SHORT COMMUNICATION

Fusion of the green fluorescent protein to amino acids 1 to 71 of bovine respiratory syncytial virus glycoprotein G directs the hybrid polypeptide as a class II membrane protein into the envelope of recombinant bovine herpesvirus-1

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It was recently shown that the class II membrane glycoprotein G of bovine respiratory syncytial virus (BRSV) is integrated into the envelope of recombinant bovine herpesvirus-1 (BHV-1) virions in the correct orientation. To verify the hypothesis that the membrane anchor of BRSV G might be suitable to target heterologous polypeptides into the membrane of recombinant BHV-1 particles, an open reading frame encoding a fusion protein between amino acids 1 to 71 of the BRSV G glycoprotein and the green fluorescent protein (TMIIGFP) was combined into the genome of BHV-1. The resulting recombinant BHV-1/eTMIIGFP had growth properties similar to those of wild-type BHV-1. Live-cell analysis of cells infected with BHV-1/eTMIIGFP indicated that the fusion protein localized to the cell surface. Immunoprecipitations and virus neutralization assays using a GFP-specific antisera proved that TMIIGFP was incorporated as a class II membrane protein into virions.

Bovine herpesvirus-1 (BHV-1), a member of the subfamily Alphaherpesvirinae with a double-stranded DNA genome of 136 kbp (Roizman et al., 1992; Schwyzer & Ackermann, 1996), is an economically important pathogen. Infectious rhinotracheitis and infectious pustular vulvovaginitis are the most common clinical symptoms induced in cattle (Gibbs & Rweyemamu, 1977). BHV-1 particles contain a number of virus-encoded glycoproteins [gB, gC, gD, gE, gH, gL, gM and gN (reviewed in Schwyzer & Ackermann, 1996)] in the viral membrane, of which gB, gD, gH and probably gL (Fehler et al., 1992; Meyer et al., 1998; Miethke et al., 1995; Schröder & Keil, 1999) are essential for infectious virus replication. BHV-1 tolerates incorporation of heterologous glycoproteins into the viral envelope. Examples include pseudorabies virus glycoproteins (Otsuka & Xuan, 1996), the class II attachment glycoprotein G of bovine respiratory syncytial virus (BRSV) and the bovine viral diarrhoea virus (BVDV) E2 glycoprotein (Kühnle et al., 1998; Schmitt et al., 1999). The presence of the BRSV G glycoprotein within virions did not significantly affect entry of the recombinant BHV-1 into cells, indicating that the events leading to membrane fusion between virus particles and target cells are not hindered by the paramyxovirus glycoprotein and it was hypothesized that the membrane anchor of the BRSV G glycoprotein can be used to target foreign polypeptides into the BHV-1 envelope (Kühnle et al., 1998). To verify this hypothesis, plasmid pEGFP-N1 (Clontech) was cleaved upstream from the open reading frame (ORF) encoding the green fluorescent protein (GFP) with BamHI, blunt-ended with Klenow polymerase and then cleaved with BglII to integrate a BglII–AatII fragment isolated from pspGsyn, (Kühnle et al., 1998) after blunt-ending at the AatII cleavage site with Klenow polymerase. This fragment encompasses codons 1 to 71 of the BRSV G ORF (Kühnle et al., 1998) and encodes the cytoplasm domain and the membrane anchor of the BRSV G glycoprotein (Lerch et al., 1990). The resulting ORF, named TMIIGFP ORF, encodes a fusion protein which consists of amino acids 1–71 of the BRSV G glycoprotein followed by the hexapeptide DPPVAT and the entire GFP. The TMIIGFP ORF was cleaved out from plasmid pTMIIGFP with BglII and NotI and inserted into the BglII–AatII cleavage site with Kühnle et al., 1998) and encodes the cytoplasm domain and the membrane anchor of the BRSV G glycoprotein (Lerch et al., 1990). The resulting ORF, named TMIIGFP ORF, encodes a fusion protein which consists of amino acids 1–71 of the BRSV G glycoprotein followed by the hexapeptide DPPVAT and the entire GFP. The TMIIGFP ORF was cleaved out from plasmid pTMIIGFP with BglII and NotI and integrated into pROMe cleaved with BglII and NotI and integrated into pROMe cleaved with BglII and NotI. The resulting plasmid pROMeTMIIGFP. As a control, the GFP ORF was isolated from pEGFP-N1 after cleavage of the plasmid with BglII and NotI and integrated into pROMe cleaved with BglII and NotI. The resulting plasmid pROMeGFP and pROMeTMIIGFP were co-transfected with purified BHV-1/80-221 DNA into MDMK cells and progeny virus was serially diluted and plated on MDMK-Bu100 cells (kindly provided by L. Bello and W. Lawrence, University of Pennsylvania, Philadelphia, PA, USA) as described. BHV-1/80-221 lacks the essential gD ORF which
Fig. 1. Expression of TMIIGFP and GFP in infected cells. MDBK-Bu100 cells were infected with approximately 50 p.f.u. of BHV-1/eTMIIGFP (a), BHV-1/eGFP (b), BHV-1/eGsyn (c) and wild-type BHV-1 (d). After development of plaques, fluorescence was photographed directly (a and b) or after fixation of the cultures with 3% paraformaldehyde in PBS followed by incubation with BRSV glycoprotein G-specific MAb 66 (Furze et al., 1994, 1997) (c) or BHV-1 gB-specific MAb 42/18/7 (d) for indirect immunofluorescence. Bound antibodies were then visualized by staining with DTAF-conjugated goat anti-mouse immunoglobulin G.

