

**RESEARCH ARTICLE****Influenza virus pathogenicity is determined by caspase cleavage motifs located in the viral proteins**

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**ABSTRACT**

Almost all influenza virus proteins are found to contain caspase cleavage motifs. Two caspase cleavage consensus sequences, EXD↓Y and D/EXXD↓Y (caspase motifs) were identified in N- and C-terminal regions of influenza virus proteins nucleocapsid NP (positions D<sub>16</sub> and D<sub>497</sub>) and ionic channel M2 (positions D<sub>23</sub> and D<sub>87</sub>). Using reverse genetics with the highly-virulent avian influenza virus A/FPV/Rostock (H7N1), as a vector precursor, these NP and M2 caspase motifs were artificially altered by site-directed mutagenesis and pathogenicity of the generated caspase mutant viruses was tested in chickens. Three main groups of virus mutants were identified. The first group of mutants was characterized by high replication in cells and low virulence in chickens. These virus mutants possessed the altered N-terminal NP and C-terminal M2 caspase motifs. The second group of virus mutants, possessing the altered N-terminal caspase motif of M2, was characterized by attenuated replication in cultured cells and reduced pathogenic properties in chickens. Third, mutations generated in the C-terminus of NP were lethal and restricted virus rescue by reverse genetics, implying a critical role of this caspase site in virus replication. Thus, these data suggested that, (i) caspase motifs in virus proteins play a significant role in virus pathogenicity; (ii) the lack of direct correlation between replication potential and pathogenicity, observed in caspase mutants of the first virus group, implied that virus caspase motifs could affect immunopathogenesis during the infection process, rather than simply controlling virus production in target cells in the chicken host.

**KEYWORDS:** Influenza virus, attenuation, caspase motifs, vaccine**INTRODUCTION**

Pathogenicity of avian influenza viruses (virus ability to cause severe disease and kill avian hosts) is a multifactorial event. There are three major viral determinants involved in the control of virus pathogenicity. One of the major pathogenic determinants is the sensitivity of the viral hemagglutinin (HA) to activation cleavage by host proteases (Steinhauer, 1999). Highly- pathogenic viruses causing a severe generalized

infection process contain a Lys/Arg multibasic cleavage site in the HA, which is hydrolyzed by ubiquitous furin-like serine proteases in the cells of different hosts. The second major determinant is a specificity of the viral HA in recognizing host cell receptors. Avian viruses are characterized by specific binding to the α2-3 type of sialic acids, whereas human viruses use α2-6 ones (Skehel and Wiley, 2000). This receptor specificity permits infection of sensitive cells and contributes to virus spreading and pathogenicity in a certain host organism. The third

pathogenic determinant is linked with the virus polymerase complex. Specific domains in the polymerase proteins PB1, PB2, PA and the nucleocapsid NP specifically regulate viral RNA synthesis in avian and mammalian cells and virus pathogenicity in certain hosts (Gabriel et al, 2007). All three viral determinants mentioned are realized through interaction with specific host factors, such as proteases, cellular receptors, *etc.*, and this virus-host concordant balance provides virus pathogenic action in certain hosts.

In this study an additional determinant of virus pathogenicity was found to be linked with caspase cleavage motifs previously identified in proteins of numerous viruses, including influenza viruses (Fischer et al, 2003). Amino acid sequences corresponding to the consensus I/L/VEX<sub>1</sub>D↓Z or D/VX<sub>1</sub>X<sub>2</sub>D↓Z (where Gly, Ala, Thr, Ser and Asn are favourable at X<sub>1,2</sub> and Z, but Glu, Asp, Lys, Arg, Trp are unfavourable at these positions) are considered as canonical caspase cleavage motifs (Thornberry et al, 1997). It is well known that caspase motifs are recognized and specifically cleaved at D amino acid by the host cell cysteine-type proteases (thus called caspases – Cysteine Aspartate-Specific Proteases (Timmer and Salvesen, 2007). We have previously shown that human influenza virus protein NP and M2 possess caspase cleavage motifs METD<sub>16</sub>↓G at the N-terminus and VD<sub>87</sub>↓G at the C-terminus, respectively (Zhirnov et al, 1999; 2002). These NP and M2 sites were found to be cleaved at the late apoptotic stage in infected cells and, as a result, the NP (56 kD) was cleaved to form the apoptotic product aNP (53 kD) and the M2 (15 kD) was processed to aM2 (13 kD) (Zhirnov and Bukrinskaya, 1981, Zhirnov et al, 1999; 2001). It is noteworthy that all avian influenza viruses were found to carry the point substitution D<sub>16</sub> → G at the NP cleavage site rendering NP resistance to caspase cleavage in infected cells (Zhirnov, 1984; Zhirnov and Bukrinskaya, 1984). Unlike the N-terminal NP caspase motif, the DVDD/G motif at the C-terminus of M2 was retained in all avian and human influenza viruses [Zhirnov et al, 2002]. Whether these NP and M2 caspase motifs play role in influenza virus replication and pathogenicity is generally not known and this idea was studied here.

