

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
23 December 2004 (23.12.2004)

PCT

(10) International Publication Number
WO 2004/111249 A2

(51) International Patent Classification⁷: **C12N 15/86**

(21) International Application Number:
PCT/EP2004/006225

(22) International Filing Date: 9 June 2004 (09.06.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
03013509.9 13 June 2003 (13.06.2003) EP

(71) Applicant (for all designated States except US):
**POLYMUN SCIENTIFIC IMMUNBIOLOGISCHE
FORSCHUNG GMBH** [AT/AT]; Nussdorfer Lände 11,
A-1190 Vienna (AT).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **EGOROV, Andrej**
[RU/AT]; Leipziger Strasse 58/8, A-1200 Vienna (AU).

(74) Agent: **BÜCHEL, KAMINSKI & PARTNER**; Austrasse
79, FL-9490 Vaduz (LI).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

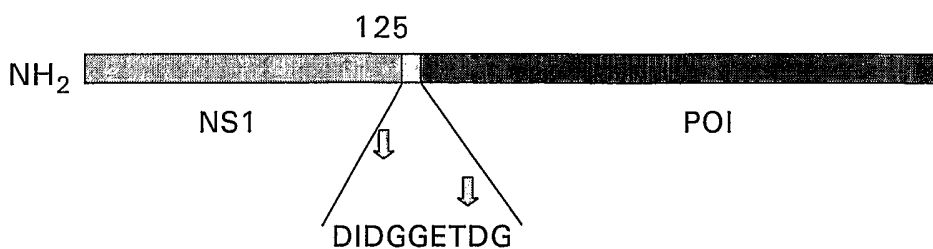
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC,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

Published:

— without international search report and to be republished upon receipt of that 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: PROTEIN CONSTRUCTS CONTAINING CASPASE RECOGNITION SITES



(57) Abstract: The present invention relates to a nucleotide sequence encoding a recombinant protein product wherein the nucleotide sequence contains at least one sequence that encodes one or more caspase recognition sites and that is located in between and links together a first part of the nucleotide sequence encoding a first part of the protein product and a second part of the nucleotide sequence encoding a second part of the protein product. The invention further relates to viral vectors and recombinant viruses containing said nucleotide sequence, as well as to vaccines, particularly attenuated live vaccines, and compositions comprising said vectors or viruses.

WO 2004/111249 A2

PROTEIN CONSTRUCTS CONTAINING CASPASE RECOGNITION SITES

FIELD OF INVENTION

5 The present invention is in the field of recombinant technology and viral vaccine production and relates to viral protein constructs and to recombinant viruses containing such constructs wherein the protein constructs contain caspase recognition sites for posttranslational
10 cleavage by caspase enzymes during infection cycles in a mammalian host or host cell. The invention further relates to a method of making such protein constructs and to their use, particularly as part of a vaccine.

15 TECHNICAL BACKGROUND

Influenza virus infection provokes the activation of caspase proteases and triggers subsequent apoptosis in infected cells (Alnemri et al., 1996). Active caspases can
20 cleave the proteins at the recognition sites which may consist of as little as a sequence of just 4 amino acids. For example, NP protein of human influenza viruses A and B was found to be cleaved in the infected cells at the amino acid sequences EXD/X and DXD/X, typical for caspase
25 proteases (Zhirnov et al., 1999).

The design of recombinant protein constructs intended for posttranslational cleavage usually requires the provision of one or more cleavage sites within the constructs in
30 order to allow for proteases to specifically cleave the amino acid sequences at these predetermined cleavage sites. Reference is made to Example 1 and Figures 2 and 3 of WO 01/64860, wherein viral vector systems are disclosed which encode a heterologous amino acid sequence comprising
35 a truncated NS1 protein of influenza A virus followed by

- 2 -

the 2A cleavage site of picornavirus, and further comprising one or more subsequent amino acid sequences of desired peptides or proteins, e.g. the Nef protein of HIV-1, the ELDKWA sequence of gp 41 of HIV-1, or an
5 interleukin, optionally preceded by a leader sequence, and optionally followed by an anchor sequence. It turned out, however, that the 2A cleavage site was not perfectly suitable for all heterologous constructs prepared by the present inventors so far. It was therefore an objective of
10 the present invention to overcome this drawback by providing an improved viral vector system that allows for superior cleavage and an increased yield of correctly cleaved protein products.

15 One of the advantages of using the influenza virus as viral vectors, in contrast to other systems such as adenoviral vectors or retroviruses, is that it does not contain a DNA intermediate and therefore is not able to integrate into the host's chromosomes. In addition, live
20 influenza virus vaccines have been developed and licensed for humans and could thus also be used for the expression of additional antigens.

In primary infected cells, one of the main strategies of
25 the influenza virus is to inhibit IFN α / β signaling to the neighboring cells, which can induce their antiviral state. In this context, the NS1 protein has been suggested to perform several important accessory functions for the effective replication of the virus in its host. First, by
30 the activity of its carboxy-terminal domain, the NS1 protein is able to inhibit the host mRNA's processing mechanisms. Second, it facilitates the preferential translation of viral mRNA by direct interaction with the cellular translation initiation factor eIF4GI. Third, by

- 3 -

binding to dsRNA and interaction with putative cellular kinase(s), the NS1 protein is able to prevent the activation of IFN-inducible dsRNA activated kinase (PKR), 2',5'-oligoadenylate synthetase system, and cytokine transcription factors such as NF- κ B or IRF 3 and c-Jun/ATF2. As a result thereof, the NS1 protein inhibits the expression of INF- α and INF- β genes, delays the development of apoptosis in the infected cells and prevents the formation of the antiviral state in the neighboring cells.

The function of the NS1 protein as an IFN- α/β antagonist was proven by the construction of several NS mutants, bearing C-terminal deletions which showed impaired growth properties in IFN competent cells such as MDCK, embryonated chicken eggs or mouse lungs, but had no attenuation effect in IFN deficient hosts such as Vero cells or PKR knock out mice. It was found, that an influenza NS mutant coding for just the first N-terminal 125 amino acids was only slightly attenuated in IFN competent systems, while mutants carrying shorter versions, respectively less than 80 aa of the NS1 protein, were dramatically attenuated in MDCK cells and mice.

