(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 October 2003 (09.10.2003)

PCT

(10) International Publication Number
WO 03/083095 A1

(51) International Patent Classification[7]: C12N 7/00, A61K 35/76, 39/155, C12N 15/62, C12P 21/06

(21) International Application Number: PCT/EP03/02520

(22) International Filing Date: 12 March 2003 (12.03.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
02076308.2 3 April 2002 (03.04.2002) EP

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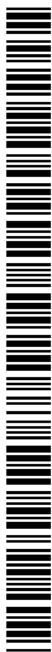

(81) Designated States (*national*): AE, AG, AL, AU, BA, BB, BG, BR, BZ, CA, CN, CO, CR, CU, CZ, DM, DZ, EC, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KE, KP, KR, LC, LK, LR, LT, LV, MA, MD, MG, MK, MN, MX, NO, NZ, OM, PH, PL, RO, RU, SD, SG, SK, TN, TR, TT, UA, US, UZ, VN, YU, ZA, ZM.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** VIRAL MUTANTS, MANIPULATED IN THE FURIN CLEAVAGE SITES OF GLYCOPROTEINS

(57) **Abstract:** The present invention relates to viruses containing glycoproteins with furin binding sites. For BRSV it has been found that the 27AA IFCSP is not essential for cell fusion activity of the F protein and the deletion or replacement of at least part of the IFCSP does not destroy F function for productive BRSV replication. Removal of Gly110 to Arg 136 and transient expression of the resulting protein (F_{ins}) resulted in formation of syncitia which were slightly smaller that those induced by wt F. thus, it has been found that oligopeptides, or bioactive proteins, can be integrated between 2 furin cleavage sites of a carrier glycoprotein, such as the furin cleavage sites of an F protein of an RSV. Due to the processing of the precursor protein, resulting in the cleavage at the furin cleavage sites, the heterologous sequence may be excised and consequently be secreted by a virus-infected cell. This concept can be applied likewise to other glycoproteins containing two furin cleavage sites, where the original sequence between the cleavage sites may replaced (in part) by a heterologous sequence or may simply be deleted. In proteins with one furin cleavage site, heterologous sequences may be inserted together with the sequence for a second cleavage site, thus creating a situation where the heterologous sequence is again flanked by two furin cleavage sites in the recombinant protein. Processing of the mutated protein will than also result in the excision of the heterologous sequence which may be secreted by virus infected cells. Viruses wherein glycoproteins are modified in one of the above-described ways, can be used for, for example vaccine purposes.

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Viral mutans, manipulated in the furin cleavage sites of glycoproteins.

The present invention relates to viruses containing glycoproteins with furin binding sites. More in particular the present invention relates to mutated Bovine Respiratory Syncytial virus (BRSV), vaccines based thereon and methods for the preparation of such vaccines. Bovine Respiratory Syncytial virus is a member of the family paramyxoviridae, and belongs to the genus of the Pneumoviruses. Paramyxoviridae belonging to this genus are human RSV, bovine RSV, ovine RSV, caprine RSV and pneumonia virus of mice. RSV is a member of the order Mononegavirales, i.e. the virus is a non-segmented negative strand RNA virus. The overall genomic organization of the non-segmented negative stranded RNA of the viruses belonging to the genus Pneumoviruses is comparable. The RNA consists of 10 genes, encoding eleven proteins, and has a length of about 15.2 kilobases. The RSV-proteins include two non-structural proteins (NS1 and NS2), four RNA-associated proteins, the nucleoprotein N, phosphoprotein P, the large, catalytic subunit L of the RNA-polymerase, a transcription elongation factor encoded by the first of two overlapping open reading frames of the M2 gene, and three envelope-associated proteins; the fusion protein F, the attachment protein G and the small hydrophobic protein SH. One characteristic shared by all pneumoviruses is the fact that they cause infections of the upper and (in most cases) lower respiratory tract. The human RVS has a worldwide distribution and has been found to be the major pediatric viral respiratory tract pathogen. Infection of bovine species with BRSV is highly comparable to HRSV infection in many respects, i.a. in the sense that it mainly causes disease in young animals. (For a review, see Van der Poel et al.; J. Inf. 29: 215-228 (1994)). Although re-infections occur frequently in both species, in cattle they usually do not cause clinical signs (Kimman and Westenbrink, Archives of Virology 1990, 112, 1-25) which suggest that a natural infection protects against clinical signs after reinfection. Mortality varies between 1% and 30%, depending on various parameters, such as virulence of the infecting strain, climate, level of animal care and occurrence of secondary infections. (Stott et al., J. Hygiene 85: 251-261 (1980), Verhoeff, et al. Vet. Rec. 115: 488-492 (1984)). The morbidity is very high (Baker et al., Am. J. Vet. Res. 46: 891-892 (1985), Baker et al., Vet. Clin. N. Am.: Food Animal Practice 1: 259-275 (1985)). Mortality due to BRSV-infection including those cases in which BRSV infection is followed by infection with other pathogens is very high, and thus the economical losses world-wide are consequently very high.

It is clear, that efficacious and reliable vaccines for both the protection against human RSV and bovine RSV are highly wanted, but vaccine development has been hampered because it is not known how a protective immune response can be induced without causing disease. First attempts to vaccinate children with formalin-inactivated vaccines led to enhanced disease after natural infection, which suggests that vaccination may even be harmful

(Anderson et al., Journal of Infectious Diseases 1995, 171, 1-7). It is known, however, that antibodies against two major surface proteins , F (a fusion protein) and G (an attachment protein), play a key role in protection (Kimman and Westenbrink, Archives of Virology 1990, 112, 1-25). However, so far vaccines based on the F- or G-protein, i.e. subunit vaccines,
5 have not been disclosed in the literature.
One way to mimic the natural infection, c.q. to efficiently trigger the host's defense mechanism is to develop a live attenuated vaccine. Such a vaccine mimics the natural triggering of the immune system, whereas due to its attenuated characteristics, it does not induce the severe clinical signs caused by the wild-type virus. The attenuated character is
10 usually obtained by mutating a gene that on the one hand plays a role in virulence, but on the other hand is not essential for viral infection and replication, and moreover plays no role in the induction of immunity.
Live attenuated vaccines must, as closely as possible, mimic the native RSV as far as their infection behavior is concerned. One of the main characteristics of native Respiratory
15 Syncytial Virus is that shortly after infection it causes formation of large syncytia. Formation of these syncytia, the result of large scale cell fusion, requires the presence of the F-, the G- and the SH-protein. (Heminway, B.R. et al., Virology 200: 801-805 (1994). Co-expression of both the F- and SH-gene, or of both the F- and G-gene gives only very low level cell-fusion (Pastey, M.K. and Samal, S.K., J. Gen. Virol. 78: 1885-1889 (1997)).
20

The fusion (F) protein is responsible for fusion of the viral and cellular membranes. Fusion of the virus and the cell membranes is thought to occur at the surface of the cell. It is necessary for penetration of the cell by the virus and to transfer the viral ribonucleoprotein into the cell cytoplasm. The F protein mediates penetration, but also promotes syncitia formation (fusion
25 of infected cell membranes with those of adjacent cells).
The F glycoprotein is synthesized as an F_0 precursor, which is cleaved post-translationally by furin-like proteases during transport to the cell surface. Cleavage at two distinct sites (prior Gly_{110} and after Arg_{136}) by the furin-like proteases is required for generation of fusion active molecules (Zimmer et al., J. Biol. Chem. 276;Gonzáles-Reyes et al., PNAS 98). The
30 resulting subunits,F1 and F2, remain linked by disulphide bonds.
This means that an 27aa peptide (Gly 110 to Arg136), the intrafurincleavagesitepeptide (IFCSP), is excised after cleavage.