We have inspected influenza virus proteins for the presence of caspase motifs and identified two additional ones in the C- and N-regions of proteins NP and M2, respectively. All four revealed caspase motifs in NP and M2 were altered by site-directed mutagenesis and virus mutants were generated by reverse genetic using a highly-pathogenic A/FPV/Rostock (H7N1) virus as a precursor vector for testing in chickens. Three groups of virus mutants were identified. In a first group, virus mutants possessing alterations in the N-terminal NP and C-terminal M2 caspase motifs did not show an effect on virus replication potential but were significantly diminished in their virulence for chickens. In a second group, virus mutant was characterized by significantly reduced replication in cultured cells and apathogenic properties in chickens. This virus mutant possessed alteration in the N-terminal caspase motif of M2. In a third group, mutations in the caspase motif at the C-terminus of NP were lethal and strongly restricted virus rescue by reverse genetics

procedure. Thus, our data suggested that caspase motifs in virus proteins could be involved in virus reproduction and in host pathways of infection immunopathogenesis, and that their artificial alterations markedly attenuated virus pathogenicity.

## MATERIALS AND METHODS

### Cells and viruses

The MDCK-II cell line (collection of Institute of Virology, Marburg) and human epithelial colon carcinoma cell line (CACO-2) (European Collection of Cell Cultures; ECACC) were cultivated in DMEM containing 10% (v/v) bovine fetal calf serum (FCS) (Gibco BRL). Cell monolayers were infected with influenza virus mutants and incubated with DMEM without serum at 37°C for different periods. To prepare virus samples, virus-containing culture fluid was clarified at 6000 x g for 20 min and then pelleted through 20 ml of 20% (w/v) sucrose prepared in PBS at 30,000 rpm (SW 50.1 rotor) for 2.5 hrs. Virus pellets were analyzed by protein gel electrophoresis.

### Generation of recombinant viruses with caspase motif mutations

Viral gene segments PB1, PA, PB2, HA, NA, NP, M and NS were cloned into the plasmid pHH21 (Wagner et al, 2005). For rescue of recombinant FPV, 1µg of each of these plasmids was transfected into 10<sup>6</sup> 293T cells along with expression plasmids encoding the subunits of the influenza virus polymerase pcDNA-PB1 (1µg), pcDNA-PB2 (1µg), pcDNA-PA (0.2µg), and pcDNA-NP (1µg) using Lipofectamine 2000 (Invitrogen) (Wagner et al, 2005). Four hours post-transfection MDCK cells (2x10<sup>5</sup>) were added and the obtained mixed 293T-MDCK cell culture was co-cultivated in DMEM containing 0.5% (v/v) FCS. Four to seven days post-transfection the supernatant containing 10<sup>3</sup>-10<sup>6</sup> HAU/ml of virus (passage 0) was removed, aliquoted and kept at -80°C. To obtain caspase mutants, site-directed mutagenesis of pHH21-FPV-NP and pHH21-FPV-M plasmids were constructed using the Quick-change mutagenesis kit (Stratagene, The Netherlands) using the following caspase site-specific primers:

M2nn<sub>23</sub>/fo: 5'cagcagaatgctgtaaacgtaacgatggctcattttg

M2nn<sub>87</sub>del/fo: 5'cagcagagtgcgtgtaattgtaaacgctcattttgtcaacat

NPgd/fo: 5'tcttatgagcagatggagaccgacggagagcgcagaatg

NPdelND/fo: 5'cttcggagacaatgcaaactatgacaattgaagaaaaatacccttg

NPdelNN/fo: 5'ggagacaatgcaaattataacaattgaagaaaaatacccttg

### Experiments in chickens

Eleven-day old chickens (Lohmann Brown line PK-13) were infected with different virus variants and housed under isolation conditions in separate rooms to exclude cross-infection; all chickens received a standard grower diet throughout the experiment. Parenteral routes of virus inoculation were found to be suitable for testing of pathogenicity of avian influenza viruses (Brugh and Beard 1986; Scholtissek and Rott 1984). Accordingly, an intramuscular (i.m.) route of infection was chosen to

provide a more accurate pathogenicity comparison of different inoculation doses of the generated recombinant viruses. Birds were infected intramuscularly with virus mutants (0.15ml per leg) and survival and disease signs, such as reduction in movement, loss of body weight, ruffled feathers, body temperature, *etc.*, were monitored daily. Titers of anti-virus antibodies in blood samples were determined by hemagglutination inhibition (HAI) test: 30µl of two-fold serially diluted chicken sera were incubated with 30µl of reference A/FPV/Ro/34 virus (8 HA units) at 22°C for 1hr followed by incubation with 50µl of 10% (v/v) chicken red blood cells at 4°C for 45min. The HAI titer was defined as the highest serum dilution inhibiting virus hemagglutination. All painful manipulations with chickens, such as bleeding, *i.m.* inoculations, *etc.*, were made under light ether narcosis.