When testing the ability of several influenza A NS1-deletion mutants to circumvent an artificial antiviral state of Vero cells due to external addition of human IFN- α , it was found that NS mutants encoding the N-terminal 125 amino acids of the NS1 protein but not mutants with an impaired RNA binding domain like delNS1 completely lacking the NS1 ORF, were fully resistant to the interferon action and were replicating as efficiently as the wild type virus under the same conditions. Thus, it could be demonstrated that the influenza virus is able to circumvent the

- 4 -

antiviral state of the interferon treated cells most likely by the activity of the NS1 protein RNA binding domain.

5 BRIEF DESCRIPTION OF THE INVENTION

Based on this finding, the present inventors established a reverse genetic system in Vero cells in which influenza virus NS transfectants could be obtained using human IFN- α as a selection drug against an IFN sensitive helper virus. The high efficiency of this system allowed to rescue a genetically stable influenza virus expressing a protein of interest (POI) such as, e.g. GFP, from the NS1 reading frame.

15 To achieve this goal, the present inventors generated influenza A viruses expressing a recombinant NS1 protein, containing caspase recognition sites inserted at or after amino acid position 125. This insertion provided a mechanism of post-translational cleavage of the NS1 protein into two parts at a late time point of infection. When general caspase inhibitor Z-VAD-FMK was added, cleavage was prevented.

25 It was further demonstrated by corresponding experiments that rescued viruses of H1N1 and H2N2 subtypes carrying such a recombinant NS gene were not restricted in growth on interferon deficient Vero cells whereas surprisingly, a temperature-sensitive (ts) phenotype was observed on MDCK cells. It was possible, however, to compensate this temperature sensitivity by treatment of infected cells with caspase 8 and 9 inhibitors. Moreover, both types of viruses were significantly attenuated in mice after intranasal immunization, resulting in at least a 10^3 -fold

- 5 -

reduction of viral titers in the lungs as compared to immunizations with the control virus comprising a 2A cleavage site instead of the caspase cleavage sites.

5 It is therefore an object of the invention to provide a method for the manufacture of recombinant peptides or proteins, particularly recombinant viral peptides or proteins, wherein the method comprises providing a suitable viral vector system comprising at least two amino
10 acid sequences linked by a protein recognition site, said protein recognition site being a caspase recognition site, preferably a combination of 2 or more identical or different caspase recognition sites, and expressing the recombinant protein or proteins, as the case may be, in a
15 suitable expression system, preferably a mammalian host cell, wherein during or after expression cleavage of the expressed protein products by one or more caspase enzymes present therein or added thereto may take place. According to the invention, each recognition site may typically
20 consist of no more than 4 amino acids.

It is another object of the present invention to provide viral vectors encoding one or more heterologous peptides or proteins, wherein the viral vector comprises nucleotide
25 sequences encoding a first peptide or protein of interest, followed by a nucleotide sequence encoding one or more caspase recognition sites, and a nucleotide sequence encoding a second peptide or protein of interest. The invention further relates to recombinant viruses
30 containing any such vector.

It is another object of the invention to provide a vaccine comprising a virus that contains a vector according to the present invention. The vaccines of the present invention
35 may be designed for application in the prophylaxis or

- 6 -

therapy of any viral infections by selecting suitable antigenic determinants of viral proteins, particularly envelope proteins, for insertion into the heterologous viral vector construct referred to above.

5

The present invention allows for the efficient manufacture of correctly cleaved peptides or proteins of interest as well as for the manufacture of attenuated live vaccines based on viruses containing recombinant viral vectors that
10 encode heterologous peptides or proteins. In a preferred embodiment, the present invention relates to a live vaccine based on attenuated, particularly temperature sensitive (ts), influenza A or B virus containing a vector system that encodes a truncated NS1 protein together with
15 caspase recognition sites and an antigenic determinant of an HIV-1 protein, e.g. the (L)ELDKWA(S) sequence of gp41 (SEQ ID NOs 2, 3, 4, 5) , or part of the Nef protein of HIV-1, or any other peptide or protein of interest from HIV or from any source other than HIV including but not
20 limited to hepatitis, tuberculosis, malaria or tumor antigens.

DETAILED DESCRIPTION OF THE INVENTION

25 In order to study caspase-dependent cleavage of an intracellular synthesized recombinant viral protein the present inventors generated influenza A and B viruses which express a recombinant NS1 protein (Fig.1) containing a pair of different caspase recognition sites (CRS), each
30 consisting of 4 amino acids and spaced apart by a glycine residue (G) in between the two sites, thus making up the aa sequence DIDGGETDG (SEQ ID NO 1). This sequence can be cleaved by caspase 3 and probably by other caspases and thus ensures postranslational cleavage of the foreign
35 sequence (i.e. peptide or protein of interest, POI) from

- 7 -

the N-terminal part of the NS1 protein. Previous constructs using 18 aa of the 2A cleavage site of picornavirus did not or not satisfactorily work in some of the POI expressing vectors.

5

Surprisingly, it was also found that recombinant influenza viruses containing caspase recognition site (CRS) DIDGGETDG (SEQ ID NO 1) appeared to be attenuated in mice after intranasal immunization. Indeed, while in vitro experiments using MDCK cells as host cells demonstrated temperature sensitivity of influenza virus containing CRS, the same virus constructs also showed very weak replication in the lungs of the experimental group of mice and, more specifically, were found to be attenuated in that group of mice by a factor of more than 100 as compared to the control group that was infected using recombinant influenza virus bearing a 2A cleavage site instead of a CRS.

20 It is concluded from the experimental results that by inserting caspase recognition sequences into influenza NS1 protein two goals have been achieved. First, attenuation of the virus in vitro and in vivo was achieved due to the impairment of the NS1 protein induced by activated caspases during the viral replication cycle. Second, more precise and efficient post-translational cleavage and separation of the foreign sequence or sequences that were inserted into the NS1 open reading frame (ORF), from the functional N-terminal part of the NS1 protein was achieved.

