N-terminal region of the PB1-F2 protein is responsible for increased expression of influenza A viral protein PB1

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Summary. – Influenza A virus (IAV) PB1-F2 protein is encoded by an alternative reading frame (+1) within the PB1 gene. PB1-F2 has been shown to contribute to the pathogenesis of influenza virus infection as well as to the secondary bacterial infection. More recently has been shown that PB1-F2 protein may regulate a viral RNA (vRNA) polymerase activity by the interaction with PB1 protein. We proved that PB1-F2 protein increased the level of expression of PB1 protein and vRNA in the infected cells. Moreover, we demonstrated that a higher level of vRNA expression resulted in the increase of expression of multiple viral proteins, including NP, M1, and NS1. Finally, we used plasmids expressing N-terminal (1–50 aa) or C-terminal (51–87 aa) region of the PB1-F2 molecule for transfection of MDCK cells co-infected with influenza A/Puerto Rico/8/34 (H1N1) virus deficient in the PB1-F2 protein expression (PR8ΔPB1-F2). These experiments clearly showed that N-terminal region of PB1-F2 protein was responsible for the increase in PB1 protein expression. C-terminal region of PB1-F2 protein had no effect. Thus, we have identified the important function for N-terminal region of PB1-F2 protein.

Keywords: influenza virus; N-terminus; PB1-F2; PB1; vRNA polymerase

Introduction

IAV is ssRNA virus with segmented genome of negative polarity. IAV plays an important role as the major human and animal respiratory pathogen, which infects millions persons and causes death of half million people each year. Although influenza A virus is commonly known as the cause of annual influenza epidemics in humans, wild aquatic birds represent a natural reservoir of this virus. Occasionally, avian influenza A viruses cross the species barrier into humans. A pandemic may arise if such viruses are able to spread efficiently from human to human and cause worldwide morbidity and mortality. The introduction of a virus of H1N1 subtype in the human population in 1918 is probably the best-known example of a pandemic with devastating consequences (de Wit *et al.*, 2008). During a systematic search for peptides recognized by CD8+T lymphocytes and encoded by alternative positive strand open reading frames (ORFs) of IAV strain A/PR/8/34 (H1N1) (PR8), (Chen *et al.*, 2001) reported the existence of a novel 87-aa protein representing the 11th defined IAV gene product. Since this protein is encoded by the second (+1) ORF of the PB1 gene, it was designated PB1-F2. The novel protein is encoded by nt 120–381 in the PB1 genomic segment (Chen *et al.*, 2004) and according to the Kozak´s analysis PB1 is the only gene of A/Puerto Rico/8/34 that does not possess a purine (adenine or guanine) nucleotide in the -3 position relative to +1 nucleotide of ORF of PB1 gene. Therefore translation initiation of PB1 is inefficient. Next initiation ATG codon surrounded by exact matching Kozak sequence is serving for PB1-F2 synthesis. The PB1-

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F2 ORF is present in most IAVs isolates typically encoding a predicted protein of 90 amino acids. Some IAV isolates, particularly those of human, avian or swine origin with hemagglutinin (HA) of H1 or H9 subtypes encode a C-terminally truncated PB1-F2 of various lengths (Pančuchárová and Russ, 2006). In addition to its mode of translation, PB1-F2 has several unique features. These include its absence from some IAV isolates (Zell *et al.*, 2007), variable expression in the individual infected cells, rapid degradation, mitochondrial localization, formation of a nonselective ion channel (Chen *et al.*, 2001, Yamada *et al.*, 2004, Henkel *et al.*, 2010), and apoptotic or pro-apoptotic properties. PB1-F2 localization in the mitochondrial membrane is mediated via C-terminal basic amphipatic alpha-helix (Gibbs *et al.*, 2003). The N-terminal portion of PB1-F2 might determine the cell type specificity of the proapoptotic function of the C-terminal mitochondrial translocating signal (Zamarin *et al.*, 2005). Further studies using mouse models support a role for PB1-F2 in the pathogenicity and lethality (de Wit *et al.*, 2008) probably by disrupting a function of alveolar macrophages (Coleman, 2007). IAVs knocked out for the expression of PB1-F2 were not attenuated in the replication in tissue culture, but their pathogenicity and lethality for mice was considerably reduced. Also, the PB1-F2-knockout viruses were cleared from the lungs more rapidly and induced earlier immune response to the infection (Zamarin *et al.*, 2006) implying that PB1-F2 played a role in suppression of immune response responsible for the viral clearance. PB1-F2 enhances inflammation during the primary viral infection of mice and increases both the frequency and severity of secondary bacterial pneumonia (McAuley *et al.*, 2007). The most recent studies show that although PB1-F2 is not required for the viral infectivity, it interacts directly with PB1. The absence of PB1-F2 results in an altered localization of PB1 (Mazur *et al.*, 2008) and decreased polymerase activity. In the present study we confirmed these observations. Moreover, our findings clearly demonstrated that not only expression of PB1 protein was enhanced by PB1-F2, but other viral proteins were affected too. Finally we demonstrated that for these enhancement was responsible N-terminus of PB1-F2 only.

