A novel protein expression strategy using recombinant bovine respiratory syncytial virus (BRSV): modifications of the peptide sequence between the two furin cleavage sites of the BRSV fusion protein yield secreted proteins, but affect processing and function of the BRSV fusion protein

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INTRODUCTION

Bovine respiratory syncytial virus (BRSV) and human respiratory syncytial virus (HRSV) are members of the genus Pneumovirus in the family Paramyxoviridae (Pringle, 1996). Both replicate in the respiratory tract and cause similar diseases in their respective natural hosts (Collins et al., 2001). BRSV encodes 11 proteins (Collins et al., 2001; Lerch et al., 1989; Mallipedi et al., 1990), which include the viral envelope-associated proteins, namely the fusion (F) protein, the attachment (G) protein and the small hydrophobic (SH) protein. Of these, only the F protein is essential for BRSV replication in cell culture (Karger et al., 2001). The BRSV F protein mediates fusion between the viral and the cellular membranes during the initial stages of the infection and de novo expression leads to fusion of adjacent cell membranes resulting in the formation of syncytia. Like the F proteins of other enveloped viruses, the BRSV F protein is synthesized as a precursor, F0, and has to be activated by cleavage by the furin endoproteinase in the trans-Golgi network (Klenk & Garten, 1994).

A so far unique feature of both HRSV and BRSV F proteins is the cleavage of the respective F0 proteins at two furin recognition sites after the Arg109 and Arg136 residues, resulting in the release of a 27 aa intervening peptide (Gonzáles-Reyes et al., 2001; Zimmer et al., 2001), which is N-glycosylated and has been named pep27 (Zimmer et al., 2001).

Recently it was shown that cleavage of the BRSV F protein...
after Arg102 and the presence of pep27 are dispensable for BRSV replication in cell culture (Zimmer et al., 2002). Recent studies by Zimmer et al. (2003) demonstrated that pep27 is further modified after cleavage, resulting in conversion to a bioactive peptide of the tachykinin family, named virokinin. Virokinin acts on specific G protein-coupled receptors, and it has been suggested that virokinin plays a role in the pathogenesis of BRSV, for example by assisting escape from the host immune response and increasing virus survival in the host (Zimmer et al., 2003).

We report here that the sequence of the intervening peptide influences intracellular transport, maturation and biological activity of the F protein and show that bovine cytokines inserted into F0 in place of pep27 are secreted into the medium of transfected and recombinant BRSV (rBRSV)-infected cells.

METHODS

Cell culture and viruses. Madin–Darby bovine kidney cell clone Bu100 (MDBK; kindly provided by L. Bello and W. Lawrence, Philadelphia, USA) was grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 5 % fetal bovine serum (FBS), 2-4 mM L-glutamine, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. Cell cultures were incubated at 37 °C in a humidified atmosphere containing 5 % CO₂. The bovine pharyngeal cell line 244 (KOP/R; provided by the Collection of Cell Lines in Veterinary Medicine, Insel Riems) was propagated in DMEM with 10 % FBS as described above. Bovine interleukin 2 (boIL2)- and BRSV replication in cell culture (Zimmer et al., 2003). For transient expression, KOP/R cells were co-transfected with 2.5 µg of the full-length plasmid pBRSV-FKat, pBRSV-FboIL2, pBRSV-FboIL4 and pBRSV-FboIFN-γ.

Transfection experiments. For transient transfection, KOP/R cells were co-transfected with 2.5 µg of the respective expression plasmid and 0.5 µg pAMB25 DNA per 5 × 10⁵ cells using the Superfect reagent (Qiagen) as recommended by the supplier. For recovery of rBRSV from cloned DNA, subconfluent BSR T7/5 cells stably expressing phase T7 RNA polymerase were transfected as described previously (Buchholz et al., 2000) with the single restriction sites SpII and ClaI, which are present in the rBRSV G/F and M7 intergenic regions, resulting in BRSV full-length plasmids pBRSV-Fsyn, pBRSV-Fpep27, pBRSV-FKat, pBRSV-FboIL2, pBRSV-FboIL4 and pBRSV-FboIFN-γ.