#### Polyacrylamide gel electrophoresis (PAGE)

Polypeptides were electrophoresed in 12% (w/v) polyacrylamide gels using Tris-glycine-SDS buffer followed by autoradiography as previously described (Zhirnov et al, 1999). For electrophoresis, cellular polypeptides were dissociated in 2% (w/v) SDS, 0.05 M dithiothreitol, 0.02 M Tris-HCl (pH 6.8) for 10min at 85°C.

#### Western blot analysis (WB)

After SDS-PAGE the polypeptides were transferred from the gel onto Protran-nitrocellulose membranes (pore-size 0.45-µm) (Schleicher and Schuell) by semidry electroblotting and processed with virus-specific antibodies as previously described (Zhirnov and Klenk, 2003). Then membranes were exposed to horseradish peroxidase (HRP)-conjugated secondary anti-species antibodies (Dako), followed by ECL visualization on Kodak film.

#### Focus assay in cultured cells

MDCK cells grown in 24-well plates were incubated with 0.4 ml/well of ten fold virus dilutions in DMEM. After 60min incubation at 37°C, the virus inoculum was removed, and cells were covered with 1.0ml of 1% (w/v) agarose (culture quality; Sigma) in DMEM. 48 hrs after infection, cells were fixed with 4% (v/v) paraformaldehyde and stained with anti-influenza virus antibody followed by visualization of virus foci with TMB insoluble substrate “True Blue” (KPL) (Zhirnov and Klenk, 2003).

## RESULTS

#### Caspase cleavage motifs in viral proteins and their alterations

As a first step we inspected proteins NP and M2 of highly-pathogenic avian influenza virus A/FPV/Ro/34 (H7N1) to identify caspase cleavage motifs. For this purpose a bioinformatics tool GrabCas predicting potential caspase cleavage sequences was applied (Backes et al, 2005). The NP was found to possess two caspase cleavage motifs: the N-terminal ETG<sub>16</sub>/G and EEYD<sub>576</sub>/N at the C-terminus, as well the M2 protein carried the motifs DSSD<sub>23</sub>/G in the N-terminal exomembrane domain and VD<sub>VDD</sub><sub>87</sub>/G in the C-terminal cytoplasmic tail. Additional inspection of other viral proteins revealed numerous caspase cleavage motifs in the proteins PB1, PA, PB2, NS1, NA and HA

(summarized in Table 1). The proteins NEP, M1, and the hypothetical NSP (Zhirnov et al, 2007) appeared to lack any caspase cleavage motifs. Wide distribution of caspase motifs in different influenza virus proteins implied that these sites could play an important role in functioning of the viral proteins and virus biology.

These observations prompted us to study the roles of the NP and M2 caspase motifs in virus replication and pathogenicity. These sites were altered by site-directed mutagenesis and virus mutants were generated using a reverse genetics approach. Two types of caspase motif alterations were generated. The first type was generated to alter the avian-like non-cleavable motif ETG<sub>16</sub>/G within the NP protein to a human-like cleavable ETD<sub>16</sub>/G (NPdg) motif. The second type was developed to alter cleavable caspase motifs EEYD<sub>576</sub>/N (NP), DSSD<sub>23</sub>/G (M2), and VD<sub>VDD</sub><sub>87</sub>/G (M2) into non-cleavable ones. For such alterations the residues E and D at positions -1, -3, and/or -4 (underlined), which are well known to be the most important for the recognition and cleavage by specific caspases (Thornberry et al, 1997), were either substituted for an N residue or deleted in the different mutants (summarized in Table 2). These complex mutations were introduced into caspase motifs to impede the appearance of wild type back revertants. For example, the mutants M2nn<sub>87</sub> and M2nn<sub>87</sub>del possessed a similar attenuated phenotype, however, the first one was less stable and reverted to the WT genotype after 1-2 passages in chicken (data not shown). To impede such reversion, the deletion mutant M2nn<sub>87</sub>del was generated and studied here in detail.