Notwithstanding the aforesaid, it shall be mentioned that previous attempts to rescue NS transfectants with inserts of more than 275 aa using plasmid only transfection systems frequently failed. Accordingly, in order to rescue

- 8 -

the GFP expressing vector it was necessary to improve the rescue system and to enhance its efficiency. This goal was achieved by applying co-transfection of additional plasmids expressing the proteins of the viral polymerase complex (RNP).

Using GFP as an example for insertion of a heterologous POI it was confirmed on one hand that the recombinant NS1-GFP virus containing caspase recognition sites was replicating to high titers and was genetically stable while passaging in Vero cells, generating 100% of fluorescent plaques. On the other hand, however, it was found that the virus, although growing and expressing GFP in IFN competent cells, was losing GFP activity by generating various deletions within the recombinant NS gene. It may be concluded therefrom that the IFN antagonizing function of the NS1-GFP virus was partially compromised although the virus had an intact N-terminal part of the NS1 protein which efficiently released from the GFP molecule by caspase cleavage.

While the NS1-GFP virus was growing in IFN competent cells it did not or only poorly replicate in wild type C57/bl mice, much like the delNS1 virus which completely lacks the NS1 ORF. However, when substituting PKR knock out mice for the wild type mice replication of NS1-GFP virus in the lungs proceeded up to a titer of equal or more than 10^4 PFU/g of lung tissue, as was also the case with delNS1 virus. Moreover, even after two days of replication in mouse lungs the virus did not lose its capacity to express GFP.

Various reasons may be considered when trying to explain this attenuating effect in vivo. In the present case, the GFP insert may have some negative influence on the

- 9 -

formation of the NS1 dimers, making NS1 function to be less efficient and therefore promoting the activation of PKR.

5 Using the method of the present invention it was unambiguously demonstrated that the influenza virus can be engineered in such a way that foreign sequences which are even longer than the NS1 protein itself can be expressed from the NS1 reading frame and that such recombinant
10 viruses can be successfully maintained and propagated to high titers in IFN deficient substrates such as Vero cells.

This new approach could be a promising strategy for the
15 generation of live antiviral, preferably influenza virus-based, vaccines and vectors, and most preferably using NS1 truncation technology as disclosed, e.g., in WO 01/64860, wherein NS1 truncation occurs at or after aa position 125 by deletion of the N-terminal part of the NS1 sequence or
20 by insertion of one or more desired POI sequences after aa position 125. In addition, the vector constructs and viruses containing such constructs according to the present invention can be used as tools for screening of antiviral drugs against desired antigens, and where the POI is a
25 marker substance, e.g. a fluorescent dye, also for tracking virus infections in animals and human subjects.

It is pointed out, however, that the concept of insertion of caspase recognition sites into a vector system for
30 recombinant protein production is of a more general nature and may likewise be applicable to vectors other than influenza virus vectors, such as e.g. baculovirus or adenovirus vectors or other vector systems known in the art.

- 10 -

BRIEF DESCRIPTION OF THE FIGURES

FIG.1: Schematic drawing of the structure of a truncated recombinant NS1 protein containing caspase recognition sites (CRS).

FIG.2: Post-translational cleavage of the recombinant viral protein NS1-Caspase-ELDKWAS-IL1.

FIG.3: Virus yields in MDCK cells at 39°C after treatment with different caspase inhibitors. Ordinate: viral titers expressed in pfu/ml; abscisse: different caspase inhibitors; "w/o" = without; "general" means inhibitor Z-VAD FMK, which inhibits all caspases.

FIG.4: Viral growth in lungs of infected mice; ordinate = viral titers in pfu/ml; abscisse = days post infection; triangles = control virus (i.e. without CRS), squares = experimental virus (i.e. with CRS).

Fig.5: Cleavage of recombinant NS1-GFP protein in infected cells. MDCK cells were infected with NS1-GFP virus in the presence (lanes 2 and 3) and absence (lane 1 and 4) of the universal caspase inhibitor Z-VAD-FMK. 12 h post infection, cells were lyzed, analyzed by Western blot and detected with anti-NS1 mouse serum (lanes 1 and 2) and followed by alkaline phosphatase staining or anti-GFP rabbit serum (lanes 3 and 4) using a chemoluminescence assay.

A = NS1-GFP fusion protein; B = cleaved GFP; C = cleaved NS1.

Fig.6: Viral growth in mouse lungs. PKR^{+/+} mice or PKR^{-/-} mice were infected intranasally with 5*10⁴ PFU/animal of the transfectant NS1-GFP virus. On day 2, mouse lungs were titrated using a limiting dilution assay on Vero cells and shown as geometric mean titer.

- 11 -

In order that the invention described herein may be more fully understood, the following examples are set forth. The examples are for illustrative purposes only and are not to be construed as limiting the present invention in any respect.

Example 1: Preparation of recombinant negative strand influenza A viruses

10 The plasmid clones containing the caspase recognition site (CRS) and gp-41 sequence have been prepared on the basis of the existing plasmid clone of influenza NS gene pUC19/NSPR (Egorov et al., 1998, J Virol 72/8, 6437-41). The sequence of CCS was inserted into the NS1 protein
15 ORF, at the position 400nt followed by the sequence of the protein of interest (designated LEI) consisting of HA leader peptide + ELDKWAS (SEQ ID NO 3) peptide sequence from GP-41 of HIV-1+ IL-1 nanopeptide. As a second step, a final recombinant plasmid was constructed harboring a
20 mutated influenza A/PR/8/34 NS gene flanked by the human polymerase I (PolI) promoter and Hepatitis Delta Virus (HDV) genomic ribozyme sequences.