It has now been found that the 27AA IFCSP is not essential for cell fusion activity of the F
35 protein and that deletion or replacement of at least part of the IFCSP does not destroy F function for productive BRSV replication.

Removal of Gly110 to Arg136 and transient expression of the resulting protein (F_{ins}) resulted in formation of syncitia which were slightly smaller that those induced by wt F.

Thus, it has been found that oligopeptides, or bioactive proteins, can be integrated between 2 furin cleavage sites of a carrier glycoprotein, such as the furin cleavage sites of an F protein of an RSV. Due to the processing of the precursor protein, resulting in the cleavage at the furin cleavage sites, the heterologous sequence may be excised and consequently be secreted by a virus-infected cell. This concept can be applied likewise to other glycoproteins containing two furin cleavage sites, where the original sequence between the cleavage sites may be replaced (in part) by a heterologous sequence or may simply be deleted.
In proteins with one furin cleavage site, heterologous sequences may be inserted together with the a sequence for a second cleavage site, thus creating a situation where the heterologous sequence is again flanked by two furin cleavage sites in the recombinant protein. Processing of the mutated protein will than also result in the excision of the heterologous sequence which may be secreted by virus infected cells.
Other viruses with glycoproteins that may be manipulated in such a way are for example bovine herpesvirus 1 or e.g. Newcastle disease virus or ILTV.
Viruses wherein glycoproteins are modified in one of the above-described ways, can be used for, for example vaccine purposes.
The mutations between the furin binding sites may result in an attenuated virus, or may be used to introduce a positive marker into a vaccine or for co-expression of other heterologous sequences.

Thus, the present invention relates to a virus having a glycoprotein with one or more furin cleavage sites, characterised in that nucleic acid sequence encoding the glycoprotein is mutated in such a way that either at least part of the naturally occurring sequence between the codons encoding two furin cleavage sites has been deleted, and/or a heterologous sequence has been inserted, wherein, if the heterologous sequence is inserted next to a furin binding site, the inserted sequence contains the heterologous sequence linked to the sequence of another furin cleavage site.

With a “heterologous sequence” a sequence is meant that does not naturally occur in this location in the viral genome (nucleic acid sequence) or in the sequence of the glycoprotein encoded thereby (amino acid sequence).

In a preferred embodiment the present invention relates to a Respiratory Syncytial Virus, preferably BRSV characterized in that at least part of the IFCSP has been deleted or

replaced by a heterologous amino acid sequence or a heterologous amino acid sequence has been inserted.
Preferably a deletion is made in the IFCSP and most preferably all of the IFCSP has been deleted.
5 The BRSV can have a deletion only, or, in the alternative, the IFCSP has been deleted and replaced by heterologous sequences.
The mutated BRSV according to the invention can be used as attenuated live vaccines. Heterologous sequences, when inserted in the IFCSP locus may encode biological important peptides, parts of proteins, or complete heterologous proteins.
10 The BRSV of the present invention may thus be used in vaccines for the protection of a host against BRSV infection.

The recombinant or mutated BRSV according to the invention can be produced by mutating the gene sequence encoding the F protein.
15

Mutations can be introduced by means of standard recombinant DNA technology. Standard recombinant DNA techniques such as making cDNA, cloning of cDNA in a plasmid, digestion of the gene with a restriction enzyme, followed by endonuclease treatment, re-ligation and homologous recombination in the host strain, are all known in the art and described i.a. in
20 Maniatis/Sambrook (Sambrook, J. *et al.* Molecular cloning: a laboratory manual. ISBN 0-87969-309-6). Site-directed mutations can e.g. be made by means of in vitro site directed mutagenesis using the Transformer® kit sold by Clontech. The full genome of a typical BRSV strain (strain ATue51908, as deposited in the GenBank database under accession no. AF092942), has been sequenced by Buchholz, U.J., S. Finke, and K.-K.Conzelmann. (J.
25 Virol. 73: 251-259 (1999)).

Methods for the preparation of non-segmented negative stranded RNA viruses have been described in EP 0702085.
Methods for the preparation of recombinant BRSV, starting from plasmids harboring the
30 various BRSV-genes as e.g. cDNA copies have been published i.a. by Collins et al., in Proc. Natl. Acad. Sci 92: 11563-11567 (1995). Mutations in the genes encoded by those plasmids can easily be made as discussed above.

RSV mutants according to the present invention are very suitable as carriers for
35 heterologous RNA sequences. This heterologous RNA can be inserted in the region that was deleted from the F -gene. A heterologous RNA sequence is a sequence that originates from a source, other than the parental (B)RSV strain. It may be derived from the DNA of another organism or may be synthetically made.

In an even more preferred form, the heterologous RNA sequence codes for a polypeptide. The heterologous RNA sequence may contain promotor sequences such that expression is under control of these sequences. These sequences may be the promotor sequences that are found to be linked to the heterologous gene coding for the polypeptide, in its native form, or it may be other promotor sequences suitable for expression in eukaryotic cells. It is obvious to those skilled in the art that the choice of a promotor extends to any eukaryotic, prokaryotic, viral or synthetically prepared promotor capable of directing gene transcription in cells infected by the BRSV mutant. Such promotors may be HRSV or BRSV promotors, but also promotors obtained from other RNA viruses, e.g. other Paramyxoviruses are suitable.

In a still even more preferred form, the heterologous RNA sequence encodes an antigen of another mammalian pathogen, which is able to elicit a protective immune response, whereby the antigen is expressed by the BRSV mutant according to the invention upon replication in the host cell. This has the advantage that a mammal can be immunized against two or more diseases: BRSV and another disease

In the most preferred form of this embodiment, the heterologous gene is selected from the group of cattle pathogens, for example, Bovine Rotavirus, Bovine Viral Diarrhoea virus, Parainfluenza type 3 virus, , Bovine Herpesvirus, Foot and Mouth Disease virus and *Pasteurella haemolytica*. Those genes of the viruses and bacteria mentioned that are involved in triggering an immunological response are known in the art.