Immunoprecipitation. Transfected or infected cells were incubated with cell culture medium lacking methionine and cysteine for 2 h
before addition of $^{35}$S-methionine (500 μCi ml$^{-1}$, 18.5 MBq ml$^{-1}$) and $^{35}$S-cysteine (250 μCi ml$^{-1}$, 9.25 MBq ml$^{-1}$). Lysis of cells and immunoprecipitation of proteins from cell lysates and culture supernatants were performed as described (Keil et al., 1985) using monospecific rabbit sera directed against boIL2 and boIL4 (Kühnle et al., 1996) or a BRSV F1-specific rabbit serum raised against a bacterially expressed F protein containing the entire amino acid sequence of the F1 subunit. Precipitated proteins were visualized by fluorography after 12.5 or 5% SDS-PAGE.

**Determination of boIFN-γ activity.** Secretion of biologically active boIFN-γ into the cell culture medium of transfected KOP/R cells was analysed by a vesicular stomatitis virus (VSV) plaque reduction assay. Supernatants from KOP/R cells expressing boIFN-γ as control were serially diluted in normal cell culture medium and added against a bacterially expressed F protein containing the entire amino acid sequence of the F1 subunit. Precipitated proteins were visualized by fluorography after 12.5 or 7.5% SDS-PAGE.

**Indirect immunofluorescence assays.** Cells were fixed with 3% paraformaldehyde in PBS for 20 min, subjected to membrane permeabilization with 0-2% Triton X-100 and sequentially incubated with F-specific mAb 19 (kindly provided by Geraldine Taylor, Compton, UK) and 5-(4,6-dichlorotiazin-2-yl)amino]fluorescein hydrochloride (DTAF)-conjugated rabbit anti-mouse IgG (Dianova).

**Analysis of cell culture characteristics.** For multi-cycle growth curves, MDBK cultures were infected with 0-1 p.f.u. per cell. At 6 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 viros. Cells were washed twice with cell culture medium and incubated for the times indicated, when supernatants and cells were harvested together and stored at −70°C. Serial dilutions were titrated on MDBK cells and cultures were incubated under semi-solid medium containing methylcellulose for 6 days. Plaques were counted after immunostaining with mAb 19 and DTAF-conjugated anti-mouse IgG under a fluorescence microscope.

**Determination of plaque diameters.** MDBK cells were infected with diluted virus stocks and incubated under semi-solid medium containing methylcellulose for 6 days. Diameters of 100 randomly selected plaques were determined after immunostaining with mAb 19 and DTAF-conjugated anti-mouse IgG under a fluorescence microscope using a graduated ocular.

**Penetration kinetics.** MDBK cells were pre-cooled at 4°C for 30 min and further incubated at 4°C for 2 h after the addition of approximately 200 p.f.u. virus to allow adsorption. Cultures were then shifted to 37°C and extracellular viros 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 semi-solid medium containing methylcellulose for 6 days. Plaques were counted after immunostaining with mAb 19 and DTAF-conjugated anti-mouse IgG under a fluorescence microscope using a graduated ocular.

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**Fig. 1.** Construction of the F protein mutants. (a) Schematic representation of the wt F protein. The signal peptide (sig) and the location of pep27 between the F1 and F2 subunits and the membrane anchor are indicated. Arrows mark the furin cleavage sites 1 and 2 (Fu1 and Fu2) after Arg109 and Arg136, respectively. The covalent linkage of the F1 and F2 subunits by disulfide bonds is indicated. (b) Scheme of the BRSV-Fsyn ORF. The location, amino acid sequence and nucleotide sequence of the region encoding pep27 are shown. The Smal and AvrII restriction sites are shown in bold. (c) Amino acid changes within the F protein mutants. Numbering of the amino acids refers to the wt sequence.
Cleavage of the BRSV F protein precursor F₀ at two furin cleavage sites results in release of pep27 (Zimmer et al., 2002), which encompasses amino acids Gly₁¹⁰ to Arg₁³⁶ (Fig. 1). This peptide is subsequently converted into virokinin, a biologically active tachykinin, which is secreted from BRSV-infected cells (Zimmer et al., 2003). To test the influence of the intervening peptide on cleavage and function of the F protein and to test whether the F protein can be used as a transporter of secreted heterologous peptides or (glyco)proteins, we constructed ORFs encoding F protein mutants (Fig. 1c) that lacked the entire pep27 (Fpep27) or in which pep27 was replaced by an arbitrarily chosen amino acid sequence (FKat), or by mature boIL2 (FboIL2), mature boIL4 (FboIL4) or mature boIFN-γ (FboIFN-γ). To analyse the modified F proteins independently from phage T7 RNA polymerase-expressing cells, we used a synthetic ORF (BRSV-Fsyn ORF), which allowed expression of the F protein via the nucleus and in which the codons for the furin cleavage sites were flanked by restriction enzyme cleavage sites for SmaI and AvrII (P. König & G. M. Keil, unpublished data). The modified ORFs were obtained by replacement of this Smal–AvrII fragment by Smal- and AvrII-cleaved fragments encompassing the desired sequences (Fig. 1). This strategy for the construction of the mutated ORFs ensured that the respective mature F₁ and F₂ subunits possessed the same amino acid sequence upon cleavage as the corresponding wt F subunits.