This plasmid clone was used for the transfection of Vero
25 cells. Synthetic chimeric NS gene transcribed by PolI polymerase in vivo (Vero cells) was rescued using special influenza virus helper viruses: PR8 DelNS (rf. WO 99/64571) or A/Singapore/1/57-del NS87 (rf. WO 02/24876). Transfected cells were infected with one of the listed
30 viruses 24 hours after transfection (moi= 1) and the virus yield was subjected for the selection procedure in Vero cells. Because of the entire property of both helper viruses to be sensitive to interferon action (as a result of NS1 deletions) human interferon-alpha (10 units/ml of
35 media) have been used as selective pressure. After 2-3

- 12 -

passages in Vero cells in the presence of interferon chimeric NS mutants PR8-NS-caspase-LEI (H1N1) and Sing-NS-caspase-LEI (H2N2) were obtained. In contrast to the helper strains these viruses were not sensitive to the
 5 interferon action because of the functional RNA binding domain coded by the first 125 aa of NS1 ORF.

In the functional map of the engineered NS1 protein of the present invention it is indicated that the insertions are
 10 introduced after aa position 125 and followed by a stop codon which has in effect that the remaining adjacent portion of the NS1 gene segment rests untranslated.

Suitable recombinant protein constructs according to the present invention are represented in general by the
 15 schematic drawing of FIG. 1, while the invention disclosed herein is explicitly exemplified for the following recombinant protein construct (SEQ ID NO 3) that was prepared following the aforementioned protocol:

20

NS1 { MLPSRRQRGGWDHAGQQKQGDKDIMDPNTVSSFQVDCFLWHVRKRVAD
 QELGDAPFLDRLRRDQKSLRGRGSTLGLDIETATRAGKQIVERILKEESD
 EALKMTMASVPASRYLTDMTLEEMSRDWSMLIPKQKVAGPLCIRMDQAIM
DIDGGETDG MKTIIALSYIFCPALGQDLP ELDKWAS VQGEESNDK
 25 | ← CRS → | ← Leading Sequence → | ELDKWAS | ← IL-1 → |

Example 2: Cleavage of a recombinant protein construct containing CRS

30

The recombinant protein construct described in Example 1 was further subjected to post-translational cleavage by caspase enzymes. Fig.2 mirrors the results of the western blot analysis, showing that the recombinant NS1 protein
 35 containing CRS was efficiently cleaved by the activated

- 13 -

caspases after 12 hours of infection (lane 4 and 5). When caspase inhibitor VAD was added (lane 6) protein cleavage was abolished.

5 MDCK cells were infected with recombinant influenza Sg/NS1-Caspase-ELDKWAS-IL1 virus and analysed by western blot using mouse anti-NS1 serum. Lane 1 shows the size of NS1/125 protein, containing just 125 NS1 specific N-terminal amino acids remaining in the present constructs.
10 Lane 3 and Lane 4 show the cleaved and uncleaved forms of the recombinant protein at 4 hours (lane 3) and 10 hours (lane 4) after infection; lane 5 shows cleavage of the NS1-Caspase-ELDKWAS-IL1 protein 12 hours after infection in Vero cells; lane 6 shows the result when caspase
15 inhibitor Z-VAD-FMK (Supplier: R&D Systems) was added to the maintenance medium, the result being failure of cleavage of the NS1-Caspase-ELDKWAS-IL1 protein 12 hours after infection of Vero cells.

20 Example 3: Attenuation in vitro

Surprisingly it was found that insertion of the caspase cleavage sites into influenza NS1 protein causes a temperature sensitivity (ts) of the virus in MDCK cells
25 (Table 1).

Vero cells and MDCK cells were infected with recombinant influenza A (H1N1) virus comprising the caspase recognition sites PR8-NS-Caspase-LEI (H1N1) or with a
30 control virus PR8-NS2A-LEI (H1N1)(control), lacking the CRS and comprising the 2A-cleavage site instead, at a moi=1. Infected cells were incubated at two temperatures 37°C and 39°C for 24 hours. Caspase inhibitors were added to the culture media at a concentration of 0.25 µM. Viral

- 14 -

yields were measured by the titration on Vero cells at the permissive temperature of 37°C.

Table 1: Temperature sensitivity of CRS-bearing
5 recombinant virus

Viruses	Vero cells		MDCK cells	
	37°C	39°C	37°	39°C
PR8-NS2A-LEI (H1N1) (control)	5×10^6	3×10^6	1×10^6	2×10^4
PR8-NS-caspase-LEI (H1N1)	2×10^7	1×10^7	2×10^6	$< 10^2$

The results presented in Table 1 demonstrate that CRS-containing virus had about a 10^4 -fold reduction in growth at the elevated temperature of 39°C as compared to the
10 optimal growth temperature of 37°C. This (ts) phenotype was expressed only in MDCK cells, but not in interferon deficient Vero cells.

Caspase 8 and 9 are probably involved in the mechanism of
15 this (ts) phenotype expression, because addition of caspase 8 and 9 as well as of the general caspase inhibitor to the tissue culture media have increased the viral titers by a factor of more than 100 as compared to the control groups where DMSO solution without inhibitor
20 or other caspase inhibitors were added (see Fig.3).

Example 4: Attenuation in mice C57 black

In order to prove whether the attenuated, i.e. ts,
25 phenotype observed in vitro caused by insertion of caspase recognition sites (CRS) into recombinant influenza virus

- 15 -

constructs would also be detectable in vivo, a mouse experiment was carried out.

Surprisingly, it was found indeed that influenza A and B recombinant viruses containing the CRS DIDGGETDG (SEQ ID
5 NO 1) appeared to be attenuated in mice after intranasal immunization. Fig.4 shows the growth curves of two similar recombinant influenza viruses, differing primarily in the presence or absence of CRS. The control virus PR8-NS-2A-LEI (H1N1) does not have this site and replicates
10 efficiently up to 3.5 log/g in the lung tissues. The experimental virus PR8-NS-caspase-LEI containing CRS displays very weak replication in the lungs and is more than 100 times attenuated in the experimental group of mice.