Furthermore, the heterologous RNA sequence may encode a cytokine. Several cytokines, e.g. interferons are known to play an important role as immune modulators. Thus it may be advantageous to include genetic information for this kind of molecule into said section.

A virus mutant according to the present invention, and in particular a live BRSV, optionally expressing one or more different heterologous polypeptides of specific pathogens can, when it has an attenuated character, be used to vaccinate mammals. Vaccination with such a live vaccine or live vector vaccine is followed by replication of the BRSV mutant within the inoculated host, expressing in vivo the BRSV polypeptides, along with heterologous polypeptides if the encoding genes are inserted. The polypeptides expressed in the inoculated host will then elicit an immune response against both BRSV and the specific pathogen. If the heterologous polypeptide derived from the specific pathogen can stimulate a protective immune response, then the mammal inoculated with the BRSV mutant according to the invention will be immune to subsequent infection by that pathogen as well as to infection by BRSV. Thus, a heterologous nucleic acid sequence incorporated into the region

of F-gene where the IFCSP has been deleted in the RSV according to the invention may be expressed in vivo during several replication cycles, providing a solid, safe and long-lasting immunity to the pathogen from which it was derived.
Therefore, another embodiment of the invention relates to vaccines for the protection of mammals against Bovine Respiratory Syncytial virus infection. Such vaccines comprise a Bovine Respiratory Syncytial virus according to the invention and a pharmaceutically acceptable carrier.

A mutant virus according to the invention containing and expressing one or more different heterologous polypeptides can serve as a multivalent vaccine.
For the preparation of a vaccine the BRSV mutant according to the present invention can be grown on susceptible cells, e.g. on a cell culture of bovine origin. The viruses thus grown can be harvested by collecting the tissue cell culture fluids and/or cells. The live vaccine may be prepared in the form of a suspension or may be lyophilized.

The viruses according to the invention may also be inactivated and, for example, be used in a vaccine in inactivated form together with a suitable adjuvant.

In addition to an immunogenically effective amount of the viral mutant according to the invention the vaccine comprises a pharmaceutically acceptable carrier or diluent. Examples of pharmaceutically acceptable carriers or diluents useful in the present invention include e.g. materials as simple as sterile water or physiological salt solution. Also, stabilizers such as SPGA, carbohydrates (e.g. sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein containing agents such as bovine serum or skimmed milk, plant hydrolysates and buffers (e.g. phosphate buffer).

Optionally, one or more compounds having adjuvant activity may be added to the vaccine. Suitable adjuvants are for example aluminum hydroxide, phosphate or oxide, oil-emulsions e.g. of Bayol F(R) or Marcol 52(R), saponins or vitamin-E solubilisate. These adjuvants have the advantage that they help to stimulate the immune system in a non-specific way, thus enhancing the immune response to the vaccine.
Thus in a preferred form of this embodiment, the vaccine comprises an adjuvant.
The useful effective amount to be administered will vary depending on the age and weight of the animal, the mode of administration and type of pathogen against which vaccination is sought. Nevertheless, since the live attenuated viruses according to the invention are self-propagating, the amount of virus initially administered is not critical. A suitable dosage can be for example about $10^{3.0}$ - $10^{7.0}$ pfu/mammal.

For administration to mammals, the virus mutant according to the invention can be given inter alia intranasally, intradermally, subcutaneously or intramuscularly.

There are several ways to store both inactivated and live organisms. Storage in a refrigerator is e.g. a well-known method. Also often used is storage at -70°C. Viruses can also be kept in liquid nitrogen. Freeze-drying is another way of conservation. Freeze-dried viruses can be stored and kept viable for many years. Freeze-drying can be done according to all well-known standard freeze-drying procedures. Optional beneficial additives, such as e.g. skimmed milk, trehalose, gelatin or bovine serum albumin can be added in the freeze-drying process. Therefore, in a more preferred embodiment, the vaccine according to the present invention is in a freeze-dried form.

Another embodiment of the present invention relates to methods for the preparation of a vaccine according to the invention. In a simple form, such methods comprise admixing a virus according to the invention and a pharmaceutically acceptable carrier or diluent. In more complex forms, such methods may comprise admixing adjuvants, freeze-drying and other preparations known in the art.

Brief description of the figures:

Figure 1: Construction of mutagenized BRSV F ORFs
Figure 2: Plaque sizes of recombinant BRSV
Figure 3: Growth curves of recombinant BRSVs
Figure 4: Penetration kinetics of recombinant BRSVs
Figure 5: Insertion of arbitrary choosen amino acid sequence and second furin cleavage site into gB of BHV-1.
Figure 6: Presence of the modified gB ORF in recombinant BHV-1/gBFu2.
Figure 7: Processing of gBFu2.

EXAMPLES

Example 1:
To analyse the importance of the 27 aa peptide excised from F_0 after cleavage, we mutagenized a synthetic BRSV F ORF which enables expression of F via the nucleus. Removal of Gly110 to Arg 136 and transient expression of the resulting F_{ins} protein resulted in formation of syncitia which were slightly smaller than those induced by wt F demonstrating that the IFCSP, although beneficial, is dispensable for F-induced cell-cell fusion.

Example 2:
To investigate whether the amino acid sequence between the furin cleavage sites effects cell fusion activity of F, codons for arbitrary designed oligopeptides Pat (ProAlaThreArgIleCysIleAla IleSerThrAsnGluThrThr), Kat
5 (LysAlaThrArgIleAsnIleSerThrProGluArgPheGluLysThr) and Vet (IleAsnThrGluArgValGluThrSerValAlaCysCysIleAsnGlu) were inserted between the codons for Gly 110 and Lys 131.
Transient expression of F_{Pat}, F_{Kat}, F_{Vet} revealed that F_{Kat} and F_{Vet} induced syncytia formation whereas in cultures expressing F_{Pat} only single cells stained positive by indirect
10 immunofluorescence.

Example 3:
To test whether entire proteins can be integrated, the ORFs encoding bovine interleukins 2 and 4 and bovine interferon gamma were integrated.
15 Secretion of the interleukins was demonstrated by radioimmunoprecipipitation, presence of biologically active boIFNgamma by inhibition of VSV plaque formation. Only F_{boIL4} induced cell-cell fusion whereas expression of F_{boIL2} and $F_{boIFNgamma}$ resulted in only single F-positive cells.

20 **Material and Methods for examples 1-3:**

Construction of mutagenized open reading frames Fins-F, Fins-Y, Fkat, Fpat and Fvet.