#### Replicative abilities of caspase virus mutants in cultured cells

Next, replicative potential of the generated virus caspase mutants in cultured cells was compared with wild type virus generated by reverse genetics (WTRG) using a focus formation test in MDCK cells. It was found that mutations in the caspase motifs, as a rule, were not lethal and most of mutants efficiently replicated in MDCK cells (Table 2) and produced normal large focuses in canine MDCK (Figure 1) and human CACO-2 cells (data not shown). This observation indicated that mutations in caspase motifs were not restrictive for virus replication in cells of different origin. Only mutations in the caspase motif located at the C-terminus of the NP appeared to be lethal and restricted virus replication. Interestingly, the mutants NPdg and M2nn<sub>87</sub>del formed large-sized focuses (500 cells and more per focus), similar to the wild type WTRG virus, whereas M2nn<sub>23</sub> produced predominantly small-sized ones. Examination under the light microscope, showed a lot of single virus-positive cells and small foci (15-50 cells per one focus) in cell cultures infected with the last mutant, implying a low ability of this mutant to assembly virions and/or infect neighbouring cells (not shown). Next, virus titers produced in MDCK cells under one-cycle growth were evaluated (Figure 2A). Consistent with the focus assay, mutants NPdg and M2nn<sub>87</sub>del, like WTRG virus, produced high titers of progeny virus while M2nn<sub>23</sub> mutant synthesized markedly lower yields. Collectively, the above data indicated that two mutants (NPdg and M2nn<sub>87</sub>del) possessed high replicative ability, similar to WTRG virus,

**Table 1.** Caspase cleavage motifs identified in influenza A/FPV/Rostock/34 virus

Protein/length (aa)	Predicted caspase cleavage motifs (*)	Responsible Caspases	Accession numbers
<b>PB1 (757)</b>	TEYD↓A (648)	Caspase-6, Caspase-8	ABI85065
<b>PB2 (759)</b>	DDVD↓Q (256)	Caspase-3, Caspase-7, Caspase-8	M21851
<b>PA (716)</b>	DISD↓L (379) IELD↓E (416) NDTD↓V (506)	Caspase-2 Caspase-6, Caspase-8, Granzyme B Caspase-8	M21850
<b>HA (536)</b>	AEED↓G (475) DLAD↓S (454)	Caspase-8 Caspase-2	M24457
<b>NP (498)</b>	AEEYD↓N (497) ETG↓G (16) (**)	Caspase-6, Caspase-8	M21937
<b>NA (447)</b>	TETD↓S (362)	Caspase-6, Caspase-8	CAA36475
<b>NS1 (230)</b>	DESD↓E (74) LDID↓T (55)	Caspase-2, Caspase-8 Caspase-4, Caspase-8	M29617
<b>M2 (97)</b>	DSSD↓P (24) VDVDD↓G (87)	Caspase -2 Granzyme B, Caspase-6, Caspase-7	M55475

(\*) Canonical caspase cleavage motifs (Thornberry et al, 1997) were identified in viral proteins with the CrabCas bioinformatics tool and those possessing the highest caspase recognition factor, and the predicted responsible caspases, were outlined [Bakes et al. 2005]. Sequence position of the scissile D in the whole polypeptide is indicated in parentheses. Sequence data of Influenza A/FPV/Rostock/34(H7N1) virus proteins were taken from GenBank.

(\*\*) The Cleavable caspase motif ETD<sub>16</sub>↓G identified in human viruses was substituted in nature for a non-cleavable ETG<sub>16</sub>/G motif and maintained in the avian virus population (Zhirnov et al, 1999).

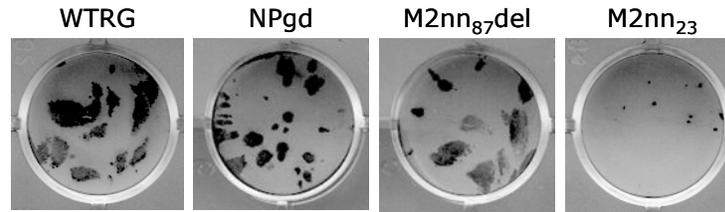
**Table 2.** Alterations in caspase cleavage motifs of NP and M2 proteins

Viral proteins	Natural caspase motifs *)	Altered caspase motifs	Mutant brief name	Replication ability of virus mutant (**)	Stability of caspase mutations (***)
<b>NP</b>	ETG <sub>16</sub> ↓G	ETD↓G	NPgd	R	M
	AEEYD <sub>497</sub> ↓N	ANYD/N ANYN/N	NPdelND NPdelNN	L L	-- --
<b>M2</b>	DSSD <sub>23</sub> ↓P	NSSN/P	M2nn <sub>23</sub>	R	H
	VDVDD <sub>87</sub> ↓G	VNVND/G VNVN/G	M2nn <sub>87</sub> M2nn <sub>87</sub> del	R R	M H