Materials and Methods

Cells and viruses. Madin-Darby Canine Kidney cells (MDCK) were grown in DMEM (Dulbecco´s Modified Eagle´s Medium, BioWitthaker) supplemented with 5% FBS (HyClone), gentamicine 40 μg/ml at 37°C, 5% CO_2 in cell incubator. Allantoic fluid containing influenza virus A/Puerto Rico/8/34 (H1N1) (PR8) and A/Puerto Rico/8/34 (H1N1) conferring site mutation in the position T120C, C153G on the 2 segment (PR8ΔPB1-F2) was kindly provided by Jack R. Bennink and Jonathan W. Yewdell (Laboratory of Viral

Diseases, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, USA), and was prepared by Peter Palese.

Expression plasmids preparation. Allantoic fluid containing PR8 virus served as source of genetic information for all the cloning procedures. RNA was extracted with NucleoSpin® RNA II Purification Kit (Macherey-Nagel) by the manufacturer instructions. RNA was reverse-transcribed using M-MuLV Reverse Transcriptase (Fermentas) and subjected to PCR using Phusion® High-Fidelity PCR Master Mix (Finnzymes) with primers designed on sequence GenBank database AC: EF467819. Upstream primer 5′-<u>GACGACGACAAG</u>ATGGGACAGGAACAGGATA CAC-3’ and downstream primer 5′-<u>GAGGAGAAGCCCG GT</u>TCTACTCGTGTTTGCTGAACAAC-3′ in the case of PB1-F2, and upstream primer 5′-<u>GACGACGACAAG</u>AT GGATGTCAATCCGACCTTACTTTT-3′ and downstream primer 5′-<u>GAGGAGAAGCCCGGT</u>CTATTTTTGCCGTCT GAGCTCTTCAA-3′ in the case of PB1. For PCR amplification and cloning of the N-terminal part of PB1-F2 upstream primer 5′-<u>GACGACGACAAG</u>ATGGGACAG GAACAGGATACAC-3′ and downstream primer 5′-<u>GAG GAGAAGCCCGGT</u>**CTA**AAACCACTTGGTTCATTGTCT TCT-3′ with introduced stop codon (bold) were used since for C-terminal part of the PB1-F2 cloning upstream primer 5′-<u>GACGACGACAAG</u>ATGCCCAAACAGATTGTG TATT-3′ and downstream primer 5′-<u>GAGGAGAAGCCCG GT</u>TCTACTCGTGTTTGCTGAACAAC-3′ were used. pTri Ex™-4 Ek/LIC Vector Kit (Novagen) was used for the cloning of amplified PCR products by the recommendations of manufacturer. Underlined parts of the primers represents overhangs necessary for cloning strategy. DNA sequencing was used for the cloning correctness confirmation of the resulted expressing plasmids. Immunofluorescence was used for the confirmation of expression ability.

Cell transfection. MDCK cells propagated on 24-well plates to 70–80% confluence. TurboFect™ *in vitro* Transfection Reagent (Fermentas) was used as recommended by the manufacturer and after 24 hrs post transfection (p.t.) the samples were used in further experiments.

Polyclonal and monoclonal antibodies. Polyclonal rabbit serum specific for the N-terminal part of PB1-F2 was prepared by the intraperitoneal immunization of the rabbit with peptide corresponding to the position 3–13 aa of the PB1-F2 conjugated to keyhole limpet hemocyanin as a carrier protein. Polyclonal rabbit serum raised against full-length PB1-F2 (PR8) was kindly provided by John Yewdell, Bethesda, NIH. Polyclonal serum against PB1 protein was prepared by the DNA vaccination. Briefly, six to eight-week old female Balb/c mice were vaccinated intramuscularly three times in two weeks interval with 100 μg DNA of the pTriEx4 PB1. Complete immune mouse serum (IMS) was prepared by intraperitoneal immunization of the female Balb/c mouse with whole purified virus PR8. Ten days after second dose, the blood was collected and serum was obtained. As a loading control, the detection of the β-actin was used by the mouse anti-β-actin monoclonal antibody (Sigma Aldrich). Monoclonal antibody (MAb)

AG55 anti-PB1-F2 (Krejnusová *et al.*, 2009) and MAb 107 anti-NP (Varečková *et al.*, 1995) were prepared by the standard hybridoma procedure. MAb M21 anti-M1 and MAb NS1 anti-NS1 were kindly provided by John Yewdell, Bethesda, NIH. Polyclonal rabbit anti mouse IgG FITC conjugate (Dako) and polyclonal goat anti-rabbit 594 alexa fluor (Invitrogene) were used as secondary antibodies in the indirect immunofluorescence. Polyclonal rabbit anti-mouse HRP conjugate (Dako) was used in Western blot analyses and immunoprecipitation assay.

