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

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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 be replaced 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 (NH_2) subunit and boIFN-α, whereas cleavage at the newly introduced site was normal. This resulted in virus particles that also contain the NH_2-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 endoprotease localized in the *trans*-Golgi network (TGN) (2, 12, 15). After cleavage, the amino-terminal subunit (NH_2 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 type 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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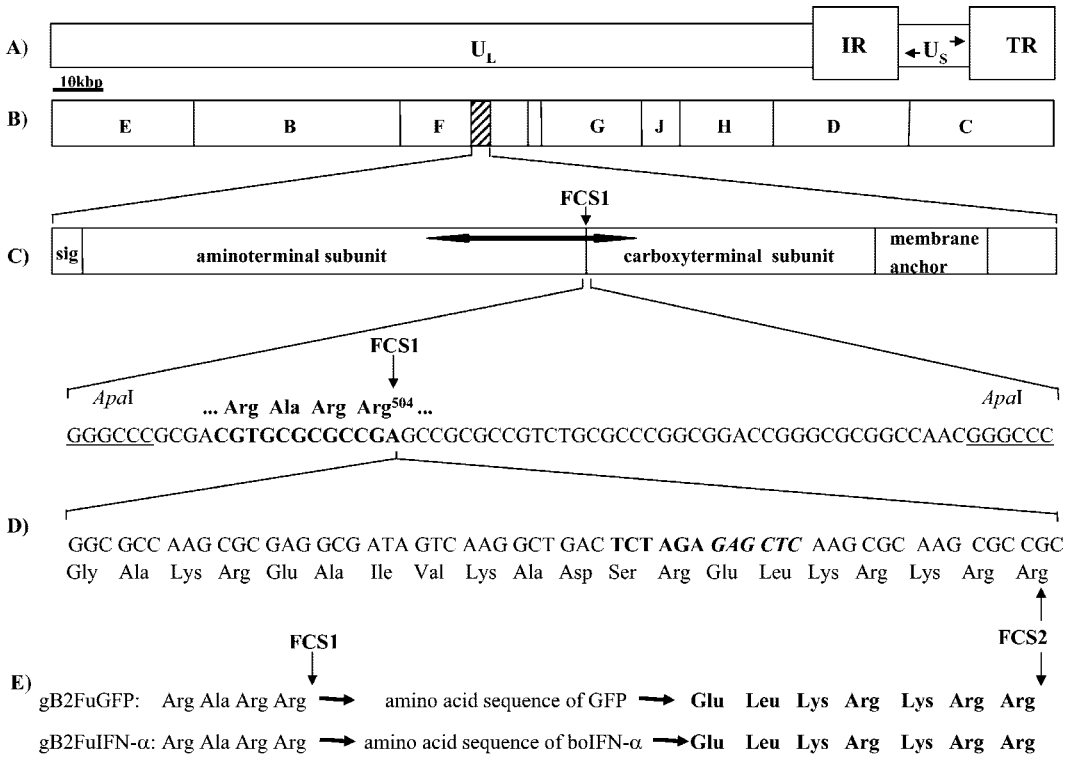

FIG. 1. Construction of the gB mutants. (A) Schematic representation of the BHV-1 genome. Unique long (U_L) and unique short (U_S) segments are indicated, as are the internal and terminal repeat sequences (IR and TR, respectively). Arrows indicate the isomerization of the U_S during replication. (B) 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$^-$ *lacZ*$^+$ BHV-1. The fragment between the ApaI cleavage sites (underlined) which encompass 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 Arg^{504} 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.