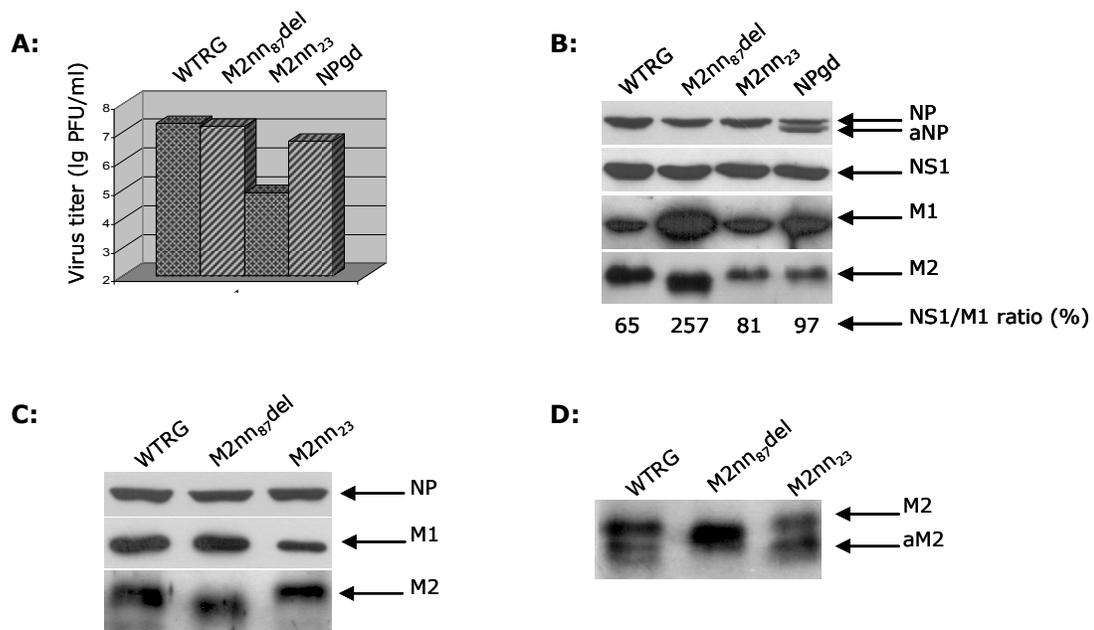
(\*) Cleavable and non-cleavable caspase motifs in NP and M2 proteins of Influenza A/FPV/Ro/34 virus are shown by “↓” and “/”, respectively. Alterations in caspase motifs were made by site-directed mutagenesis and virus mutants were generated using a reverse genetic approach.

(\*\*) Replication ability of virus caspase mutants in MDCK cells was identified as R (replicable mutant virus) or L (mutation was lethal and virus progeny was not observed).

(\*\*\*) Stability of generated mutations was evaluated by genome sequencing of virus passaged in cultured cells (at MOI 0,01) and chickens. H (high) – mutations were retained during at least 3 passages in cell cultures and chickens. M (middle) – mutation was stable in cultured cells and returned back to the wild type after 1-2 passages in chickens.



**Figure 1.** Foci formed by virus caspase mutants in MDCK cells. MDCK monolayers were infected with 10-fold dilutions either of WTRG, NPgd, M2nn<sub>23</sub>, or M2nn<sub>87del</sub> viruses. Virus stocks were passaged 1 time in MDCK cells were used as inoculum. At 45 h.p.i. cells were fixed and stained with anti-FPV antibodies and TMB-insoluble dye. Plate wells displaying similar numbers of virus foci were photographed.



**Figure 2.** Virus growth and polypeptide profiles in cells infected with different caspase mutants. MDCK cells were infected with mutant viruses (MOI ~1), thoroughly washed and incubated in DMEM lacking FCS. 15 hpi virus titers in culture fluid were determined by immune focus assay in MDCK cells, mean titers from 3 parallel titrations, (A) and equivalent numbers of infected cells were scrapped and the proteins NP, M1, NS1, and M2 were detected by PAGE-WB (B). WB membrane was scanned using the TINA program and the M1 ratio as a per cent of the NS1 (100%) was calculated (below the membrane B). Virions from culture fluid were clarified by sedimentation through a sucrose cushion and the virus polypeptides NP, M1, and M2 were analyzed by PAGE-WB (C). M2/aM2 polypeptide profiles in infected cells at 30 h.p.i. were also tested by PAGE-WB (D).

but that another one (M2nn<sub>23</sub>) displayed low replication in cultured cells.

#### Protein profiles in virions and cells infected with caspase virus mutants

The major viral proteins NP, M1, NS1, and M2 were analyzed as markers of virus synthesis in infected cells. Levels of these proteins synthesized by WTRG and mutant viruses in infected cells and in virions were compared. All proteins were detected in infected cells and their amounts were similar to those in WTRG-infected cells (Figure 2B) and WTRG virions (Figure 2C). The polypeptide profile of virus synthesized in NPgd-infected cells was also similar

to WTR virus (not shown). The mutated viral protein NPgd was found to be cleaved in infected cells and its apoptotic form aNP was clearly detected (Figure 2B; lane NPgd) supporting a critical role of D16 in this cleavage.