15

Example 5: Rescue of influenza virus expressing gfp from the NS1 reading frame

Viruses and cells:

20 Vero and MDCK cell lines originated from the American Type Culture Collection (ATCC). Vero cells were adapted to and further cultivated in DMEM/Ham's F12 (Biochrom F4815) with 4 mM L-glutamine and protein-free supplement. MDCK cells were cultivated in DMEM/Ham's F12 medium containing 2 %
25 heat inactivated fetal calf serum (FCS, HyClone SH30071) and 4 mM L-glutamine. Influenza virus A/PR/8/34 (H1N1) (PR8) and NS mutants A/PR8/delNS1 (delNS1), A/PR8/NS1-125 (NS1-125) and A/PR8/NS1-del40-80 (NS1-del40-80) (Fig.1) were obtained in Vero cells by using the transfection
30 protocol as described in Egorov et al.,1998. All viruses were grown and titrated in Vero cells at 37° C.

Construction of plasmids:

Viral RNA was extracted from influenza PR8, reverse
35 transcribed and served as a template for subsequent

- 16 -

amplification of the NS gene by PCR. The NS sequence was blunt end cloned between the PolI promoter and hepatitis delta virus (HDV) genomic ribozyme and designated as pPolI-NS-HDV. Oligonucleotides coding for the caspase
5 recognition site, i.e. 5'-ATTGATGGAGGTGAACTGATGGG-3' (SEQ ID NO 6) and 5'-CCCATCAGTTTCACCTCCATCAAT-3' (SEQ ID NO 7), were synthesized, annealed and blunt end ligated into plasmid pPolI-NS-HDV after nucleotide position 400 of NS. The resulting plasmid was named pPol-NS125Casp-HDV.

10

Insertion of the GFP encoding sequence (Quantum's SuperGlo™ GFP, extracted from Vector pQBI25-fc1, Qbiogene) into plasmid pPol-NS125Casp-HDV downstream the CRS resulted in the plasmid pPol-NS-Casp-GFP-HDV. The
15 expression plasmids for PB1, PB2, PA and NP influenza proteins were obtained by cloning the corresponding PR8 genes into the vector pTriEx-1 (Novagene) under the chicken actin promoter; they were designated pTriEx-PB1, pTriEx-PB2, pTriEx-PA and pTriEx-NP, respectively.

20

Generation of transfectant viruses:

90% confluent Vero cells were transfected with 1µg of each plasmid pPol-NS-Casp-GFP-HDV, pTriEx-NP, pTriEx-PA, pTriEx-PB1 and pTriEx-PB2 using the nucleofection
25 technique (Amaxa), according to the manufacturer's instruction manual. 24 hours later, cells were examined for GFP expression and infected with delNS1 helper virus (MOI = 0,1). Eight hours post infection (p.i.), 3 U of human IFN-α (NIBSC 1st International Standard 1999, human
30 leukocyte derived) per ml culture medium were added and cells were incubated 48 hours at 37°C. The supernatant was passaged twice in Vero cells in the presence of IFN-α at a concentration of 3 U/ml and plaqued in Vero cells. Plaques containing the correct NS1-GFP gene were identified by RT-

- 17 -

PCR. Following two rounds of plaque purification, in which
plaques were routinely examined for GFP expression, a pure
virus could be isolated. This virus was designated as
A/PR8/NS1-125GFP (NS1-GFP), analyzed by RT-PCR and the
5 correct sequence was confirmed by nucleotide sequence
analysis.

Viral replication in mice lungs:

Four to six weeks old C57BL/6 mice or PKR knock out mice
10 (PKR-/-), which were derived from C57BL/6 mice by the
targeted deletion of PKR (Yang et al., 1995) were infected
intranasally (i.n.) with 5×10^4 PFU/animal of the virus
under ether anesthesia. At day two, mice were sacrificed
and lungs aseptically removed. A 10% tissue extract in PBS
15 was prepared by grinding the tissue sample with a rotor
homogenizer. The suspension was centrifuged at 2,000 g for
five minutes and the viral yield of the supernatants was
determined by limiting dilution assay in Vero cells.

20 Polyacrylamide gel electrophoresis and Western blot analysis:

SDS PAGE of virus preparations was carried out using Tris-
Glycine 16% gels (Anamed). Western blotting was performed
by electrophoretic transfer of the proteins from the gel
25 to a polyvinyl difluoride membrane (Millipore) for two
hours at 400 mA. After overnight blocking in TPBS (PBS
with 0.1% Tween 20) containing 3% of skim milk, the
membrane was incubated for one hour with specific mouse
anti-NS1-GST or rabbit anti-GFP antisera (Clontech) and
30 diluted 1:5,000 in TPBS buffer containing 1% of skim milk.
After washing with TPBS, the membrane was either incubated
for one hour with anti-mouse alkaline phosphatase or anti-
rabbit peroxidase labeled conjugate (Sigma) diluted
1:40,000 in TPBS containing 1% of skim milk. The blots
35 were developed in staining buffer (100 mM Tris HCl; 100 mM

- 18 -

NaCl; 5 mM MgCl₂, pH 9.5) with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate or by ECL plus Western Blotting Detection system (Amersham) for chemoluminescence, respectively.

5 Results:

Growth of influenza NS mutants in Vero cells treated with human IFN- α :

To determine the IFN sensitivity of NS mutants, Vero cells were infected with the wild type PR8 virus or three different NS mutants containing either only 125 N-terminal aa (NS1-125), lacking NS1 ORF (delNS1) or harboring a 40 aa deletion (NS1-del40-80) in the N-terminal part of the molecule (Table 2).

15 Table 2: Growth comparison of influenza virus NS1 mutants

Mutant	NS1 modification	Titer with IFN [PFU/ml]	Titer w/o IFN [PFU/ml]
PR8	N -----230	8×10^6	2×10^7
NS125	N -----125	5×10^6	4×10^7
Del40-80	N ---40 80-----230	<10	8×10^5
delNS1	N ---10	<10	2×10^6

Table 2 shows the growth of recombinant virus mutants containing different NS1 truncations on Vero cells in the absence or presence of externally added human IFN- α .
 20 Titers of influenza PR8 virus and NS mutants grown in the presence or absence of human IFN- α in Vero cells were determined 36 h post infection using a plaquing assay on Vero cells.