The ORFs encoding wt and mutated F proteins were integrated into pelcas, which enables protein expression under control of the MCMV early 1 promoter in the presence of the MCMV major immediate-early protein pp89, encoded by plasmid pAMB25 (Bühler et al., 1990; Koszinowski et al., 1986). Plasmid pelcas also contains the polyadenylation signal from the BHV-1 gD gene for efficient polyadenylation of transcripts (Kühnle et al., 1996). Transient expression of the F variants was achieved by co-transfection of the respective pelcas plasmid together with pAMB25 into KOP/R cells. Cultures expressing wt F protein were fixed 28 h p.t. due to an early cytopathic effect, whereas the mutant F-expressing cells were fixed at 44 h p.t. Expression of the F proteins was monitored by indirect immunofluorescence using F-specific mAb 19 (Fig. 2). Wt F protein induced large syncytia, which started to detach from the culture dishes at about 30 h p.t. Syncytia were also found in cultures expressing Fpep27⁻. However, the size of the

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**Fig. 2.** Influence of the intervening peptide on F-mediated syncytium formation. Transfected KOP/R cells expressing the indicated F proteins were fixed at 28 (wt F) or 44 (mutants) h p.t. Cultures were incubated with F-specific mAb 19. Bound antibody was visualized by DTAF-conjugated rabbit anti-mouse IgG using a fluorescence microscope.
fused cells and the number of nuclei within the syncytia were significantly reduced. Cultures expressing FKat and FboIFN-γ contained mainly single F-positive cells and the few detected syncytia contained only three to five nuclei. No fused cells were found in cells expressing FboIL2 and FboIFN-γ, even when cultures were fixed at 72 h p.t. These results showed that pep27 is not essential but is beneficial for F-mediated syncytium formation and that the cell fusion activity is influenced by the amino acid sequence between the furin cleavage sites.

To analyse whether the F mutants were processed correctly and to elucidate the fate of boIL2 and boIL4 contained within FboIL2 and FboIL4, transfected-cell proteins were metabolically labelled with [35S]methionine/[35S]cysteine and immunoprecipitated proteins were visualized by fluorography after SDS-PAGE. From lysates expressing wt F, Fpep27 or FKat protein, a polyclonal rabbit serum specific for the F 1 subunit precipitated the respective uncleaved F 0 precursor molecules with apparent molecular masses of 71 kDa (wt F), 61 kDa (Fpep27) and 67 kDa (FKat), and the F 1 (48 kDa) and F 2 (20 kDa) subunits (Fig. 3a), which remain covalently linked by disulfide bonds after furin cleavage. As expected, the mobilities of the F 1 and F 2 subunits did not differ among wt F and the mutants. Thus, replacement of pep27 by an artificial amino acid sequence did not impede cleavage. Comparison of the steady state F 0-to-F 1 ratios indicated that processing of Fpep27 may be less efficient than processing of FKat and wt F. Relatively more F 0 molecules were also found for FboIL2, FboIL4 and FboIFN-γ (Fig. 3b), indicating an inhibitory effect of the respective intervening proteins on processing. This interpretation was supported by the results of pulse-chase experiments (Fig. 4). Transfected cells were incubated with [35S]methionine/[35S]cysteine for 30 min and chased for the times given in Fig. 4 with normal cell culture medium. The F 0 molecules of the F variants were detected after the labelling period, with wt F 1 and F 1 of FKat already being visible at the end of the pulse (Fig. 4a). The majority of wt F 0, F 0Kat and F 0boIL4 was cleaved after 60 min chase (Fig. 4a) but processing of the latter appeared slightly delayed. In contrast, cleavage of F 0pep27, F 0boIL2 and F 0boIFN-γ was much more delayed, which was reflected by the later appearance of the corresponding F 1 proteins and the high levels of the respective F 0 molecules at the end of the chase period. In the panels showing processing of Fpep27, FKat, FboIL2, FboIL4 and FboIFN-γ, proteins of minor abundance that migrate between the respective F 0 and F 1 proteins also appeared to be post-translationally processed. The nature of these proteins is not clear. They may represent