For introduction of the envisaged mutations into the BRSV-F encoding open reading frame
25 (ORF) contained in plasmid pspFsyn between the SmaI and AvrII cleavage sites, the respective DNA fragments were generated by hybridization of synthetic oligonuclotides (Fig. 1). All cloning steps were done according to established procedures (Fritsch, Sambrook etc). Correct mutagenesis was verified by sequencing.

30 *Construction of F ORFs containing the open reading frames for FboIL2 and FboIL4.*

For introduction of the bovine interleukin 2 and 4 ORFs into the BRSV-F encoding open reading frame (ORF) contained in plasmid pspFsyn between the SmaI and AvrII cleavage sites, the respective DNA fragments were generated by PCR with primers as listed below:
35

PCR primers for amplification of bovine interleukins 2 and 4
Il2 + primer taaggcgcccgggcgaagcgcggcgcacctacttcaagctctacg
Il2 - primer taagagctcctaggaagcggcgcttgcgcttcttgccagtcattgttgagtagatgc
Il4 + primer taaggcgcccgggcgaagcgcggcggacacaagtgtgatattacc
40 Il4 - primer taagagctcctaggaagcggcgcttgcgcttcttgccacacttggagtatttctccttc

PCR was performed using plasmids promeIL2 and promeIL4 (Kühnle et al., Journal Gen.Virol,, 77,2231-2240,1996), respectively. All cloning steps were done according to established procedures (Fritsch, Sambrook etc). Correct mutagenesis was verified by sequencing.

5

Cloning of expression plasmids

For transient expression of wildtype and mutagenized BRSV F, the respective ORFs were isolated after cleavage of the plasmids with BglII and integrated into plasmid prome

10 downstream from the murine cytomegalovirus early 1 promoter (Kühnle et al. Journal Gen.Virol,, 77,2231-2240,1996).

Integration of the F ORFs into rBRSV.

15 The F ORFs were isolated after cleavage of the respective pspF plasmids with NcoI and EcoRI and integrated into NcoI and EcoRI-cleaved plasmid pGsGe to provide BRSV-specific gene start and gene end sequences (Buchholz et al.) The resulting plasmids were cleaved with ClaI and SphI and the F expression cassettes were used to replace the respective fragment in pBRSV.

20

Transfection experiments and recovery of recombinant viruses

BSR T7/5 cells (U. Buchholz et al. J. Virol) stably expressing T7 RNA polymerase were grown overnight to 80% confluency in 32 mm-diameter dishes in Dulbecco's modified

25 Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were transfected with a plasmid mixture containing 10µg of full length plasmid (p rBRSV), 4µg of pN, 2µg of pM2, and 2µg of pL. Transfection experiments were carried out with a mammalian transfection kit ($CaPO_4$ transfection protocol, Stratagene). Five days after transfection cells were split at a ratio of 1:3. Until a total cpe was observed (d21-28 post

30 transfection) cultures were split every 4-5 days. Virus was released by freezing and thawing. Cellular debris was removed by pelleting at 800×g. Supernatants were used for production of high-titre virus stocks in Madin-Darby bovine kidney (MDBK) cells. The medium was adjusted to 100 mM $MgSO_4$ and 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 7,5), and the highly cell-associated virus was released by freezing and thawing.

35 This material from the first MDBK passage was used for further experiments.

Cells and viruses

Madin-Darby bovine kidney (MDBK) cells were grown in DMEM supplemented with 5% FCS, 100 units of penicillin per ml, 100µg of streptomycin per ml, and 2.4 mM L-glutamine. Stock titrations of serially diluted samples were carried out under plaque conditions. MDBK cells were grown in 15 mm-diameter dishes under semi-solid medium overlay. Foci of infected cells were counted on d6 post infection (p.i.).

1. Plaque diameters

MDBK cells were infected with approximately 100 plaque forming units (pfu) and overlaid with semi-solid medium containing 0.75% methylcellulose. After a 6 days incubation the cultures were fixed with 3% paraformaldehyde in phosphate buffered saline without Mg^{2+} and Ca^{2+} (PBS) and 0.2% Triton X-100. Cells were sequentially incubated with the appropriate dilutions of mab anti-RSV F clone 18B2 (Institut Pourquier, Montpellier, Fr.), peroxidase (POD) conjugated goat anti-mouse immunoglobulin G (Ammersham) and 2mM 3-amino-9-ethyl-carbazole (AEC) in 50 mM sodium acetate (pH 5.0, Sigma) with 0.03% H_2O_2. Plaque diameters were measured microscopically and indicated as mean values of at least 50 plaques.

Kinetics of viral spread were monitored according to the same protocol. Cultures were fixed at the various times indicated.

2. Multiple-step growth kinetics

MDBK cells were infected with rBRSV at a multiplicity of infectivity (moi) of 0.04. After 2h of incubation at 37°C inocula were removed, the cultures were rinsed twice with PBS, and supplemented with cell culture medium. At the times indicated the supernatants were collected respectively the entire cultures were frozen at –70 °C. Infectivity was determined by titration of serial dilutions on MDBK cells . Plaques were counted after a 6 days incubation under semi-solid medium overlay.

3. Penetration kinetics

Prechilled MDBK cells were incubated with approximately 300 pfu of rBRSV, Fins-F BRSV, for 2h at 4 °C. Non-adherent virus was removed by thoroughly rinsing with icecold PBS. After shifting the incubation temperature from 4 °C to 37 °C penetration was terminated at the times indicated. Cell-associated extracellular virus was inactivated through low pH with citric buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0). Plaques were counted after 6d of incubation under semi-solid medium. The plaque counts of untreated control cultures were defined as 100% penetration.

4. Transient expression analyses

Bovine pharyngeal cells (KOP-R) were grown overnight in 3,2 cm-diameter plastic dishes to 80% confluence. Transient transfection experiments were performed with 2 µg of the BRSV F expression plasmids with 0,5 µg of the transactivator plasmid pAMB 25, encoding the 89 K MCMVie phosphoprotein (Koszinowski et al., J.Virol, 58, 59-66,1986). Superfect® transfection reagent was added according to the manufacturer's protocol (Qiagen, Hilden,

Germany). After 24 h the transfection medium was replaced by cell culture medium. 36-48 h *post transfectionem* cells were fixed with 3% paraformaldehyde in phosphate buffered saline (PBS) and permeabilized with 0,2% Triton X-100. The cultures were sequentially incubated with appropriate diluted mab anti-RSV F clone 18B2 (Institut Pourquier) and with DTAF
5 (5-4,6-dichlorotrazin-2yl aminofluorescein)-conjugated goat anti-mouse immunoglobulin G (Dianova, Hamburg, Germany).