- 19 -

The infected cells were incubated either without addition of human IFN- α to the cell culture medium or in the presence of 3 U/ml human IFN- α , whereupon the viral titers were compared 36 h post infection. It was found, that
5 mutants delNS1 and NS1-del40-80 containing an impaired RNA binding/dimerization domain were dramatically attenuated with regard to growth under the pressure of IFN- α , whereas the wild type virus as well as the NS1-125 mutant containing an intact RNA binding domain showed almost
10 similar titers when incubated in the presence or absence of IFN- α (Tab.2). Thus, the first N-terminal 125 amino acids of the NS1 protein may possibly serve as a resistance factor responsible for antagonizing IFN action.

15 Rescuing GFP expressing influenza NS vector:

Vero cells were transfected with plasmids expressing the components of the influenza RNP complex and the plasmid pPol-NS-Casp-GFP-HDV encoding chimeric vRNA in which GFP was cloned as the protein of interest (POI) into the NS1
20 reading frame after aa 125, according to the general concept depicted in Fig.1. In addition, an artificial caspase recognition site (DIDGGETDG) was introduced between those two proteins in order to provide posttranslational separation of the molecules.

25

Transfected cells were infected with an IFN-sensitive helper virus (delNS1) and the virus yield was harvested 48 h post infection. This yield contained at least 10^2 PFU/ml progeny of the "fluorescent virus" and 10^5 PFU/ml of the
30 helper virus, determined by limiting dilution assay in Vero cells without IFN- α treatment. Two passages in Vero cells in the presence of IFN- α were performed to enrich the population of the transfectant virus and a pure GFP expressing plaque was isolated. After a further two rounds

- 20 -

of plaque purification in Vero cells, in which plaques were routinely examined for GFP expression, a pure clone of GFP expressing virus was isolated and designated as A/PR8/NS1-125GFP (NS1-GFP).

5

This virus, when applied at MOI=1, was able to induce expression of GFP as early as six hours post infection in Vero and MDCK cells with full manifestation 24h post infection. By Western blot analysis it was confirmed that
10 insertion of the CRS indeed resulted in posttranslational cleavage of the GFP from the N-terminal part of the NS1 protein (Fig.5). Addition of general caspase inhibitor Z-VAD-FMK (R&D Systems) to the medium inhibited cleavage, thus confirming that cleavage occurred due to host cells
15 caspase activity.

Growth capacity of NS-GFP expression vector:

As expected, the recombinant NS1-GFP strain had an IFN resistant phenotype and was capable of replicating in MDCK
20 cells and ten days old embryonated chicken eggs as efficient as in Vero cells up to titers of 10^6 - 10^7 PFU/ml. However, the genetic stability of the construct was not equal in IFN deficient and in IFN competent cell substrates. In contrast to Vero cells, passages in MDCK
25 cells caused evolution of NS1-GFP deletion mutants at a fairly high rate accompanied by the loss of GFP expression after several passages.

Replication in mice:

30 Virus NS1-GFP revealed a full attenuation in the wild type mice (C57BL/6) as compared to the NS125 mutant, which reached a titer of 5×10^4 PFU/g in the lung tissues two days post infection (Fig.6). However, the infection of PKR knock out (PKR-/-) mice, previously shown to support the
35 replication of the delNS1 virus, resulted in the

- 21 -

replication of the GFP expressing vector in lung tissues to 4×10^4 PFU/g, detected two days post infection. Furthermore, even after two days of replication in mouse lungs, the virus was still expressing GFP as confirmed by
5 titration in Vero cells.

- 22 -

References:

- Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G,
Thornberry NA, Wong WW, Yuan J. 1996. *Human ICE/CED-3*
5 *protease nomenclature*. Cell. 18;87(2):171.
- Egorov A, Brandt S, Sereinig S, Romanova J, Ferko B,
Katinger D, Grassauer A, Alexandrova G, Katinger H and T
Muster. 1998. *Influenza A transfectant viruses with long*
10 *deletions in the NS1 protein grow efficiently in Vero cells*.
J Virol. 72 (8):6437-6441.
- Zhirnov OP, Konakova TE, Garten W and H-D Klenk. 1999.
Caspase-dependent N-terminal cleavage of influenza virus
15 *nucleocapsid protein in infected cells*.
J Virol. 73 (12):10158-63.
- Yang, Y. L., Reis, L. F., Pavlovic, J., Aguzzi, A., Schafer,
R., Kumar, A., Williams, B. R., Aguet, M., and Weissmann, C.
20 (1995). Deficient signaling in mice devoid of double-
stranded RNA-dependent protein kinase. Embo J 14(24), 6095-
106.

- 23 -

CLAIMS

1. A nucleotide sequence encoding a recombinant protein product characterized in that the nucleotide sequence contains at least one sequence section that encodes one or more caspase recognition sites and that is located in between and links together a first part of the nucleotide sequence encoding a first part of the protein product and a second part of the nucleotide sequence encoding a second part of the protein product.
2. The nucleotide sequence according to claim 1, wherein said first part of the nucleotide sequence encodes a truncated NS1 protein, preferably the first 125 N-terminal amino acids of the NS1 protein.
3. The nucleotide sequence according to claim 1 or 2, wherein said second nucleotide sequence encodes a peptide or protein of interest (POI), said POI preferably being a tumor antigen or an antigenic determinant of a viral or bacterial protein, the viral or bacterial protein preferably being selected from the group consisting of gp41 of HIV-1, gp120 of HIV-1, Nef protein of HIV-1, HA of influenza virus, NA of influenza virus, TBC antigen, and malaria antigen.
4. The nucleotide sequence according to any one of claims 1 to 3, wherein said sequence section comprises two caspase recognition sites spaced apart by a glycine residue, the sequence section preferably being SEQ ID NO 1.
5. A recombinant nucleotide vector, preferably a viral recombinant vector, comprising a nucleotide sequence as defined in any one of claims 1 to 4.

- 24 -

6. The vector according to claim 5, characterized in that it renders a virus containing said vector attenuated, particularly temperature sensitive, in MDCK cells but not in interferon deficient Vero cells.