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**Fig. 3.** Proteolytic processing of wt F and F mutants. (a) Transfected KOP/R cells expressing wt F (lane 1), Fpep27− (lane 2) and FKat (lane 3) were incubated with [35S]methionine/[35S]cysteine at 20 h p.t. for 4 h. Labelled proteins were immunoprecipitated from lysed cells using a polyclonal rabbit anti-F 1 serum and visualized by fluorography after size separation by 12.5% SDS-PAGE. The positions of the respective F 0, F 1 and F 2 proteins are indicated. (b) Transfected KOP/R cells expressing FboIFN-γ (lane 1), FboIL2 (lanes 2 and 4) and FboIL4 (lanes 3 and 5) were incubated with [35S]methionine/[35S]cysteine at 20 h p.t. for 4 h. Labelled proteins were immunoprecipitated from lysed cells using the polyclonal rabbit anti-F 1 serum (lanes 1–3), the polyclonal rabbit anti-boIL2 serum (lane 4) or the polyclonal rabbit anti-boIL4 serum (lane 5). (c) Metabolically labelled proteins from the culture medium of transfected KOP/R cells expressing FboIL2 (lane 1) and FboIL4 (lane 2) and from KOP/R cells infected with BHV-1/ieIL-2 (lane 3) and BHV-1/ieIL-4 (lane 4) and incubated with [35S]methionine/[35S]cysteine from 4 to 16 h p.i. were immunoprecipitated using the polyclonal rabbit anti-boIL2 serum (lanes 1 and 3) or the polyclonal rabbit anti-boIL4 serum (lanes 2 and 4). Precipitated proteins were separated by 12.5% SDS-PAGE and visualized by fluorography. The relative mobilities of marker proteins are indicated in kDa on the right of each panel.
differentially glycosylated precursor molecules of the corresponding F mutants.

The pulse–chase analyses indicated that cleavage of the F protein occurred after further modification of the F0 protein to a slower-migrating form, F0/C3 (Fig. 4b), which has not been described so far. In wt F-expressing cells, F0/C30 migrated as a diffuse band and appeared to be rapidly cleaved. In contrast, F0/C30 accumulated in Fpep27-expressing cells (Fig. 4b), which corresponded to the lower abundance of the respective F1 form. F0/C30 molecules were also clearly detectable during maturation of FKat and allusively during the processing of FboIL4 (Fig. 4a).

The fate of boIL2 and boIL4 after transport by FboIL2 and FboIL4 to the trans-Golgi network and cleavage was analysed by immunoprecipitation of cell-associated and secreted proteins using rabbit sera against boIL2 and boIL4 (Kühnle et al., 1996) and supernatants from KOP/R cells infected with boIL2- and boIL4-expressing BHV-1 recombinants BHV-1/ieIL-2 or BHV-1/ieIL-4 (Kühnle et al., 1996) as controls. From the culture medium of cells transfected with pBoIL2, the anti-boIL2 serum precipitated the 22 kDa boIL2 protein, which migrated at a distance comparable with mature boIL2 secreted into the supernatant of KOP/R cells infected with BHV-1/ieIL-2 (Fig. 3c, lanes 1 and 3), whereas F0/boIL2 (Fig. 3b, lane 4), but not mature boIL2, could be precipitated from lysed cells (not shown). An equivalent result was obtained after transfection with pBoIL4 followed by immunoprecipitations in which the anti-boIL4 serum precipitated the 20 kDa boIL4 protein, which co-migrated with BHV-1/ieIL-4-expressed boIL4. Again, boIL4 was detected only in the transfected cell culture medium (Fig. 3c, lanes 2 and 4), but not in cell lysates, from which the anti-boIL4 serum exclusively precipitated the uncleaved F0/boIL4 (Fig. 3b, lane 5). Thus, boIL2 and boIL4 were efficiently secreted from the transfected cells after cleavage of their respective precursors. The additional bands seen in all lanes in Fig. 3(c) were due to non-specifically precipitated proteins, and faster-migrating polypeptides present in Fig. 3(c, lanes 1 and 3) probably represented breakdown products of boIL2 (Kühnle et al., 1996).