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% CO_2. Bovine pharyngeal cell line 244 (KOP-R) and bovine kidney cell line PT (kindly provided by Roland Riebe, The Collection of Cell Lines in Veterinary Medicine, Insel Riems, Germany) were maintained under the same conditions with the exception that the culture medium contained 10% fetal bovine serum. Wild-type BHV-1 strain Schönböken (BHV-1) was kindly provided by O. C. Straub (Tübingen, Germany). The gE-negative BHV-1 strain GKD, a commercially available vaccine virus (Bovilis IBR marker; Intervet International, Boxmeer, The Netherlands) was described in detail by König et al. (13). The gB-negative mutant gB$^-$ BHV-1/GKD was constructed by replacing the ApaI fragment within the gB open reading frame (ORF) (Fig. 1) by a GFP expression cassette (unpublished data). gB$^-$ BHV-1/GKD and gB$^-$ *lacZ*$^+$ BHV-1 (kindly provided by Frank Fehler) were propagated on BHV-1 gB-expressing cell line pMT-gI (20). BHV-1/gB^{rev} was isolated after cotransfection of purified gB$^-$ *lacZ*$^+$ BHV-1 DNA with pSPgB on noncomplementing MDBK cells and plaque purification (unpublished data). Vesicular stomatitis virus strain Indiana was kindly provided by Horst Schirrmeier (Insel Riems, Germany).

Construction of plasmids. All cloning procedures were carried out by standard methods (21). To obtain plasmid p2Fu, primers gB2Fu-1+ (GAGGCGCCACG AGCGAGGCGATAGTCAAGGCTGACTCTAGAGAGCTCAAG) and gB2Fu-1− (CGCCGGGCGCAGACGGCGCGGCGCGGCGCTTGCGCTTGAGCTCTC TAGAG) were subjected to PCR with *Taq* polymerase (1 cycle of 94°C for 1 min and 33 cycles of 94°C for 30 s and 60 and 72°C for 1 min each). The amplification product was used as the template for a second PCR with primers gB2Fu-2+ (TAAGAATTCGGGCCCGCGACGTGCGCGCCGAGGCGC C AAGC G C G AGGCG) and gB2Fu-2− (AGTAAGCTTGGGCCCGTTGGCCGCGCCCGG TCCGCCGGGCGCAGACGGCG), using the same conditions. The amplified fragment which contained the ApaI-flanked sequence shown in Fig. 1 was cleaved with EcoRI and HindIII and integrated into plasmid vector pSP73 cleaved with the same enzymes.

For insertion of the coding sequence for mature boIFN-α, the respective 167 codons were isolated by PCR from plasmid pieIFNα$_{syn}$, which contains an artificial ORF encoding boIFN-α subtype C (25) that has been assembled from synthetic oligonucleotides by using the codon preference of glycoprotein B of bovine herpesvirus 1 (unpublished; accession number AJ784928). This ORF had been integrated into plasmid pie1cas, a derivative of plasmid pROMie (16). PCR amplification of the fragment encoding mature boIFNa was performed with *Pfx* polymerase as recommended by the supplier. The primers, which also provided adapter sequences for in-frame insertion into gB, were matIFNa+ (TAAGGG CCCGCGACGTGCGCGCCGATGCCACCTGCCGCACACGCACAGC) and matIFNa− (TAAGAGCTCGTCCTTGCGGCGGAAGCTCTCCTGCAG). The amplification product purified after 1 cycle of 95°C for 2 min and 33 cycles of 95°C for 30 s and 55 and 72°C for 1 min each was blunt ended with Klenow polymerase, cleaved with SacI, and integrated into the EcoRI-cleaved, blunt-ended, and SacI-cleaved plasmid pSP2Fu, resulting in plasmid pSP2FuIFNα. Plasmid pSP2FuGFP was constructed accordingly by using gB2FuGFP+ (TAAGGGCC CGCGACGTGCGCGCCGAATGGTGAGCAAGGGCGAGGAGCTG) and gB2FuGFP− (TAGAAGCTTGAGCTCCTTGTACAGCTCGTCCATGCC) as primers and pEGFP-N1 (Clontech, Palo Alto, Calif.) as the template. Primer sequences were derived from the sequence provided with the plasmid.

The ApaI fragments contained in pSP2Fu, pSP2FuGFP, and pSP2FuIFNα were subsequently used to replace the ApaI fragment encompassing the coding sequence for the furin cleavage site (Fig. 1) within pSPgB, which contains a

3.8-kbp NotI fragment encompassing the entire gB gene. The resulting plasmids were named pSPgB2Fu, pSPgB2FuGFP, and pSPgB2FuIFN-α.