was replaced by a lacZ expression cassette. Therefore, only viruses which have acquired the gD ORF by replacement of the lacZ cassette by the insert of the recombination plasmids should be lacZ+ and able to replicate on non-complementing cells (Kühnle et al., 1996). Virus isolates from plaques that did not stain blue under a Bluo-Gal-containing agarose overlay (Fehler et al., 1992) were again titrated on MDBK-Bu100 cells and isolates which produced only ‘white’ plaques were characterized further by Southern blot hybridizations using 32P-labelled DNA probes from the gD ORF, the lacZ and the gE ORF to verify that the recombinants BHV-1/eTMIIGFP and BHV-1/eGFP were generated as envisaged. In vitro growth properties of the recombinants did not significantly differ from those of wild-type BHV-1.

To test for GFP expression, MDBK-Bu100 cultures were infected with approximately 50 p.f.u. of the isolates BHV-1/eTMIIGFP or BHV-1/eGFP and used for live-cell analysis after development of plaques. In contrast to the very intense and homogeneous BHV-1/eGFP-induced fluorescence (Fig. 1 b), cells infected with BHV-1/eTMIIGFP exhibited a weaker fluorescence (Fig. 1 a) with a distribution indistinguishable from the allocation of BHV-1/eGsyn (Kühnle et al., 1998) expressed glycoprotein G of BRSV (Fig. 1 c) or BHV-1 gB (Fig. 1 d) or BHV-1 gD (not shown) after visualization by indirect immunofluorescence. To quantify the differences in the fluorescence intensities, MDBK-Bu100 cells were infected with BHV-1/eGFP and BHV-1/eTMIIGFP at an m.o.i. of 5. Cells were harvested 16 h later and relative fluorescence was determined by flow cytometry using a Becton-Dickinson FACS-Calibur or with a Fluoroscan II (Labsystems). Both methods revealed that the BHV-1/eGFP-induced fluorescence was about 6-fold higher than that induced by BHV-1/eTMIIGFP. Since Northern blot analyses with RNA from cells infected with the recombinants demonstrated comparable amounts of transcripts encompassing the respective GFP ORF (not shown), it was assumed that the reduced fluorescence is due to the expression of GFP as a membrane protein. This assumption was supported by the results of the immunoprecipitations shown in Fig. 2, which demonstrated that comparable amounts of GFP-related proteins were expressed in BHV-1/eTMIIGFP- and BHV-1/eGFP-infected cells.

The BHV-1/eTMIIGFP-encoded fusion protein and precursor/product relationships were identified by pulse-chase experiments (Fig. 2 a, b, c). In contrast to GFP, for which no processing of the 28 kDa apparent molecular mass protein could be observed during the chase period (Fig. 2 a), the
apparent molecular mass of TMIIGFP started to shift from 35 kDa after the pulse to 37 kDa after 30 min chase. Processing of the 35 kDa precursor, whose size is in good agreement with the calculated molecular mass of 35.4 kDa, appeared to be slow and was not completed even after 120 min chase (Fig. 2b). Conversion of the 63 kDa gD precursor molecules to the mature 72 kDa gD in BHV-1/eTMIIGFP- (Fig. 2c) and BHV-1/eGFP- (not shown) infected cells was nearly complete after 90 min chase, which is also found for gD in wild-type BHV-1-infected cells, indicating that expression of TMIIGFP does not detectably interfere with processing of gD. Attempts to demonstrate that glycosylation of the TMIIGFP precursor molecules results in conversion to the 37 kDa form by digestion of immunoprecipitated TMIIGFP with N-glycosidase F or neuraminidase and O-glycosidase were not successful. According to the enzymes used, electrophoretic mobilities of gD increased as expected (Kühne et al., 1998; van Drunen Littel-van den Hurk & Babiuk, 1986), which demonstrated that enough enzymatic activity for complete deglycosylation was added to the respective probes (data not shown). This situation is similar to that found for the BHV-1-expressed BRSV glycoprotein G, for which the presence of O-glycosidase-resistant O-glycans (Kurilla et al., 1995) or addition of other side chains (Kühne et al., 1998) has been discussed previously.