Secretion of boIFN-γ was not directly monitored due to the lack of specific antibodies. Evidence that biologically active boIFN-γ was released from FboIFN-γ-expressing cells was obtained by the results of VSV plaque reduction assays, which revealed that supernatants from cells transfected with pBoIFN-γ contained 10–20 U IFN-γ (ml medium)−1 (data not shown). These results demonstrated that the BRSV F protein can be used as a vehicle to transport proteins into the extracellular space.

To study the effect of the mutations on the function of the F protein for productive virus replication, plasmids expressing wt or modified anti-genomic RNAs and support plasmids encoding the BRSV N, P, L and M2 proteins were co-transfected into BSR T7/5 cells (Buchholz et al., 1999).
Infectious rBRSV was efficiently recovered from transfections with the pBRSV plasmids containing the ORFs encoding wt F, Fpep27, FKat, FboIL2 and FboIL4, whereas attempts to rescue infectious virus expressing FboIFN-γ were repeatedly unsuccessful, suggesting that FboIFN-γ interferes with a vital function of BRSV. It appears, however, unlikely that the anti-viral activity of boIFN-γ precluded isolation of BRSV-FboIFN-γ, since recombinant human RSV, which expresses IFN-γ classically from a gene cassette, could be recovered from plasmids (Bukreyev et al., 1999).

Viral stocks of wt F-expressing BRSV-Fsyn and mutant F-expressing BRSV-Fpep27, BRSV-FKat, BRSV-FboIL2 and BRSV-FboIL4 were prepared on MDBK cells for further characterization. Infection of KOP/R cells with the isolates revealed that all induced syncytium formation, irrespective of the ability of the F proteins to mediate cell fusion in transient expression experiments (not shown). Direct sequencing of RT-PCR fragments of the respective F ORFs using RNA from partially purified virions as templates revealed that the ORFs encoding wt F, Fpep27 and FKat were as expected, whereas the ORFs encoding FboIL2 and FboIL4 carried mutations resulting in an exchange of Val152 to Glu within the F protein sequence of BRSV-FboIL2 and of Gly69 to Arg within the boIL4 protein sequence of BRSV-FboIL4. Whether these mutations were beneficial for the viability and/or cell fusion activity mediated by BRSV-FboIL2 and BRSV-FboIL4 needs to be clarified.

Cell culture characteristics of the isolates were analysed on MDBK cells. For multi-cycle growth curves, cells were infected with 0.1 p.f.u. per cell, non-penetrated virions were inactivated by low-pH treatment at 6 h p.i. and cultures were stored at the times indicated in Fig. 5(a) at −70 °C until titration. BRSV-Fsyn and BRSV-FboIL4 replicated almost identically, whereas reproduction of BRSV-Fpep27 and BRSV-FKat was initially delayed but reached wt titres by 48 h p.i. (Fig. 5a). In contrast, infectious BRSV-FboIL2 was detected only at 48 h p.i. and final titres reached by 144 h p.i. were approximately 100-fold lower than the titres obtained for the other isolates. Thus, an intervening peptide is not required for infectious replication of BRSV and its amino acid sequence can influence virus yield, which, with regard to pep27, is in accordance with results published by Zimmer et al. (2002). To test for the influence of the intervening peptide on direct cell-to-cell spread of BRSV,
 MDBK cultures were infected with appropriate dilutions of virus stocks and incubated under methylcellulose-containing semi-solid medium. At day 6 p.i., cells were fixed and immunostained using F-specific mAb 19 and DTAF-conjugated anti-mouse IgG. The diameters of 100 plaques of each isolate were measured using a fluorescence microscope and a graduated ocular. Fig. 5(b) shows that direct spreading of BRSV was not affected by the deletion of pep27, whereas its replacement by the Kat peptide or the bovine cytokines resulted in reduced cell-to-cell spread.