Construction of BHV-1 recombinants. MDBK cells were cotransfected with 5 μg of recombinant plasmid and 1 μg of purified gB$^-$ *lacZ*$^+$ BHV-1 or gB$^-$ BHV-1/GKD DNA as described previously (8). Virus progeny from the culture supernatants was titrated on MDBK cells. Cultures were incubated under a 0.6% agarose overlay containing Bluo-Gal (8) until plaques appeared. Infected cells were isolated by aspiration, resuspended in culture medium, frozen at −70°C, and further plaque purified to homogeneity.

Immunoprecipitation. Cells were infected and proteins were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine as indicated. Immunoprecipitation of proteins from cell lysates were performed as described previously (8), using a monospecific rabbit serum directed against a synthetic peptide representing amino acids Leu^{910} to Asn^{928} of the carboxy terminus of BHV-1 gB (anti-gB serum). Labeled proteins were visualized after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by fluorography or with a Fuji FLA3000 fluorescence scanner and Aida two-dimensional (2-D) gel evaluation software.

Western blotting. Proteins were separated by SDS–10% PAGE, transferred to nitrocellulose, and probed with anti-GFP monoclonal antibodies (MAbs) (Roche, Mannheim, Germany), using the Supersignal West Pico chemiluminescence kit (Pierce, Rockford, Ill.) as recommended by the supplier.

Determination of boIFN-α activity. Secretion of biologically active boIFN-α into the cell culture medium was analyzed by use of a vesicular stomatitis virus (VSV) plaque reduction assay. Supernatants from KOP/R cells infected with BHV-1/gB2FuIFN-α or BHV-1/GKD as a control were harvested 24 h after infection. The supernatants were serially diluted in normal cell culture medium after ultracentrifugation and sterilization by UV light and were added to KOP/R cells in six-well plates. Cultures were incubated for 24 h at 37°C and then infected with approximately 100 PFU of VSV. Supernatants were removed at 1 h postinfection (p.i.), and semisolid methylcellulose-containing medium was added. Plaques were counted after 24 h of incubation at 37°C. Comparison of boIFN-α activities with or without UV light treatment revealed no differences between the samples (not shown).

Indirect immunofluorescence assay. Cells were fixed with 3% paraformaldehyde in phosphate-buffered saline for 20 min, subjected to membrane permeabilization with 0.2% Triton X-100, and sequentially incubated with gB-specific MAb 42/18/7 (8) and tetramethyl rhodamine isocyanate-conjugated rabbit anti-mouse 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 methylcellulose. Plaques were counted after 2 days.

(i) Determination of plaque diameters. MDBK cells were infected with diluted virus stocks and incubated under semisolid medium containing methylcellulose for 2 days. Diameters of 100 randomly selected plaques were determined under a microscope by using a graduated ocular.

(ii) Penetration kinetics. MDBK cells were precooled at 4°C for 30 min and further incubated at 4°C for 2 h after addition of about 200 PFU to allow adsorption. Cultures were then shifted to 37°C, and extracellular virions were inactivated at the indicated times by incubation of the monolayers with low-pH citrate buffer for 2 min. Cells were washed twice with cell culture medium and incubated under semisolid medium containing methylcellulose. Plaques were counted after 2 days. The plaque count of untreated cultures was set as 100% penetration.