To determine whether the membrane anchor of the BRSV glycoprotein G is indeed sufficient to target the hybrid protein into the viral membrane, [35S]methionine/cysteine labelled proteins from infected cells and purified BHV-1/eGFP and BHV-1/eTMIIGFP virus particles were immunoprecipitated with a GFP-specific antiserum (Clontech) and, to control the infection and the purity of the virus preparations, BHV-1 gD-specific MAb 21/3/3 precipitated the 28 kDa GFP from BHV-1/eGFP-infected cells (Fig. 2d, lane 4) and only the mature 72 kDa gD from the respective virions (Fig. 2d, lanes 5 and 6). The anti-GFP serum precipitated the 28 kDa GFP from BHV-1/eGFP-infected cells (Fig. 2d, lane 4) and proteins with apparent molecular masses of 37, 35 and 28 kDa after infection with BHV-1/eTMIIGFP (Fig. 2d, lane 3). Proteins with the same mobility were also detected among purified BHV-1/eTMIIGFP virion proteins (Fig. 2d, lane 7), suggesting that both mature and incompletely processed TMIIGFP are associated with virus particles. However, the presence of polypeptides migrating at a similar position as GFP among the anti-GFP reactive proteins from BHV-1/eTMIIGFP virions indicates that some degradation of the fusion protein occurs during virion purification and immunoprecipitation. It is also possible that the 28 kDa protein found in BHV-1/eTMIIGFP-infected cells and in virions represents an intracellular cleavage product of TMIIGFP. Whether this protein had retained the BRSV G membrane anchor remains to be determined because BRSV G-specific
polyclonal antibodies (Kühnle et al., 1998) did not react with either form of TMIIGFP. Comparison of the relative intensities between gD and TMIIGFP from infected cells and virions indicates that gD and TMIIGFP were incorporated into cellular and viral membranes with comparable efficiencies, suggesting that gD is not preferentially integrated into the viral envelope.

The faint band at 28 kDa detected after precipitation of proteins from BHV-1/eGFP virions by the anti-GFP serum indicated that GFP molecules were associated with BHV-1/eGFP particles. This assumption was supported by infection of MDBK-Bu100 cells in the presence of cycloheximide (100 µg/ml) to prevent de novo protein synthesis with purified virus preparations of BHV-1/eTMIIGFP and BHV-1/eGFP at an m.o.i. of 10 followed by flow cytometry at 3 h post-infection. The maxima of the relative fluorescence intensities corresponded to 1–2 × 10^4 for BHV-1/eTMIIGFP-infected cells and 2–2 × 10^1 for BHV-1/eGFP-infected cells using the maximal relative fluorescence determined for wild-type BHV-1 as zero (data not shown). Assuming a comparable particle to p.f.u. ratio and taking into account that GFP fluorescence is more efficient than TMIIGFP fluorescence, approximately 30-fold more molecules of the fusion protein than GFP molecules were associated with virus particles. Although not further analysed, it is assumed that GFP constitutes a component of the tegument of BHV-1/eGFP virions and is released into infected cells after fusion of viral and cellular membranes. Whether this association is linked with the synthesis of GFP as a BHV-1-encoded protein or simply due to the over-expression of the protein needs to be clarified. The presence of cryptic packaging signals within GFP cannot be excluded either.

To demonstrate that TMIIGFP is integrated into the viral envelope in the correct orientation and to provide evidence that GFP is not a component of the membrane of BHV-1/eGFP virus particles, BHV-1/eTMIIGFP and BHV-1/eGFP virions were tested for susceptibility to neutralization by the GFP-specific antiserum (Fig. 3, open symbols) and, as a control, by MAb 21/3/3 (Fig. 3, closed symbols). Without complement, the anti-GFP serum did not neutralize the recombinants at a 1:100 dilution and had no effect on the infectivity of wild-type BHV-1 at dilutions of 1:100 and higher irrespective of the presence of complement (data not shown). With complement, the anti-GFP serum efficiently neutralized BHV-1/eTMIIGFP virions (Fig. 3, open circles) but not BHV-1/eGFP virions (Fig. 3, open squares). Thus, TMIIGFP is integrated in the viral envelope as a class II membrane protein, whereas GFP appears to be a constituent of the tegument of BHV-1/eGFP virions. Both BHV-1 recombinants were comparably neutralized by MAb 21/3/3 (Fig. 3), indicating that the presence of TMIIGFP in the viral membrane does not interfere with binding of MAb 21/3/3 to gD.

In summary, our results demonstrate that amino acids 1–71 of BRSV glycoprotein G, which encompass the cytoplasmic domain and the membrane anchor, are sufficient to target proteins into the envelope of BHV-1 without notably affecting the in vitro replication of BHV-1. This raises the possibility of the construction of virions with altered biological properties for potential use in vaccine development. In addition, the results described here and the findings that alphaherpesviruses efficiently incorporate heterologous membrane glycoproteins like human CD4, BVDV E2 or BRSV G (Dolter et al., 1993; Kühnle et al., 1998; Schmitt et al., 1999) into the envelope of recombinant virions indicate that membrane association is not necessarily dependent on herpesvirus-specific targeting signals. This suggests that the presence of any membrane protein within the relevant compartment at the appropriate stage in virus replication is sufficient for inclusion into the viral envelope.

Recombinant BHV-1/eTMIIGFP and plasmid pROMe-TMIIGFP provide helpful tools to prove or disprove this hypothesis which is under discussion (Brideau et al., 1998).

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References


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