To determine whether the intervening peptide affected entry of BRSV into the host cell, approximately 200 p.f.u. of the respective viruses were allowed to adsorb to MDBK cell cultures at 4°C for 2 h. Cultures were then shifted to 37°C and extracellular virions were inactivated by low-pH treatment at the times indicated in Fig. 5(c). Cultures were overlaid with methylcellulose-containing semi-solid medium and plaques were counted 6 days later. BRSV-Fsyn and BRSV-Fpep27− penetrated comparably and required between 75 and 90 min for 50% penetration, demonstrating that pep27 is also dispensable for efficient virus–cell fusion. BRSV-FKat, BRSV-FboIL4 and BRSV-FboIL2 entered the cells significantly more slowly and needed approximately 120, 150 and 200 min for 50% entry, respectively. This result is surprising because, with the exception of BRSV-FboIL2, the mature F proteins within the envelope of the different virions should be identical in their amino acid sequence. In addition, comparison of the relative mobilities of the virion-associated F1 subunits gave no indication of different post-translational modifications (Fig. 6, left panel). Whether the migration of the corresponding F2 subunits was also unaffected could not be answered unequivocally, due to a number of breakdown products of the size of the F2 subunit, probably generated during immunoprecipitation.

To demonstrate that boIL2 and boIL4 are released from cells infected with BRSV-FboIL2 and BRSV-FboIL4, newly synthesized proteins were metabolically labelled with [14C]methionine/[35S]cysteine from 12 to 72 h p.i. with BRSV-Fsyn, BRSV-Fpep27−, BRSV-FKat, BRSV-FboIL2 and BRSV-FboIL4. Immunoprecipitations of proteins from the culture medium with the boIL2- or boIL4-specific antisera showed that the respective cytokines were released from cells infected with BRSV-FboIL2 or BRSV-FboIL4, respectively (Fig. 6, right panel), which demonstrated that the F protein of BRSV can be used to express secreted proteins as intervening peptides by rBRSV.

DISCUSSION
The F proteins of HRSV and BRSV possess a unique feature among furin-cleavable F proteins. They contain two consensus sequences for furin cleavage, which flank a small peptide of 27 aa that is removed from the F protein precursor molecule by proteolytic cleavage (Gonzáles-Reyes et al., 2001; Zimmer et al., 2001). This peptide of the BRSV F protein was named pep27 and it has been shown recently that pep27 is further modified to virokinin, a biologically active tachykinin, which is subsequently secreted from infected cells (Zimmer et al., 2003) and thus may play a role in the pathogenicity of BRSV in cattle. To elucidate whether an intervening amino acid sequence between the two furin cleavage sites is important for the biological activity of the BRSV F protein and the replication of BRSV, we constructed ORFs encoding F proteins that lacked the entire pep27 or in which pep27 was replaced by 23 aa, named the Kat peptide. To test for the possibility of using the F protein as a transporter for furin-excisable secreted proteins, we also constructed ORFs in which the coding sequence for pep27 was replaced by the coding sequence for mature boIL2, boIL4 or boFN-γ. Due to the mutagenesis strategy, all

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*Fig. 6.* Secretion of boIL2 and boIL4 from infected cells. KOP/R cells were infected with BRSV-Fsyn (lanes 1 and 6), BRSV-Fpep27- (lanes 2 and 7), BRSV-FKat (lanes 3 and 8), BRSV-FboIL2 (lanes 4 and 9) and BRSV-FboIL4 (lanes 5 and 10) with approximately 1 p.f.u. per cell. Cultures were incubated with [35S]methionine/[35S]cysteine from 12 to 72 h p.i. Culture supernatants were clarified by low-speed centrifugation and virions were pelleted by ultracentrifugation for 1 h at 45 000 r.p.m. in a Beckman TLA 45 rotor. Labelled proteins were immunoprecipitated from lysed virions with the polyclonal rabbit anti-F, serum (lanes 1–5) or from the ultracentrifugation supernatant with the polyclonal rabbit anti-boIL2 serum (lanes 6–9) or the polyclonal rabbit anti-boIL4 serum (lane 10). Precipitated proteins were analysed by 7.5% (lanes 1–5) or 12.5% (lanes 6–10) SDS-PAGE and visualized by fluorography.
mature F proteins consisted of F₁ and F₂ subunits with identical amino acid sequences. Transient transfection experiments revealed that removal of pep27 or replacement by the Kat peptide resulted in a reduction in F protein-mediated cell fusion activity. Cultures expressing FboIL4 showed only a few small syncytia and no syncytium formation was observed after expression of FboIL2 and FboIFN-γ, which correlated and was consistent with the strongly delayed cleavage of their respective precursors. Whether this delay also influenced the cell surface expression of the respective F proteins needs to be clarified. Indirect immunofluorescence assays on non-permeabilized cells did not reveal major differences.