RESULTS

Insertion of a second furin cleavage site and an intervening peptide into gB. After transport into the endoplasmic reticulum and removal of the signal peptide, the primary gB translation 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, 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^{504} (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 introduction 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 consensus sequence for furin cleavage corresponds to cleavage site Fu2 of the bovine respiratory syncytial virus fusion protein (14). For expression by BHV-1, plasmid pSPgB2Fu was cotransfected with purified DNA of gB$^-$ *lacZ*$^+$ BHV-1 into MDBK cells. In this gB-negative mutant, amino acids Gln^{331} to Ala^{603} of gB are replaced by a *lacZ* expression cassette (Fig. 1). Since gB is essential for BHV-1 replication, only recombinant virions that have acquired a functional gB could give rise to infectious progeny. The transfected-cell supernatant was titrated on MDBK cells after development of a complete cytopathic effect. Virus contained in "white" plaques under a Bluo-Gal-containing agarose overlay was picked and plaque purified until homogeneity. The isolate BHV-1/gB2Fu was analyzed further. Unfortunately, indirect-immunofluorescence assays revealed that MAb 5B4 did not react with BHV-1/gB2Fu-infected cells (data not shown), which were, however, recognized by anti-BHV-1 gB MAb 42/18/7. Since MAb5B4 reacted with vaccinia virus-infected cells but failed to bind gB2Fu after transient expression and also after cell-free synthesis, we assume that the MAb 5B4 epitope is not correctly presented in the gB2Fu context. To demonstrate that BHV-1/gB2Fu contained the modified gB ORF in which an XbaI 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 patterns after ethidium bromide staining and hybridization to a gB-specific probe revealed that the BamHI F fragment of BHV-1/gB2Fu but not that of wild-type BHV-1 was cleaved by XbaI (data not shown). The resulting two fragments were of the expected sizes, indicating that the modified gB ORF had been integrated into the BHV-1/gB2Fu genome as envisioned. The presence of the codons for the intervening peptide and FCS2 within the gB2Fu ORF of BHV-1/gB2Fu was confirmed by sequencing of a PCR fragment encompassing the modified ApaI fragment (Fig. 1). Thus, insertion of the additional amino acids with a second furin cleavage site into gB did not abolish gB function for BHV-1 replication. Analysis of single-step growth, direct spreading, and entry revealed no significant differences between BHV-1/gB2Fu and BHV-1/gB^{rev} (data not shown), indicating that the processes of gB-mediated fusion between viral and cellular membranes were not affected by the sequence modification. To analyze the effect of the intervening peptide and FCS2 on transport and processing of gB2Fu, KOP-R cells were infected with BHV-1/gB2Fu and BHV-1/gB^{rev}, incubated with [^{35}S]methionine-[^{35}S]cysteine for 30 min, and then chased with normal cell culture medium for the times given in Fig. 2. Labeled proteins were immunoprecipitated from cell lysates with anti-gB serum, a polyclonal rabbit serum

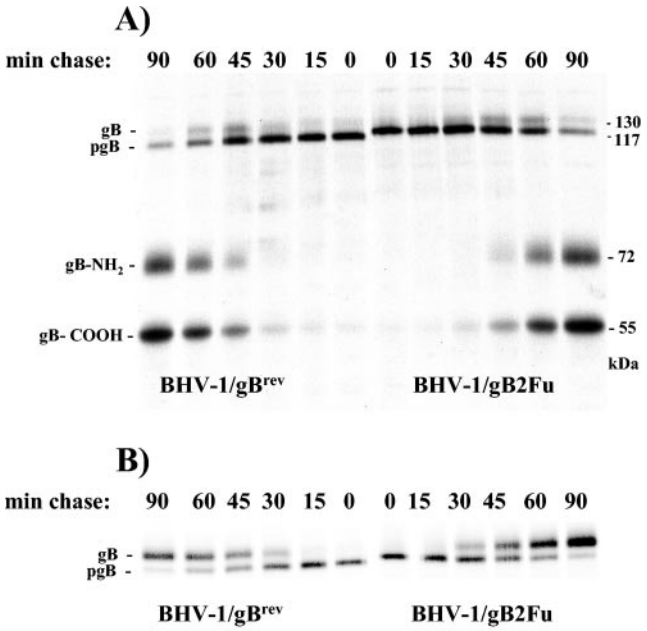

FIG. 2. Maturation of wild-type gB and gB2Fu. KOP/R cells were infected with BHV-1/gB^{rev} and BHV-1/gB2Fu at 10 PFU/cell in the absence (A) or presence (B) of the furin inhibitor dec-RVKR-CMK (100 μM), and proteins were labeled with [^{35}S]methionine-[^{35}S]cysteine at 6 h p.i. for 30 min, washed, and further incubated with normal cell culture medium as indicated. Cell lysates were incubated with anti-gB serum, and immunoprecipitated proteins were separated by SDS–7.5% PAGE. The positions of the precursor form (pgB), the uncleaved form (gB), and the NH_2 and COOH subunits of wild-type gB are shown on the left. The corresponding apparent molecular masses are indicated on the right.

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/gB^{rev}, 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 NH_2 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 NH_2 and COOH subunits of both gBs also become visible and again migrate indistinguishably (not shown in Fig. 2).

