1/gB2FuIFN-α were about one order of magnitude lower than those released from cells infected with BHV-1/GKD or wild-type BHV-1 strains (8, 16).

Direct cell-to-cell spread of BHV-1/gB2FuIFN-α also appeared to be hindered, because the sizes of plaques formed under methycellulose-containing semisolid medium on MDBK and PT cells were only around 50% of those achieved by BHV-1/GKD (Fig. 8B). These results might indicate that the incomplete cleavage of the gB2FuIFN-α precursor interferes with release and the gB function for direct spreading.

To assess whether the cleavage at both furin cleavage sites results in release of biologically active boIFN-α, the medium of cells infected for 24 h with BHV-1/GKD or BHV-1/gB2FuIFN-α was tested by a VSV plaque reduction assay for the presence of antiviral activity in comparison to a recombinant IFN-α standard (11) containing 2 × 10⁶ U/ml (kindly provided by Alfred Metzler, Zürich, Switzerland) (19). The results are shown in Fig. 9. In contrast to the medium of BHV-1/GKD-infected cells, which did not contain detectable antiviral activity, the medium of BHV-1/gB2FuIFN-α-infected cells inhibited VSV plaque formation in a dose-dependent manner and contained approximately 5 × 10⁴ U of boIFN-α per ml. Thus, significant boIFN-α activity was released from BHV-1/gB2FuIFN-α-infected cells although only a minor fraction of the precursor molecules were correctly cleaved, dem-
onstrating that biologically active glycoproteins also can be utilized in this novel approach for the expression of secreted polypeptides.

**DISCUSSION**

The conventional way to produce secreted recombinant proteins by viral vectors relies on expression from gene cassettes, which usually requires integration of open reading frames flanked by transcription control elements such as promoters and polyadenylation signals. We recently reported an alternative approach for the expression and secretion of bovine cytokines by the pneumovirus BRSV (14), which is based on the so-far unique property of the RSV F protein among furin-cleavable proteins that it contains two consensus sequences for cleavage. The two furin cleavage sites flank an intervening peptide which is secreted after cleavage by furin as a biologically active tachykinin named virokinin (28, 29). Replacement of the virokinin polypeptide by the amino acid sequence for mature boIL-2 and -4 within the F precursor molecule did not destroy the function of the F protein for BRSV replication and resulted in secretion of the bovine interleukins from cells infected with the respective BRSV recombinants (14). However, the amount of interleukins produced was relatively low in comparison to that of boIL-2 and bo-IL-4 secreted from cells infected with BHV-1 recombinants expressing these interleukins from gene cassettes (16). This led us to test whether the approach to express secreted proteins as furin-excisable integrates within precursors of glycoproteins is in principle also applicable for glycoproteins which are cleaved by furin only once and to analyze whether such modifications may affect processing and function. For that we selected gB of BHV-1, which is essential for BHV-1 replication and represents an abundant and immunodominant component of the viral envelope. Introduction of a second furin cleavage site, FCS2, 20 amino acids downstream from the authentic cleavage site FCS1 into the gB precursor was compatible with BHV-1 replication and had no detectable effect on the penetration, single-step growth, direct cell-to-cell spread, and virus yield of the resulting recombinant, BHV-1/gB2Fu. Intracellular processing and transport of gB2Fu also appeared to be unaffected. That the amino acid sequence between the two furin cleavage sites may influence intracellular transport was indicated by analysis of the intracellular transport of gB2FuGFP, which was transported to the Golgi clearly less efficiently than wild-type pgB or pgB2Fu. The reduced transport consequently resulted in a delayed and less abundant appearance of the NH2 and COOH subunits. Furin cleavage of pgB2FuGFP appeared to be largely unimpaired. Glycosylation of gB also seemed to be generally unaffected. Although not analyzed in detail, the identical migration behaviors of the NH2 and COOH subunits of gB2FuGFP suggest that N and O glycosylations are comparably realized. In addition, coprecipitation of the NH2 and COOH subunits by the anti-gB serum demonstrated that even with the uncleaved IFN-α present, gB is still expressed as a disulfide-linked dimer. The retarded transport of pgB2FuGFP, however, had no detectable effect on the biological properties of BHV-1/gB2FuGFP in cell culture. The recombinant grew to the same final titers with kinetics comparable to those of BHV-1/gBrev and entered the cells and spread directly from cell to cell as efficiently as BHV-1/gBrev. Analysis of the fate of the intervening GFP revealed that biologically active GFP accumulated in

![FIG. 8. Cell culture characteristics of BHV-1/gB2FuIFN-α. A) Growth curves. MDBK cells were infected with BHV-1/GKD (stars) and BHV-1/gB2FuIFN-α (squares) at 10 PFU per cell. Cells were treated with low-pH buffer to inactivate nonpenetrated virions at 2 h p.i. Cells (closed symbols) and supernatants (open symbols) were harvested at the times indicated and titrated on MDBK cells. Cultures were overlaid with methylcellulose-containing medium, and plaques were counted 2 days later. Arrows indicate the time point when extracellular BHV-1/GKD (open arrow) or BHV-1/gB2FuIFN-α (solid arrow) infectivity surpasses the respective intracellular infectivity. B) Plaque diameter determination. MDBK cells (solid bars) and KOP/R cells (open bars) were infected with appropriate dilutions of the indicated viruses and incubated for 2 days under methylcellulose-containing medium. Diameters of 100 plaques from each virus were measured by using a graduated ocular. Average diameters are shown in arbitrary units.](http://jvi.asm.org/)
expresses boIL-2 classically via an expression cassette (to be published elsewhere). Analysis of the cell culture properties of BHV-1/gB2FuIFN-α revealed that the size of the plaques formed by the recombinant was about 50% reduced, that single-step growth was delayed, and that the virus yield was reduced about 90%, which might be caused by structural alterations of gB2FuIFN-α due to boIFN-α insertion and/or reduced cleavage at FCS1. However, these effects cannot be unequivocally attributed to a possible misfolding of gB2FuIFN-α, since preincubation of MDBK cells with 10^4 U of recombinant IFN-α results in a similar inhibition of wild-type BHV-1 (1, unpublished results).

In summary, our results have demonstrated that BHV-1 gB can be engineered to function as a transporter for secretion of heterologous proteins and glycoproteins into the extracellular space in biologically active forms. The examples presented here show that the amino acid sequence of an intervening polypeptide may influence transport or furin cleavage but in no case destroyed gB function for BHV-1 replication, proving that the integrity of the furin cleavage site domain of BHV-1 gB and the distance between the subsequent NH2 and COOH subunits are not important for overall folding and dimerization of gB within the endoplasmic reticulum. We assume that this novel protein expression approach may be of particular interest not only for development of new vaccines but also for gene therapy applications, especially when biologically active oligopeptides need to be secreted. We speculate that this strategy will be applicable for other furin-cleaved proteins and hypothesize that it can be also applied to proteins without furin cleavage sites.

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