Example 4

To demonstrate that in principle a second furin cleavage site (FCS2) can be introduced into
10 a glycoprotein, which is only cleaved once by furin in its wild type form, and to show that the intervening peptide is excised during processing, the codons for 15 arbitrary chosen amino acids and a furin cleavage motif were integrated adjacent to the sequence encoding the original furin cleavage site (FCS1) within the gene encoding the essential glycoprotein B (gB) of bovine herpesvirus 1 (BHV-1) (Figure 5). This nucleotide sequence contained a XbaI
15 recognition site not present in wild type gB to enable diagnostic restriction enzyme analysis. (The resulting ORF will be referred to as gBFu2 ORF.)

To analyse whether the modification of the gB amino acid sequence interferes with the function of gB, the gBFu2 ORF was used to rescue a gB-negative mutant of the laboratory
20 strain BHV-1/Horst, by cotransfecting purified viral DNA and plasmid pgBFu2 DNA into bovine pharyngeal cell line KOP/R. A single plaque isolate from the infectious progeny virus (referred to as BHV-1/gBFu2) was used for further characterization.

Diagnostic cleavage of purified viral DNA and Southern blot hybridization showed that only
25 the fragment containing the gB gene of BHV-1/gBFu2, but not from the wild type BHV-1 strain Schönböken was cleaved by XbaI, which verified presence of the modification within the gB ORF of the recombinant. The results are depicted in figure 6: Purified viral DNA of wild type BHV-1 strain Schönböken (lanes 1,2,5 and 6) and recombinant BHV-1/gBFu2 (lanes 3, 4, 7 and 8) was cleaved with BamHi (lanes 1,3,5 and 7) or with BamH1 and XbaI
30 (lanes 2,4,6 and 8). After size separation on agarose gels and photography of the ethidium bromide stained gel (lanes 1 to 4), fragments were transferred to nitrocellulose filters and hybridised to ^{32}P-labelled DNA from the BHV-1 gB gene. Bound radioactivity was visualised by autoradiography (lanes 5-8). The closed arrow indicates the BamHI fragment that contains the gB gene which is cleaved by XbaI only in DNA from BHV-1/gBFu2 (open
35 arrows).

The influence of the intervening peptide and the second furin cleavage site on protein processing was studied by immunoprecipitation of ^{35}S-labelled gB from cells infected with or

without brefeldin A. Brefeldin A destroys the trans Golgi network and thus inhibits cleavage of glycoproteins by furin. Comparison of the mobilities of the immunoprecipitates showed that the gBFu2 precursor, as expected, exhibited a slightly higher apparent molecular weight than wild type uncleaved gB precursor molecules (pgB) whereas the fully processed and 72K and 55K subunits appeared identical in size, indicating that pgBFu2 was cleaved at both furin sites resulting in removal of the intervening peptide. The results are depicted in Figure 7; The ^{35}S-labelled proteins from cells infected with wild type BHV-1 (lanes 1 and 3) or recombinant BHV-1/gBFu2 (lanes 2 and 4) in presence (lanes 3 and 4) or without (lanes 1 and 2) brefeldin A were immnoprecipitated with a rabbit anti-gB serum and proteins were visualized by autoradiography after SDS-PAGE. The position of the pgB and the 72K and 55K cleavage products are indicated.

Determination of the cell culture properties of BHV-1/gBFu2 demonstrated that the function of gB for direct spreading was not inhibited by the mutation whereas penetration of BHV-1/gBFu2 was slightly impared.

Material and Methods for Example 4.

Construction of mutagenized open reading ferame gB/Fu2.
For introduction of the 15 codons for the abitrarily chosen peptide sequence and the 5 codons for the second furin cleavage site into the BHV-1 gB open reading frame a first DNA fragment was generated by PCR using oligonucleotides gbfu2-1+ and gbfu2-1- which was then used as template in a second PCR together with oligonucleotides gbfu2-2+ and gbfu2-2-. The resulting DNA fragment

TAAGAATTC**GGGCCC**GCGACGTGCGCGCCGAGGCGCCAAGCGCGAGGCGATAGTCAA
GGCTGACTCTAGAGAGCTCAAGCGCAAGCGCCGCGCCGCGCCGTCTGCGCCCGGCG
GACCGGGCGCGGCCAAC**GGGCCC**AAGCTTACT (cleavage sites for *Apa*I are in bold)

was cleaved with *Apa*I and used to replace the corresponding fragment (nucleotides 1491 to 1557 in Whitbeck et al., 1988, Journal of Virology 62, 3319 – 3327) in plasmid pgB containing the wild type gB gene.
The resulting plasmid was named pgB/Fu2. PCR and cloning were done according to established procedures (Fritsch, Sambrook etc.).

PCR oligonucleotides:
gbfu2-1+: gaggcgccaagcgcgaggcgatagtcaaggctgactctagagagctcaag
gbfu2-2+: taagaattcgggcccgcgacgtgcgcgccgaggcgccaagcgagcggcg

gbfu2-1-: cgccgggcgcagacggcgcggcgcggcgcttgcgcttgagctctctagag
gbfu2.2-: agtaagcttgggcccgttggccgcgcccggtccgccgggcgcagacggcg

Generation and characterization of recombinant BHV-1/gBFu2.

5 KOP-R cells were cotransfected with 1 µg purified gB-negative BHV-1 DNA and 5 µg DNA of pgB/Fu2. Infectious progeny from the transfection was titrated. Virus from single plaques were plaque purified and one isolate named BHV-1/gBFu2 was further characterized by Southern blotting of purified viral DNA cleaved with BamHI and BamHI/XbaI using a ^{32}P-labelled probe from the BHV-1 gB-gene.

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Transfection, plaque purification, virus DNA isolation and Southern blotting was performed as described previously (Schmitt and Keil, 1996, Journal of Virology 70, 1091 – 1099).

15 *Immunoprecipitation.*

Immunoprecipitation of ^{35}S-labelled proteins from BHV-1 infected cells using a polyclonal anti-gB serum was done as described (Fehler et al., 1992, Journal of Virology 66, 831-839). To inhibit transport of gB into the trans Golgi network brefeldin A was added to a final concentration of 5µg/ per ml.

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Determination of the cell culture properties.

Determination of penetration kinetics and direct spreading of BHV-1/gBFu2 was done as described (Fehler et al., 1992, Journal of Virology 66, 831-839, Schröder and Keil, 1999, Journal of General Virology 80, 57 – 61).

25

CLAIMS

1. A virus having a glycoprotein with one or more furin cleavage sites, characterised in that the gene sequence encoding the glycoprotein is mutated in such a way that either at least part of the naturally occurring sequence between the codons encoding two furin cleavage sites has been deleted, and/or a heterologous sequence has been inserted, wherein, if the heterologous sequence is inserted next to a furin binding site, the inserted sequence contains the heterologous sequence linked to the sequence of another furin cleavage site.