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*$^+$ 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/gB^{rev} 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/gB^{rev} (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 gB2FuGFP 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 NH_2 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 MAbs. KOP/R cells were infected with BHV-1/gB^{rev} and BHV-1/gB2FuGFP. Cells and culture media were harvested 20 h later. Among BHV-1/gB2FuGFP-infected cell proteins, the anti-GFP MAbs 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/gB^{rev}. 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 supernatants after infection with BHV-1/gB2FuGFP than in the infected cells. Only background fluorescence levels were observed after infection with BHV-1/gB^{rev}. 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

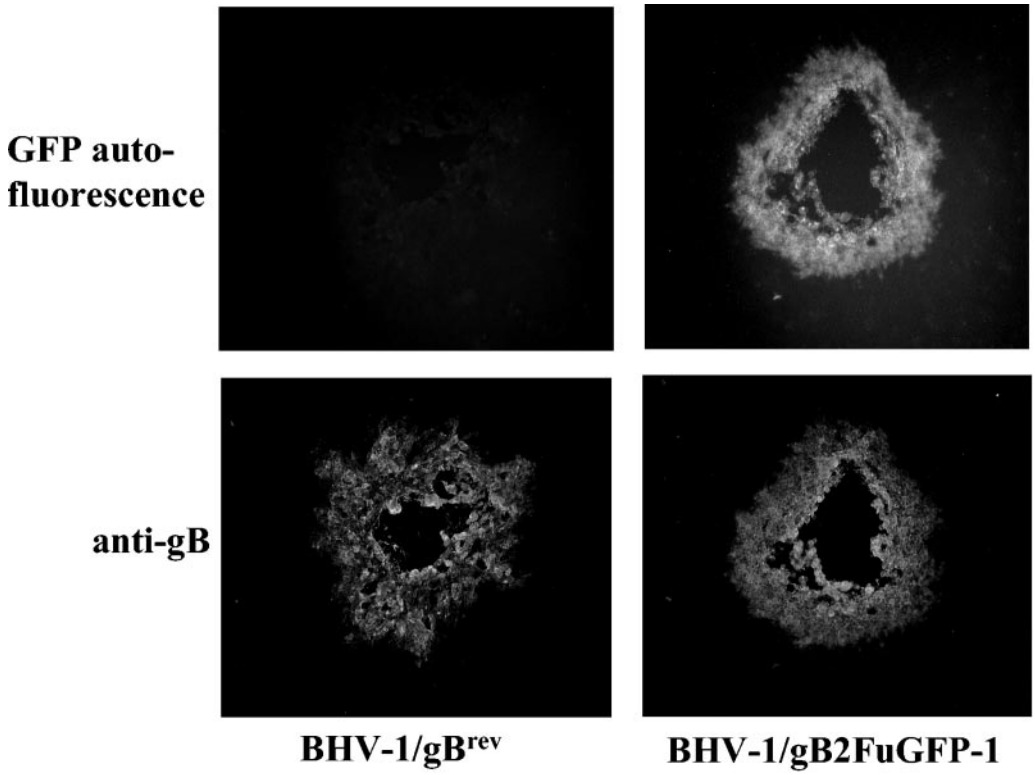

FIG. 3. Expression of GFP by BHV-1/gB2FuGFP. MDBK cells were infected with diluted stocks of BHV-1/gB^{rev} and BHV-1/2FuGFP and incubated under methylcellulose-containing medium for 2 days. Cells were fixed and stained with gB-specific MAb 42/18/7 and tetramethyl rhodamine isocyanate-conjugated anti-mouse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

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-

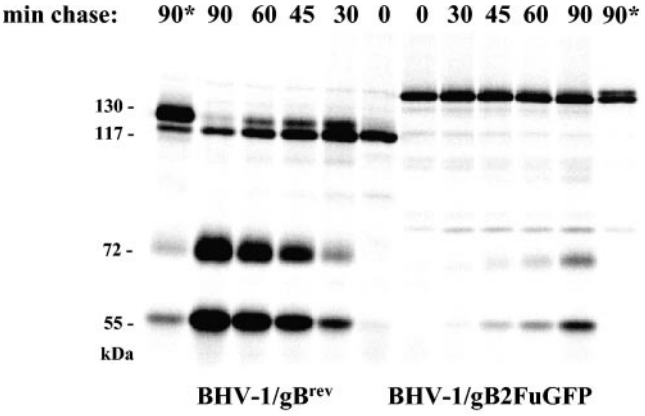

FIG. 4. Maturation of gB2FuGFP. Pulse-chase analysis was performed with BHV-1/gB^{rev} and BHV-1/gB2FuGFP as described in the legend to Fig. 2A, with the exception that the sample marked 90* was incubated in presence of 100 μM dec-RVKR-CMK during the labeling and chase period to inhibit furin cleavage.