Conversely, the mutated protein M2nn<sub>87del</sub> was not cleaved in infected cells as late as 30hpi, when WTRG M2 protein was clearly cleaved to form aM2 (Figure 2D). Interestingly, aNP and aM2 appeared in infected cells sequentially at 15 and 30 h.p.i., respectively, suggesting that NP was less resistant to caspase cleavage in infected cells than M2. Thus, sufficient levels of mutant NP and M2 proteins in infected cells and virions indicated that

mutations in caspase sites did not affect significantly intracellular synthesis and maturation of the mutated proteins. It was noted that synthesis of M1 was markedly increased in cells infected with M2nn<sub>87del</sub> mutant (Figure 2B, bottom). In this mutant M2-specific caspase mutations did not overlap the M1 open reading frame but modified a 3' non-translated region of the M1 mRNA that could elevate its intracellular stability and translation efficacy.

#### Viremia in chickens infected with caspase mutants

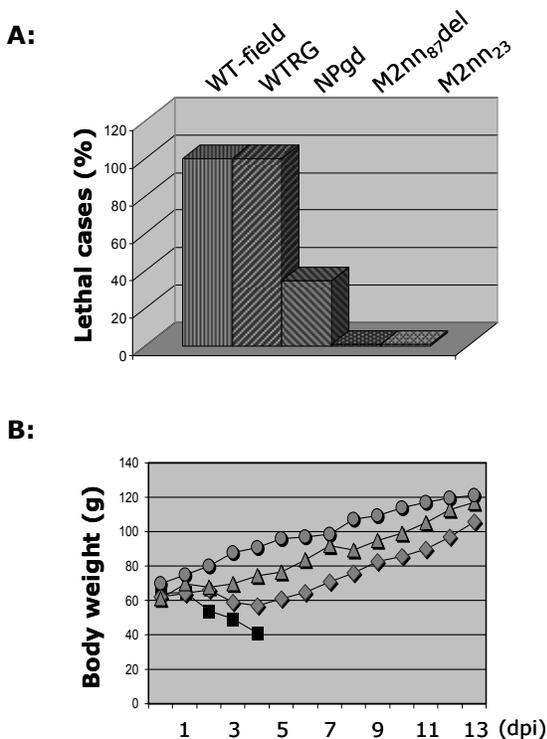
Influenza FPV virus is well known to replicate in all organs of infected chickens and develop a pantropic infection disseminating throughout the body by viremia (Feldman et al, 2000). Thus, viremia was chosen as an indicator of the infectious process and virus spreading in the chicken. Accordingly, titers of mutant viruses in blood were estimated by focus assay on days 3 and 5 after intramuscular infection of chickens. Virus was regularly detected on days 3 and 5 in the blood of WTRG-infected birds in high concentrations,  $10^5$ - $10^6$  pfu/ml of blood. Markedly lower virus titers, such as  $10^2$ - $10^4$  pfu/ml, appeared in blood of NPgd-infected chickens on day 3 pi. In chickens infected with M2nn<sub>87del</sub> and M2nn<sub>23</sub> mutants viremia was not detected by the focus assay used, indicating that virus titers in these birds were less than the threshold level of  $10^2$  pfu/ml of blood. On day 5 pi viremia was not revealed in all birds infected with virus mutants. Thus, in chickens infected with caspase mutants, virus titers in blood were markedly lower than in WTRG-infected ones. These data suggest that wild type WTRG virus effectively replicated and disseminated in chickens, whereas caspase mutant viruses were markedly attenuated in the chicken.

#### Pathogenic properties of caspase virus mutants in chickens

Pathogenic properties of the obtained FPV caspase mutants were assayed. Groups of 11-day-old chickens were infected either with WTRG, NPgd, M2nn<sub>23</sub> or M2nn<sub>87del</sub> virus mutants. It was found that the WTRG variant displayed the highest (100%) virulence for chickens (Figure 3A). Markedly lower lethality (~40%) was observed in the NPgd-infected group. M2nn<sub>23</sub> and M2nn<sub>87del</sub> did not kill chickens either at a low dose of 300 pfu (Figure 3A) or even at the massive infection dose of  $10^5$  pfu (data not shown).

To evaluate this further the pathogenic potential of these caspase mutants, the dynamics of disease symptoms caused by the different virus mutants was studied. Chickens were infected with a low dose (~100 pfu/animal) of each virus mutant and signs of illness were monitored. The severity of disease symptoms was found to correlate with the lethal ability of the virus mutant. Chickens infected with WTRG virus displayed high fever (42-43°C), rapid loss of body weight and death within 4-5 days post-infection. Both M2nn<sub>23</sub> and M2nn<sub>87del</sub> mutant viruses were almost apathogenic and did not cause visible symptoms or loss of body weight (Figure 3B). The NPgd mutant induced disease of an intermediate severity. During 4-7 days post-infection, chickens infected with this mutant displayed signs of disease, such as fever (41-42°C), ruffled feathers, lowered activity, delay of body weight gain, and

then infected birds rapidly recovered (Figure 3B). These data supported the concept that caspase mutant viruses succumbed to host defense mechanisms and displayed attenuated virulence in chickens.