That the intervening peptide plays a role in intracellular transport and maturation of the F protein was indicated by the results of pulse–chase experiments. Wt F₀ protein was readily cleaved, and the F₀Kat and F₀boIL4 were transported and cleaved with a comparably slightly delayed; however, processing of F₀pep27, F₀boIL2 and F₀boIFN-γ was clearly delayed. A possible explanation for these drastic effects could be that the amino acid sequence between the furin cleavage site influences cleavability through conformational changes of the F₀ precursor. Two-dimensional gel analysis of the F variants, however, revealed no evidence for significant differences in charge and overall structure (data not shown). In addition, the pulse–chase experiments indicated the formation of F₀⁺, a form of F₀ that, to our knowledge, has not been described previously. We assume that F₀⁺ represents the Golgi-associated F protein precursor, which contains complex-type N-glycans and also O-linked carbohydrates and which is subsequently cleaved by furin in the trans-Golgi network.

Analysis of the fate of the bovine cytokines present in the precursor molecules of FboIL₂, FboIL₄ and FboIFN-γ showed that boIL2 and boIL4 were secreted into the culture medium of transfected cells and that they migrated at a distance comparable with recombinant BHV-1-expressed mature boIL2 and mature boIL4, respectively, indicating that these cytokines were properly glycosylated. Secretion of boIFN-γ could not be analysed directly. However, the presence of IFN-γ activity in the culture medium of cells expressing FboIFN-γ provided good evidence that boIFN-γ was also correctly processed and cleaved from the precursor molecules.

The role of pep27 and the intervening peptides for the replication of BRSV was analysed after generation of rBRSVs expressing the synthetic F ORFs in place of the parental F protein. Recombinant viruses expressing wt F, Fpep27, FKat, FboIL2 and FboIL4 were isolated repeatedly, whereas generation of rBRSV containing the FboIFN-γ ORF always failed, suggesting that insertion of the amino acids for boIFN-γ into the F protein was not compatible with BRSV replication. Isolation of rBRSV lacking the entire pep27 is in accordance with results published by Zimmer et al. (2002), who reported that removal of aa 106–130 from the F protein had no significant effect on rBRSV-F(A106–130) growth. They observed, however, that the deletion mutant showed a drastically reduced syncytium formation activity in Vero cells and concluded that the mutations introduced at the C terminus of the F₂ subunit might interfere with cell-to-cell fusion. Our findings have shown that deletion of pep27 in presence of the authentic F₂ sequence had no effect on cell-to-cell spread. Expression of FKat, FboIL2 and FboIL4 by BRSV, however, negatively influenced not only direct spreading of the recombinants by cell–cell fusion, but also virus entry, as these viruses penetrated significantly more slowly into their target cells, suggesting that F protein-mediated membrane fusion processes involved in entry and direct spreading share mechanistic requirements.

In summary, our results have demonstrated that the amino acid sequence between the furin cleavage sites of the BRSV F protein influences intracellular transport, maturation and F protein-mediated syncytium formation, and affects the membrane fusion activity of recombinant virions. They further indicate that, in principle, the F protein expressed by rBRSV can be used as a transporter for expression and secretion of cytokines and probably other physiologically important heterologous polypeptides. This may be of particular interest for the development of novel vaccines, especially if the assumption that pep27 assists BRSV to escape the immune system and thus increases virus survival in the host (Zimmer et al., 2003) proves to be correct.

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