2. A virus according to claim 1, characterized in that the virus is a Bovine Respiratory Syncytial Virus wherein at least part of the IFCSP coding sequence has been deleted from the viral genome or has been replaced by a nucleic acid sequence encoding a heterologous amino acid sequence.

3. BRSV according to claim 3 wherein the complete IFCSP encoding sequence has been deleted.

4. Bovine Respiratory Syncytial Virus according to any of the preceding claims, characterized in that an heterologous RNA sequence has been inserted which encodes an antigen of another pathogen, said another pathogen being selected from the group of cattle pathogens, consisting of Bovine Rotavirus, Bovine Viral Diarrhea virus, Parainfluenza type 3 virus, Bovine Herpesvirus, Foot and Mouth Disease virus and *Pasteurella haemolytica,* and/or a cytokine.

5. Vaccine for the protection of cattle against BRSV-infection, said vaccine comprising a virus as described in claims 1-4 and a pharmaceutically acceptable carrier.

6. Vaccine according to claim 5, characterized in that it additionally comprises an adjuvant.

7. Vaccine according to claim 5 or 6, characterized in that it is in a freeze-dried form.

Fig. 1: Construction of mutagenized BRSV F ORFs

F ORF — SmaI AvrII

cc cgg gcg aag cgc ggc atc ccg gag ctg atc cac tac aag cgc aac agc acg aag aag ttc tac ggc ctg atg ggc aag aag cgc aag cgc cgc tt**c cta gg**

Arg Ala Lys Arg Gly_{110} … Lys Lys Arg Lys Arg Arg Phe_{127}

Oligonucleotides for:

Fins-F gggcgaagcgcttc and ctaggaagcgcttcgccc → **cc cgg** gcg aag cgc tt**c cta gg**

Arg Ala Lys Arg Phe_{127}

Fins-Y gggcgaagcgctac and ctaggtagcgcttcgccc → **cc cgg** gcg aag cgc ta**c cta gg**

Arg Ala Lys Arg Tyr_{127}

FKat gggcgaagcgcggcaaggccacgcgcatcaacatcagcacgcccgagcgcttcgagaagacgaagaagcgcaagcgccgcttc and ctaggaagcggcgcttgcgcttcttcgtcttctcga agcgctcgggcgtgctgatgttgatgcgcgtgccttgccgcgcttcgccc

cc cgg gcg aag cgc ggc aag gcc acg cgc atc aac atc agc acg ccc gag cgc ttc gag aag acg aag aag cgc aag cgc cgc tt**c cta gg**

Arg Ala Lys Arg Gly Lys Ala Thr Arg Ile Asn Ile Ser Thr Pro Glu Arg Phe Glu Lys Thr **Lys Lys Arg Lys Arg Arg Phe**

Fig.: 1 continued

FPat gggcgaagcgcggccccgccacccgcatctgcatcgccatcagcaccaatgagaccacgaagaagcgcaagcgccgcttc and ctaggaagcggcgcttgcgcttcttcgtggtct
cattggtgctgatggcgatgcagatgcgggtggcggggccgcgcttcgccc

cc cgg gcg aag cgc ggc ccc gcc acc cgc atc tgc atc gcc atc agc acc aat gag acc acg aag aag cgc aag cgc cgc tt**c cta gg**
Arg Ala Lys Arg Gly Pro Ala Thr Arg Ile Cys Ile Ala Ile Ser Thr Asn Glu Thr Thr **Lys Lys Arg Lys Arg Arg Phe**

FVet gggcgaagcgcggcatcaacaccgagcgcgtcgagaccagcgtggcctgctgtatcaacgagaagaagcgcaagcgccgcttc and ctaggaagcggcgcttgcgcttcttctcgtt
gatacagcaggccacgctggtctcgacgcgctcggtgttgatgccgcgcttcgccc

cc cgg gcg aag cgc ggc atc aac acc gag cgc gtc gag acc agc gtg gcc tgc tgt atc aac gag aag aag cgc aag cgc cgc tt**c cta gg**
Arg Ala Lys Arg Gly Ile Asn Thr Glu Arg Val Glu Thr Ser Val Ala Cys Cys Ile Asn Glu **Lys Lys Arg Lys Arg Arg Phe**