7. The vector according to claim 5 or 6, characterized in that it renders a virus containing said vector attenuated in vivo.

8. A recombinant virus, preferably recombinant influenza virus, characterized in that it comprises a nucleotide sequence as defined in any one of claims 1 to 4 or a vector as defined in any one of claims 5 to 7.

9. The virus according to claim 8, characterized in that it is attenuated, particularly temperature sensitive, in MDCK cells but not in interferon deficient Vero cells.

10. The virus according to claim 8 or 9, characterized in that it is attenuated in vivo.

11. A vaccine comprising a vector as defined in any one of claims 4 to 6 or a virus as defined in any one of claims 7 to 9.

12. The vaccine according to claim 11, characterized in that it is attenuated in vivo.

13. The vaccine according to claim 11 or 12, for prophylactic or therapeutic application against tumors or viral infections.

14. Use of a nucleotide sequence encoding a caspase recognition site, preferably encoding a combination of two or more caspase recognition sites, for providing a cleavage site to a nucleotide vector, for post-

- 25 -

translational cleavage of at least one peptide or protein of interest from a protein product encoded by said vector.

15. A composition comprising a nucleotide sequence as defined in any one of claims 1 to 4.

16. A composition comprising a vector as defined in any one of claims 5 to 7.

17. A composition comprising a virus defined in any one of claims 8 to 10.

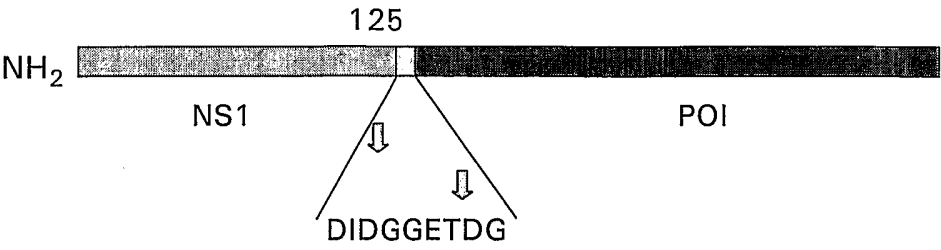


FIG. 1

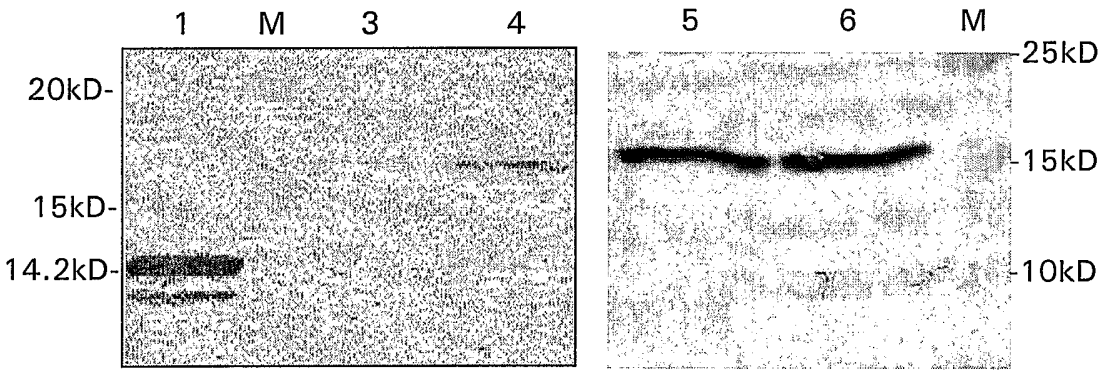


FIG.2

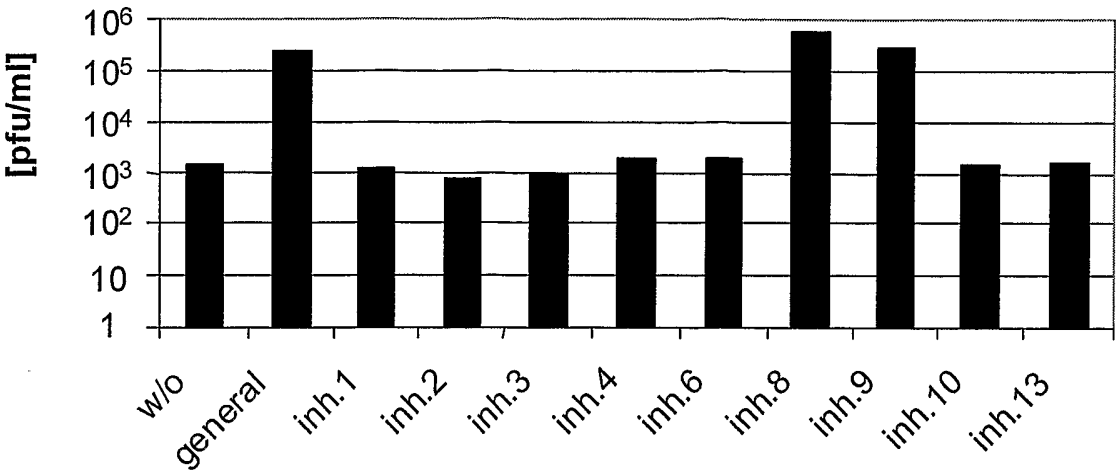


FIG. 3

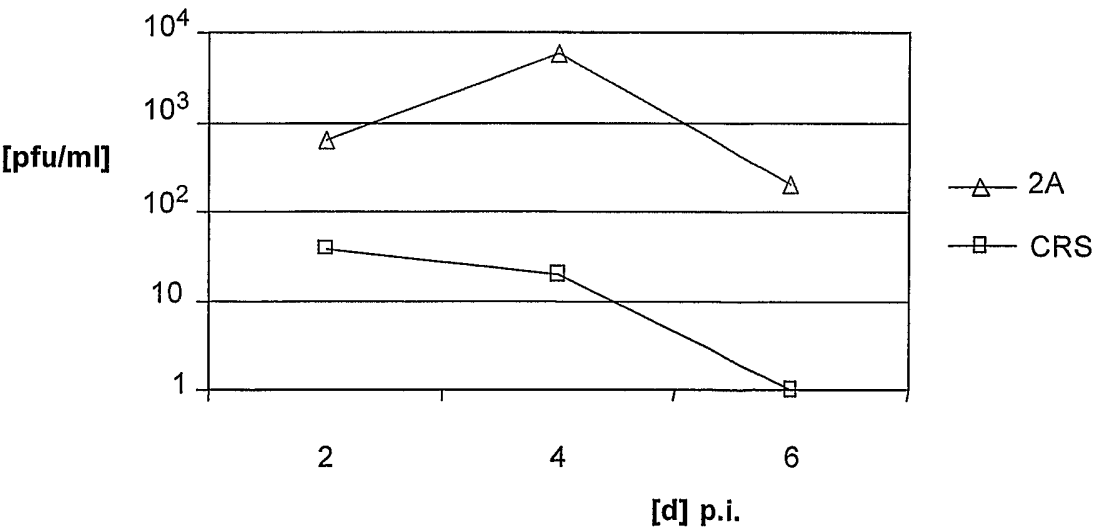


FIG. 4

3/3

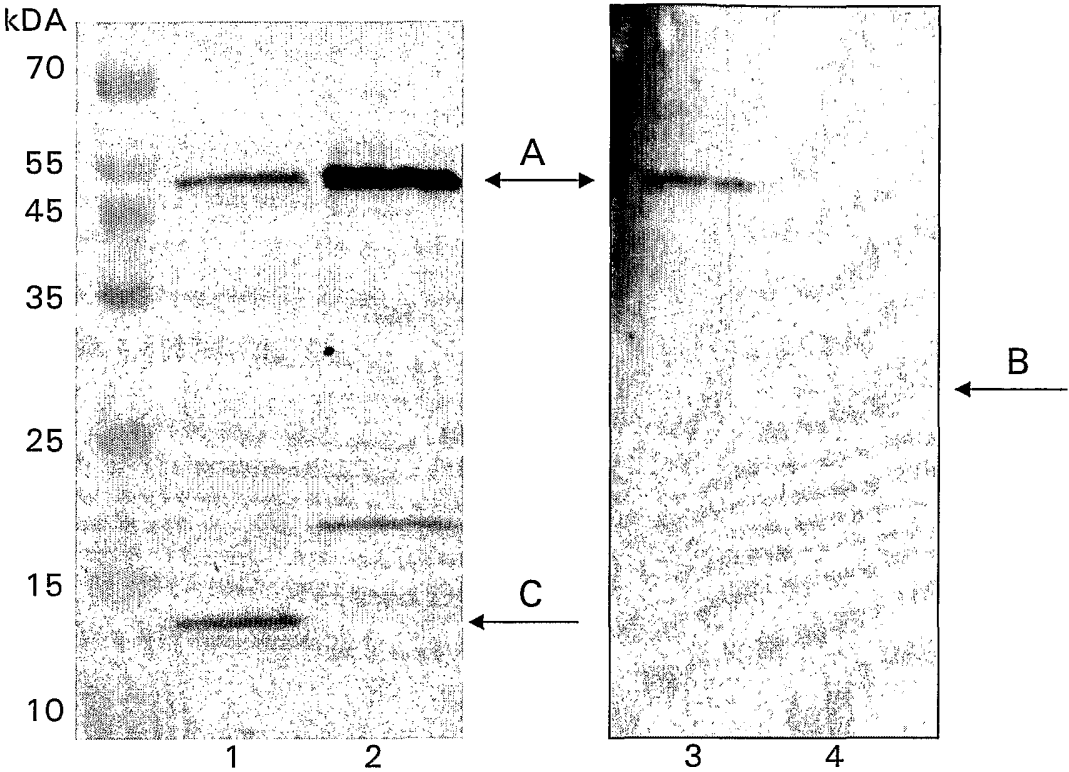


Fig.5

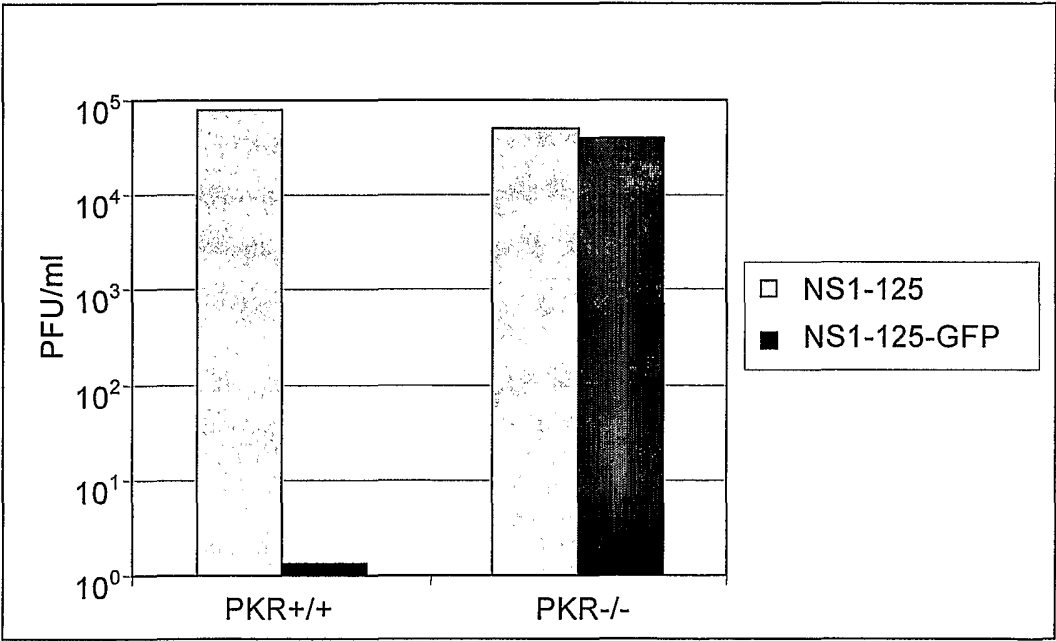


Fig. 6