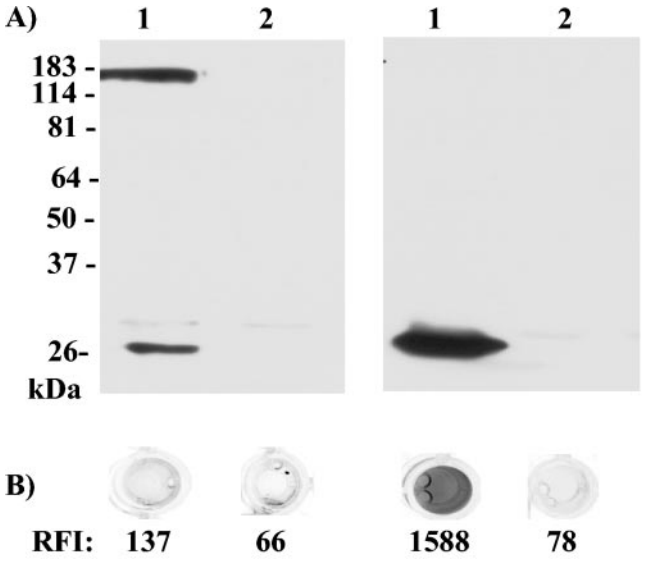

FIG. 5. Secretion of GFP from BHV-1/gB2FuGFP-infected cells. (A) KOP/R cells were infected with BHV-1/gB2FuGFP (lanes 1) and BHV-1/gB^{rev} (lanes 2). Proteins from cells (left panel) and culture media (right panel), harvested at 20 h p.i., were separated by SDS–12.5% PAGE and analyzed by Western blotting with anti-GFP MAbs. 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.

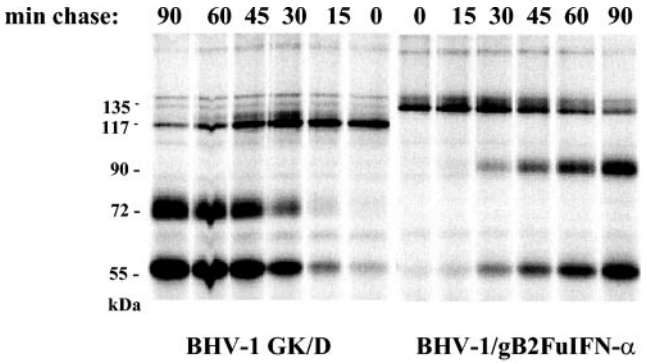

FIG. 6. Maturation of gB2FuIFN-α. Pulse-chase analysis was performed with BHV-1/GKD and BHV-1/gB2FuIFN-α as described in the legend to Fig. 2A.

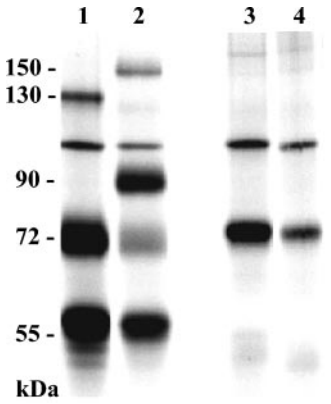

FIG. 7. Incorporation of the gB-NH_2/IFN-α fusion protein into virions. KOP/R cells were infected with BHV-1/GKD (lanes 1 and 3) and BHV-1/gB2FuIFN-α (lanes 2 and 4) at 5 PFU/cell and incubated with [^{35}S]methionine-[^{35}S]cysteine from 4 to 40 h p.i. Cell culture supernatants were clarified by low-speed 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.