**Figure 3.** Lethality and dynamics of body weight gain in chickens infected with virus caspase mutants. Eleven-days-old chickens were infected intramuscularly with 300 p.f.u. of different virus mutants, using 7 birds in each virus group. Chickens were examined daily after infection to measure body weight and monitor the number of deaths. Data on the cumulative deaths on day 15 p.i. are outlined in panel A and body weights (mean values of all chickens in the group) are shown in panel B (■ WTRG; ◆ NPgd; ▲ M2nn<sub>87del</sub>; ● M2nn<sub>23</sub>).

#### Protective and antibody responses in chickens infected with caspase virus mutants

In order to investigate the antibody responses induced by caspase mutant viruses, 11-day-old chickens were given a primary boost with 50-100 pfu of either the NPgd, M2nn<sub>23</sub> or M2nn<sub>87del</sub> mutant virus. On day 20 after the booster infection chickens were challenged with a high, lethal dose ( $10^3$  LD<sub>50</sub> /chicken) of field FPV virus. Sera from these birds were collected on day 10 after challenge infection and analyzed in the HAI test. First, chickens vaccinated with NPgd or M2nn<sub>87del</sub> caspase mutants did not display any signs of disease after challenge infection; in the M2nn<sub>23</sub>-boosted group chickens had minor transient symptoms of disease during days 3-6 after challenge and fully survived after challenge; placebo non-boosted birds rapidly died during 3-5 days after FPV challenge. Second, chickens initially infected with caspase mutant viruses displayed high HAI antibody titers that reached values of

$2^9$ - $2^{12}$  on day 10 after challenge infection. High antibody titers were retained in chickens for at least 30 days (the period of observation). There was a clear correlation between the booster virus dosage and the antibody titer developed in birds; the higher the booster dose of virus inoculated, the higher the post-challenge antibody titers appearing in the blood of birds (Table 3). Thus, these data show that non-lethal booster infection with caspase mutant viruses induced high antibody titers effectively protecting chickens against a lethal dose of field virus.

**Table 3.** Antibody responses in chickens depends on virus booster dose

Dose of booster virus per chicken (*)	Antibody titer after challenge infection (**)
$10^4$ p.f.u	$2^{14}$ $2^{13}$ $2^{12}$
$10^3$ p.f.u	$2^{11}$ $2^{11}$ $2^{12}$
$10^2$ p.f.u	$2^{10}$ $2^9$ $2^8$

(\*) Groups of 3 11-day-old chickens were intramuscularly infected with different doses of M2n<sub>87</sub>del virus (booster infection). 20 days after booster all chickens were infected with  $10^3$  p.f.u. of wild type A/FPV/Ro/34 virus (challenge dose). (\*\*) On day 9 after challenge specimens of blood were taken and serum antibody titers were determined by HAI test. Serum dilution factor (the last serum dilution inhibiting reference virus agglutination) was outlined in the column.

## DISCUSSION

This study shows that almost all proteins of influenza A viruses share caspase cleavage motifs. Similar caspase motifs were identified in proteins of influenza B and C viruses. Thus, the wide distribution of caspase motifs in viral proteins seems to be a universal property of the orthomyxovirus family. It implies that caspase motifs in viral proteins can play a significant role in virus biology and pathogenicity. This role, as shown here, could be linked with virus replication as well as with the regulation of mechanisms involved in virus pathogenicity in the host organism. Certainly, reduced virus replication in animals infected with caspase mutants facilitated survival of these animals. However, the primary reason for animal survival after infection with caspase mutants seems to be linked with the virus's inability to overcome (or to switch off) host defense mechanism(s) in the infected organism. This virus-host interplay is not yet clear but it is tempting to speculate that caspase cleavage motifs in the viral proteins can be recognized by host cell ligands, such as caspases or granzymes, to initiate their interaction and subsequent signaling of caspase-dependent pathways responsible, for example, for inflammasome orchestration in pathogen-provoked inflammation process (Martinon and Tschoop, 2007). According to our data, these host factors could be

recruited either by M2 or NP caspase motifs to switch on/off host pathways interfering with virus immunopathogenesis. These hypothetical mechanisms of host pathway signaling by viral caspase motifs remain to be discovered.