Fig. 2: Plaque size determination

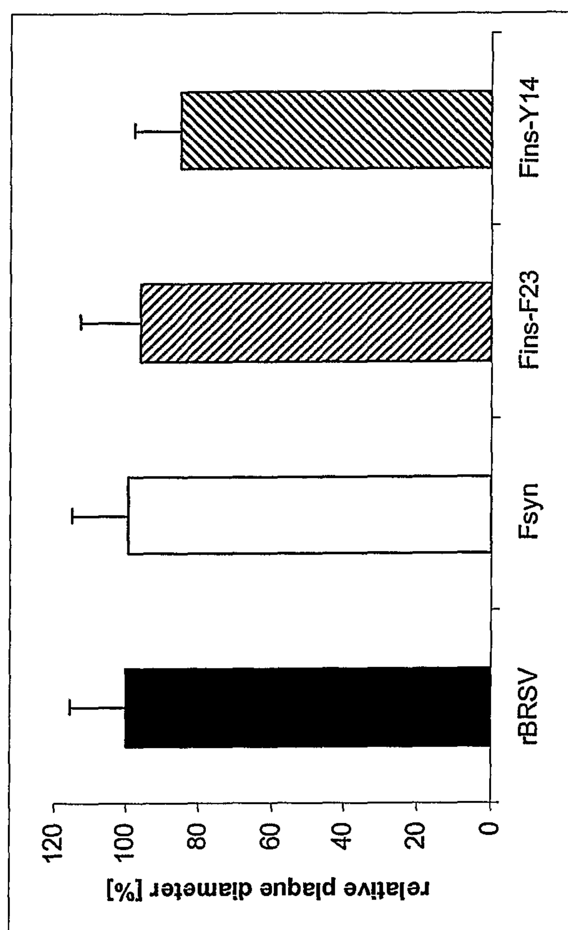


Fig. 3: Growth curves

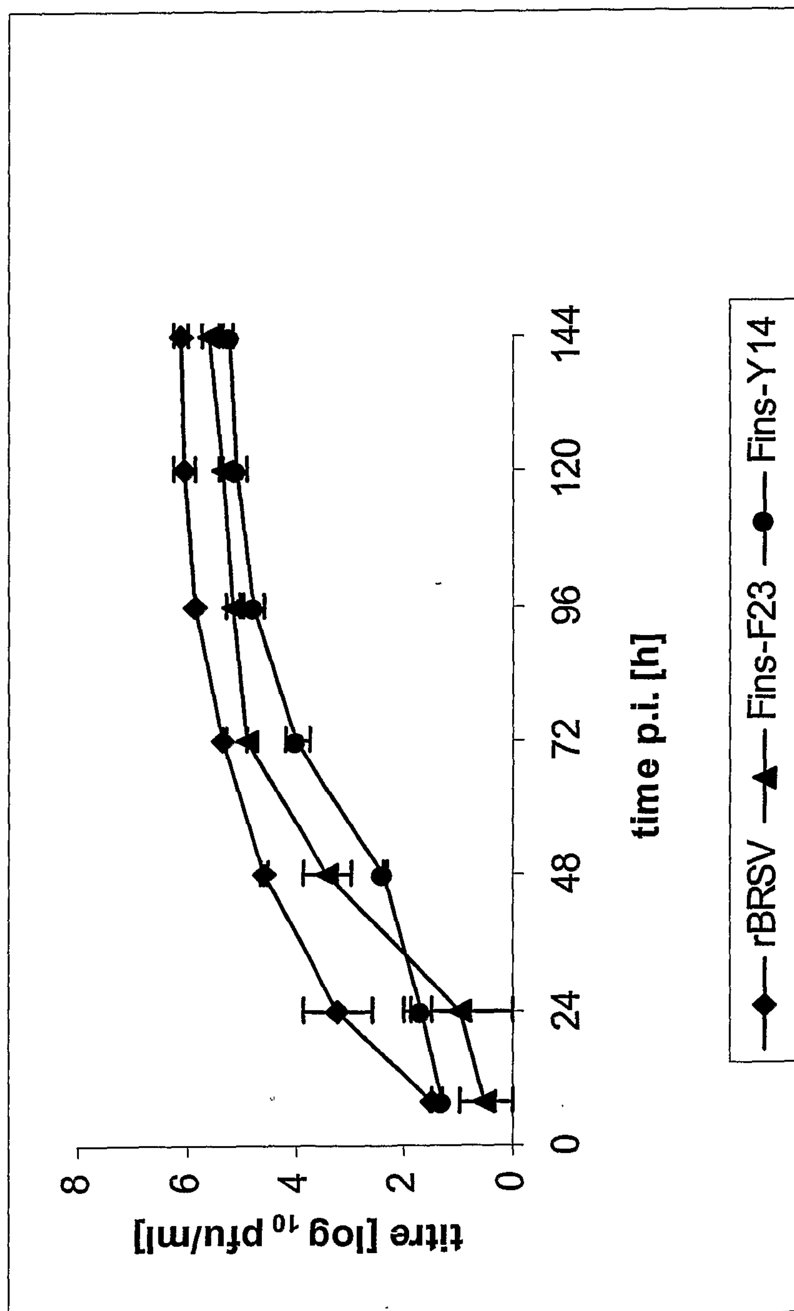


Fig. 4: Penetration kinetics

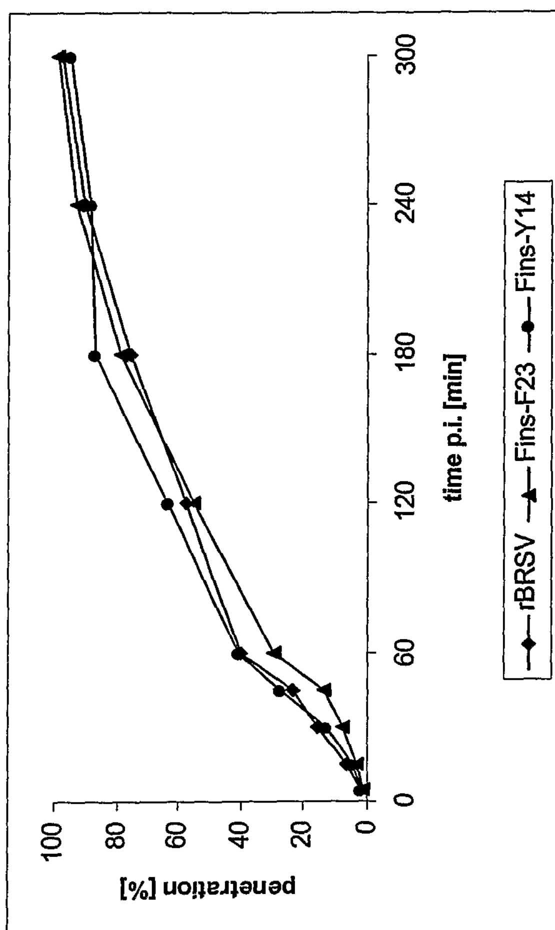
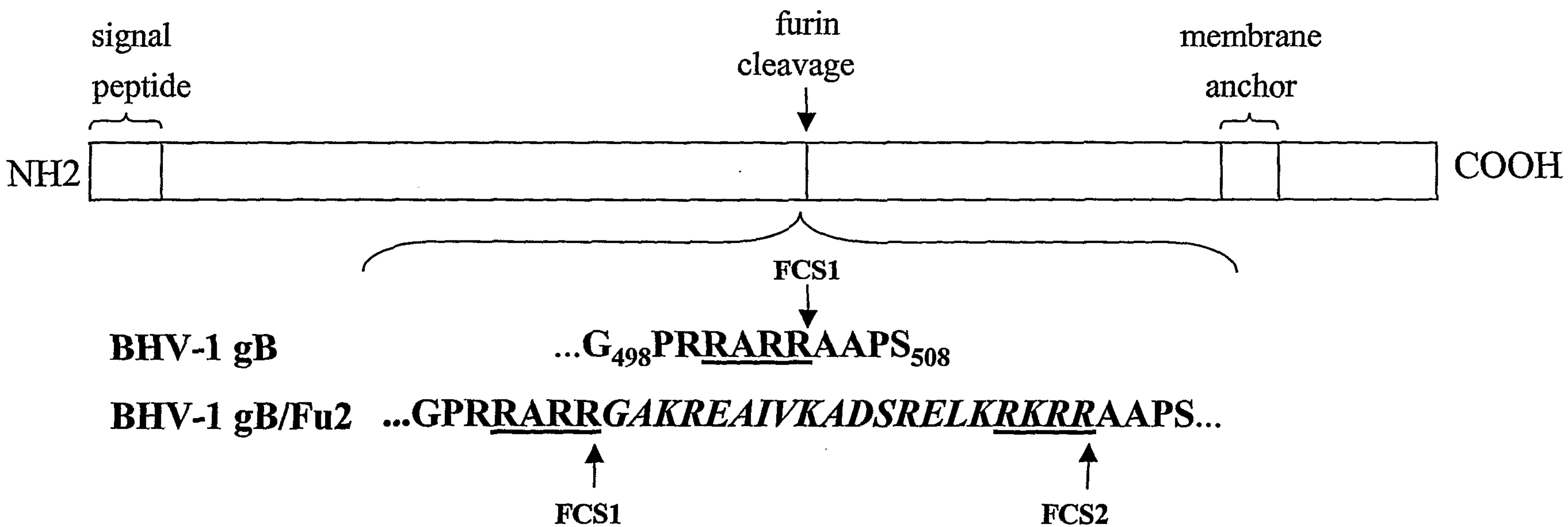