nants (13). Comparison of the intracellular transport of BHV-1/GKD gB and gB2FuIFN-α by pulse-chase experiments (Fig. 6) showed that transport of the two precursor molecules into the TGN was comparable, indicating that the IFN-α sequence did not negatively influence modification and processing within the endoplasmic reticulum and the Golgi. The kinetics of the generation of the 55-kDa subunit, which was visible already after the pulse period in cells infected with BHV-1/GKD and BHV-1/gB2FuIFN-α, also were similar (Fig. 6), indicating that furin cleavage at the introduced FCS2 was not significantly affected. In contrast, the 72-kDa amino-terminal gB subunit of gB2FuIFN-α was not detected even after 90 min of chase. A slower-migrating form with an apparent molecular mass of about 90 kDa was generated instead, with kinetics comparable to those for the formation of the 72-kDa subunit of BHV-1/GKD. Additional proteins with apparent molecular masses of about 140 and 150 kDa and with constant intensities probably represent nonspecific coprecipitates.

Since sequence analysis proved that genomic DNA of BHV-1/gB2FuIFN-α contained the correct sequence for FCS1, mature boIFN-α, and FCS2, we conclude that furin fails to cleave gB2FuIFN-α at the authentic FCS1 and that the 90-kDa protein, which is also found in purified virions (Fig. 7), is a fusion protein between the NH_2 subunit and boIFN-α. Labeling of infected-cell proteins for 18 h followed by immunoprecipitation revealed the presence of a small amount of the 72-kDa NH_2 subunit of gB2FuIFN-α, indicating that cleavage at FCS1 is not completely abolished (data not shown). Analysis of the gB forms associated with virus particles by immunoprecipitation showed that BHV-1/gB2FuIFN-α virions contain the uncleaved gB, the COOH subunit, the NH_2-subunit/IFN-α fusion protein, and small amounts of the 72-kDa NH_2 subunit (Fig. 7, lane 2). Comparison of the intensities of wild-type gB and gB2FuIFN-α obtained from virions shows that noticeably less gB was precipitated from BHV-1/gB2FuIFN-α virions than from BHV-1/GKD virions (Fig. 7, lanes 1 and 2). A comparable result was obtained when MAb 21/3/3 (8) was used to precipitate the 72-kDa BHV-1 glycoprotein D (Fig. 7, lanes 3 and 4), indicating that fewer virus particles were released from BHV-1/gB2FuIFN-α infected cells (see also Fig. 8A).

To elucidate whether the incomplete cleavage affects growth of BHV-1/gB2FuIFN-α, single-step growth was analyzed on 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 methylcellulose-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 2×10^6 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 5×10^4 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-

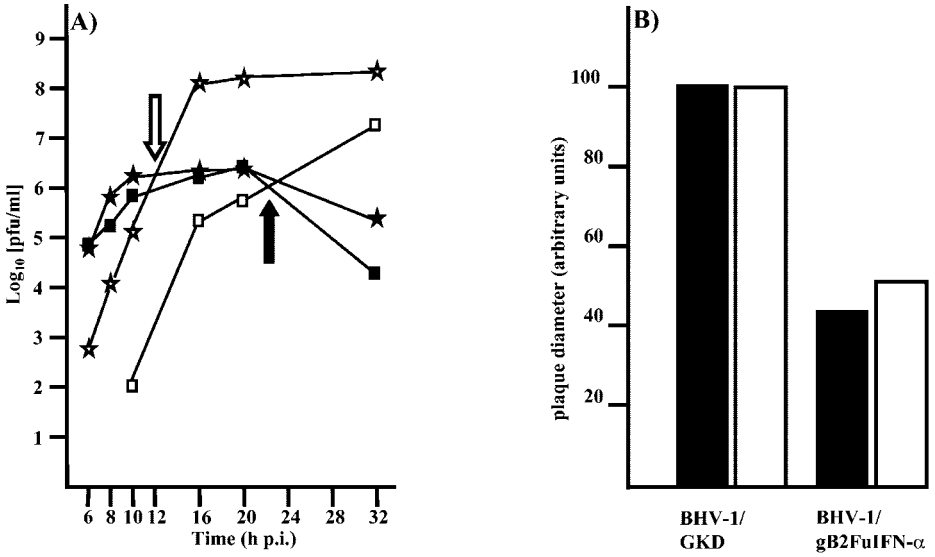

FIG. 8. Cell culture characteristics of BHV-1/gB2FuIFN-α. A) Growth curves. MDBK cells were infected with BHV-1/GKD (stars) and BHV-1/gB2FuIFN-α (squares) at 10 PFU per cell. Cells were treated with low-pH buffer to inactivate nonpentrated virions at 2 h p.i. Cells (closed symbols) and supernatants (open 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.