We previously found that influenza virus proteins NP and M2 possess short specific sequences characteristic for caspase cleavage (caspase cleavage motifs) specifically located in the terminal regions of these viral proteins (Zhirnov et al, 1991; 2001). Cleavage of these sites was shown to produce truncated apoptotic forms aNP and aM2 in infected cells at the terminal apoptotic stage. Earlier the NP caspase motif ETD16/G in human influenza virus A/WSN/33 (H1N1) was altered to the "avian" type ETG/G (NPdg) and the generated chimeric NPdg virus mutant was found to display low reproduction and quick reversion to wild "human" genotype after 1-2 passages in cultured cells and hen eggs (Zhirnov et al, 2003). As shown here, the opposite "human-like" mutation G16D in avian FPV NP failed to reduce virus replicative potential. However, both the "avian" D16G mutation in human virus NP (Lipatov et al, 2006) and the "human" G16D mutation in avian virus, as shown here, markedly attenuated virus virulence. Thus, these data show that caspase-dependent cleavage of different viral proteins, such as NP and M2, may play opposite up- or down-regulating roles, respectively, in virus attenuation. Whether the caspase-dependent cleavage of NP performs the attenuation function or this N-terminal NP caspase motif by itself is responsible for the attenuation effect is not known and remains to be discovered. Another caspase motif at the C-terminus of NP could probably play an important role in virus replication and, for this reason, its alterations were found to be restrictive for this process. It seems likely that the caspase site in the NP C-terminus is mainly involved in the virus replication process; whether or not this motif plays a role in virus pathogenesis remains to be discovered.

Influenza A viruses carry M2 protein characterized by the caspase cleavage motif VD~~V~~DD87↓G at the C-terminal cytoplasmic tail and DSSD↓P in the N-terminal extracellular domain. Both motifs are universal and widespread among all human and avian influenza A viruses. Here we show that mutation in the cytoplasmic caspase motif does not disturb the reproductive abilities of virus in cultured cells and hen eggs but significantly reduces virus dissemination and lethality in chickens. The mechanism behind such a dramatic effect of the M2 cytoplasmic caspase site on virus virulence is not yet clear. Earlier it was found that the M2 cytoplasmic tail was involved in the virus assembly process and that extended deletions caused growth defects in cultured cells (McCown and Pekosz, 2005; 2006; Iwatsuki-Horimoto et al, 2006). It seems likely that mutation in this M2 caspase site attenuates virus in chickens due to its aberrant interaction with host caspase(s) with resulting abnormal immunopathogenesis during the infection process, rather than simply as a result of defective assembly of virus taking place in target cells in chicken organs.

Attenuation of virus virulence via alteration of caspase motifs in the viral proteins NP and M2 opens a new

avenue for the design of live vaccines. This caspase motif-targeted vaccine approach is applicable for influenza A, B, and C viruses and has several advantages. First, point mutations in the NP, and especially in the M2, caspase motifs markedly reduced virus virulence and rendered virus non-lethal for chickens. Second, only a single injection of a low virus dose induced high titers of antiviral antibodies reliably protecting animals against the field virus. Third, virus mutants containing altered caspase motifs in M2 and NP were relatively stable and retained these mutations during passage in cultured cells and chickens. Additional attenuation of caspase mutants to enhance safety of a proposed vaccines is possible: (i) through complex mutations in the caspase motifs of several viral proteins; (ii) via modification of the cleavage site in the HA protein to exchange the activating protease sensitivity of the HA (Stech et al, 2005; Gabriel et al, 2007); (iii) through a deletion of the anti-interferon NS1 protein, attenuating virus growth in different organisms (Ferko et al, 2004; Richt et al, 2006); (iv) via modification of viral polymerase proteins restricting virus replication and pathogenicity in certain hosts (Subbaro et al, 1993; Gabriel et al, 2005; Newmann and Kawaoka, 2006). This type of multi-attenuated virus should be considered as a safe effective candidate for a live vaccine against influenza, including the threatening avian flu H5N1.

## CONCLUSIONS

Caspase cleavage motifs are widely distributed in the proteins of influenza A, B, and C viruses. Alterations of these caspase motifs lead to attenuation of virus pathogenicity in the host organism. Artificial modification of viral caspase motifs opens a new avenue in the design of antiviral vaccines.

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## COMPETING INTERESTS

None declared.

## LIST OF ABBREVIATIONS

FPV: Fowl plague virus  
 HA: Hemagglutinin  
 NP: Nucleocapsid protein  
 M1: Matrix protein  
 M2: Ionic channel protein  
 PB1: Polymerase basic protein 1  
 PB2: Polymerase basic protein 2

PA: Polymerase acidic protein  
 pfu: Plaque forming unit  
 hpi: Hours post-infection  
 WTRG: Wild type virus generated by reverse genetics  
 MDCK: Madine Derby canine kidney cells; CACO-2: Human epithelial colon carcinoma cells  
 WB: Western blot  
 HAI: Hemagglutination inhibition test

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