Figure 5 . Insertion of an arbitrarily chosen amino acid sequence and a second furin cleavage site into glycoprotein B of BHV-1



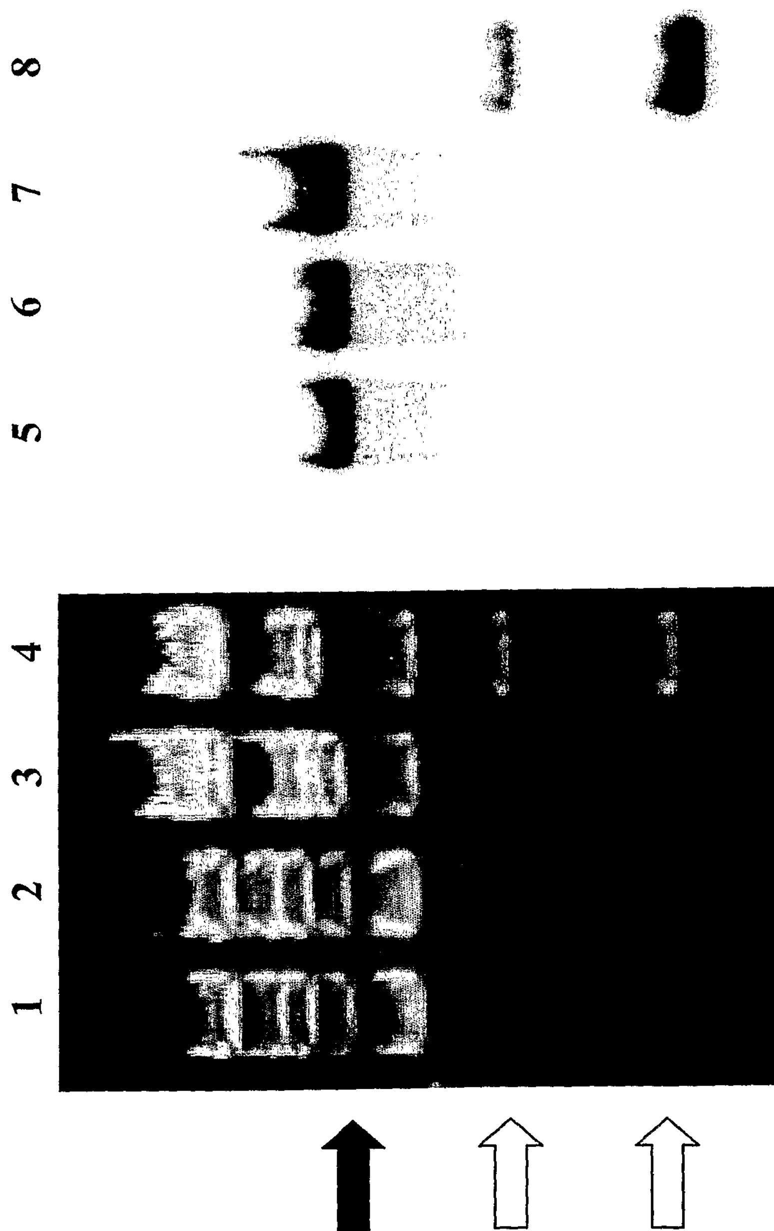
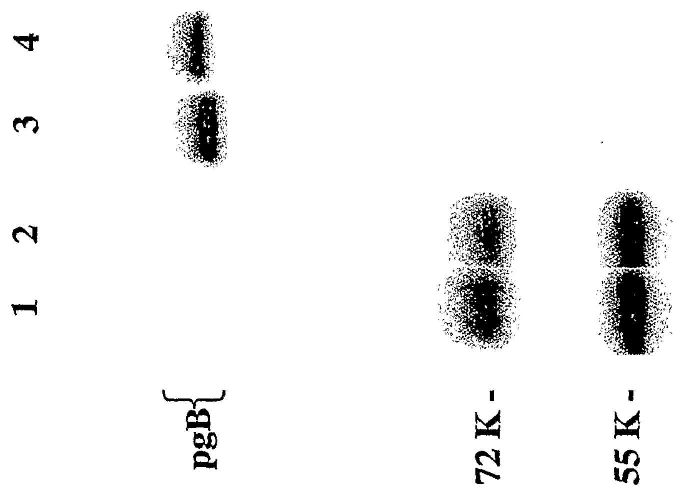


Figure 6. Presence of the modified gB open reading frame in recombinant BHV-1/gBFu2

Figure 7. Processing of gBFu2



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/02520

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N7/00 A61K35/76 A61K39/155 C12N15/62 C12P21/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, MEDLINE, EMBASE, EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	ZIMMER GERT ET AL: "Cleavage at the furin consensus sequence RAR/KR109 and presence of the intervening peptide of the respiratory syncytial virus fusion protein are dispensable for virus replication in cell culture." JOURNAL OF VIROLOGY, vol. 76, no. 18, September 2002 (2002-09), pages 9218-9224, XP009002025 September, 2002 ISSN: 0022-538X the whole document --- -/--	1-7

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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30 April 2003	09/05/2003

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/02520

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	SINGH JASBIR ET AL: "Characterization of a panel of insertion mutants in human cytomegalovirus glycoprotein B." JOURNAL OF VIROLOGY, vol. 74, no. 3, February 2000 (2000-02), pages 1383-1392, XP001118928 ISSN: 0022-538X page 1383, right-hand column, paragraph 1	1
X	ZIMMER GERT ET AL: "Proteolytic activation of respiratory syncytial virus fusion protein: Cleavage at two furin consensus sequences." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 276, no. 34, 24 August 2001 (2001-08-24), pages 31642-31650, XP002225142 ISSN: 0021-9258 page 31643, paragraph 3	1
A	the whole document	2-7
X	BOLT GERT ET AL: "Cleavage of the respiratory syncytial virus fusion protein is required for its surface expression: Role of furin." VIRUS RESEARCH, vol. 68, no. 1, June 2000 (2000-06), pages 25-33, XP001118927 ISSN: 0168-1702 page 31, left-hand column, paragraph 1	1
A	the whole document	2-7
A	SUGRUE RICHARD J ET AL: "Furin cleavage of the respiratory syncytial virus fusion protein is not a requirement for its transport to the surface of virus-infected cells." JOURNAL OF GENERAL VIROLOGY, vol. 82, no. 6, June 2001 (2001-06), pages 1375-1386, XP001120815 ISSN: 0022-1317 the whole document	1-7