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 NH_2 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 NH_2 and COOH subunits of gB expressed by BHV-1/gB^{rev} and BHV-1/gB2FuGFP suggest that N and O glycosylations are comparably realized. In addition, coprecipitation of the NH_2 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/gB^{rev} and entered the cells and spread directly from cell to cell as efficiently as BHV-1/gB^{rev}. Analysis of the fate of the intervening GFP revealed that biologically active GFP accumulated in

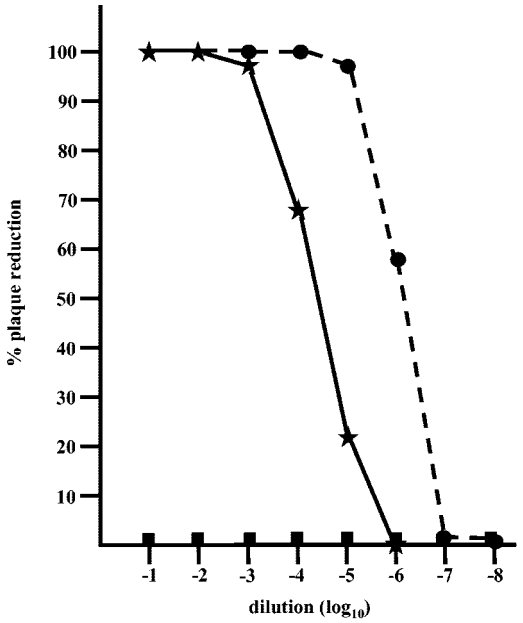

FIG. 9. Secretion of biologically active IFN-α from cells infected with BHV-1/gB2FuIFN-α. Culture media from KOP/R cells infected with BHV-1/GKD (squares) or BHV-1/gB2FuIFN-α (stars) were clarified by ultracentrifugation and sterilized by UV irradiation. The supernatants and a standard containing 2×10^6 U of recombinant IFN-α per ml (circles) were serially diluted and added to confluent KOP/R cells. Approximately 100 PFU of VSV was added to each culture 24 h later. At 1 h p.i. the inoculum was replaced by semisolid methylcellulose-containing medium, and plaques were counted at 24 h p.i.

the medium of BHV-1/gB2FuGFP-infected cells, indicating that it was efficiently secreted after removal from uncleaved gB. Thus, although the insertion of 246 amino acids into pgB hinders intracellular transport, it apparently does not interfere with the overall processing leading to functional gB.

An influence of the intervening polypeptide on furin cleavage was observed for gB2FuIFN-α, which was transported to the *trans*-Golgi network as efficiently as wild-type gB. Furin cleavage, however, occurred predominantly only at FCS2, resulting in an NH_2 subunit the size of a fusion protein between the authentic NH_2 subunit and mature IFN-α. Long-term labeling and analysis of the gB content of virions showed that cleavage also occurred to a low extent at FCS1. We assume that the amino acid sequence downstream from FCS1 interferes with the cleavage activity of furin at this site. The structural requirements to achieve full cleavage need to be identified. Despite the inefficient removal of IFN-α, we found a substantial amount of IFN-α activity in the culture medium of cells infected with BHV-1/gB2FuIFN-α, which was close to the activity reached with IFN-α-expressing replication-defective adenoviruses (4). Thus, these results demonstrate that the engineered gB can also be used to transport secreted glycoproteins, and we speculate that if full cleavage at FCS1 can be achieved, significantly larger amounts of IFN-α will be produced by cells with accordingly engineered BHV-1/gB2FuIFN-α. This assumption is supported by preliminary results obtained with a BHV-1 recombinant that secretes approximately fivefold-higher levels of mature boIL-2 as furin-excisable protein than does BHV-1/GKD-boIL-2 (13), which 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 NH_2 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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