Indirect ELISA. 96-well plate was coated with 200 ng of purified PR8 per well overnight, washed, and blocked with 1% of BSA (Sigma-Aldrich), washed twice with 0,5% Tween 20, incubated 90 mins at RT with primary antibody, washed as previously, and incubated with secondary antibody conjugated with HRP for 90 mins at RT. After washing, OPD (o-phenylenediamine dihydrochloride) containing solution with hydrogen peroxide was added as a chromogenic substrate and the reaction was stopped with 2M H_2SO_4 5 mins later. Absorbance at 492 nm was measured.

Western blot. Cells for Western blot analysis were lysed directly on the Petri dishes with 5x sample buffer, loaded onto a 12% SDS PAGE gel. Separated proteins were transferred on the nitrocellulose membrane. The blot was then washed in PBS, blocked overnight in 5% nonfat dried milk at 4°C, incubated with primary antibody for 90 mins at RT, washed in PBS tree time for 15 mins, incubated for 90 mins with secondary antibody at RT, washed three times in 0.2% NP-40 in PBS, developed with luminol (Sigma-Aldrich) solution, and exposed to the photography film.

Co-immunoprecipitation detected by the Western blot. The cells were lysed with ProteoJET™ Mammalian Cell Lysis Reagent (Fermentas). Cell lysates were incubated for 1 hr with specific antibody at RT and subsequently with protein G Sepharose 4 Fost (Pharmacia LKB) beads for 30 mins. After successive multiple washing with PBS, the samples were subjected to SDS-PAGE, 12% gel and the separated proteins were transferred on the nitrocellulose membrane and probed with the specific antibodies. None cross linking agent was used in this study.

Indirect immunofluorescence. MDCK cells were grown on glass cover slips to 60–70% confluence. The cells were transfected with plasmid DNA using Turbofect (Fermentas) or infected with PR8ΔPB1-F2 with MOI. 10 PFU or transfected and co-infected with PR8ΔPB1-F2. 24 hrs p.t. and 3–7 hrs post infection (p.i.), the cells were fixed with 3% paraformaldehyde (Sigma) in PBS for 10 mins and permeabilised with 1% Triton X-100 (Koch-Light) for 60 secs. Samples were washed three times with PBS and incubated with the mixture of the primary antibodies for 1 hr at room temperature. Subsequently, washing step was repeated as described previously, and the cells were incubated for 1 hr with the mixture of secondary antibody. After final wash step, the samples were mounted with DAPI (4′,6-diamino-2-phenylindole) containing mounting medium (Santa Cruz Biotechnologies). Fluorescence was visualized with confocal microscope LSM Zeiss 510 Meta.

Quantitative PCR. For vRNA quantification, the whole RNA from PR8 or PR8ΔPB1-F2-infected or transfected and co-infected MDCK cells was extracted with Total RNA Isolation Kit (SbsBio). The acquired RNA was reverse-transcribed as described previously. Quantification was performed in triplicate for each sample on Quantica real time thermal cycler (Techne) using Dynamo HS cyber green QPCR kit (Finnzyme) with upstream primer NS1 For 5′-GCTGGAAAGCAGATAGTGGA-3′, downstream primer NS1 Rev 5′-GCCTGGTCCATTCTGATACA-3′ (AC: CY035146). For normalization purpose, the β-actine was used with upstream primer Betacanis For 5′-CTGAATC CCAAAGCCAACCGT-3′ and downstream primer Betacanis Rev 5′-CCCCTCGTAGATGGGCACAGTG-3′ (AC: NM_001003349). Cycling conditions were 95°C for 10 mins, 94°C for 15 secs, 58°C for 30 secs, 72°C for 30 secs repeated forty times, 72°C for 5 mins. After final polymerization a melting curve analysis was performed. The Relative Standard Curve Method was applied to the statistical data evaluation.

Results and Discussion

DNA vaccination pTriEx4 PB1

For DNA vaccination, the PB1 gene was cloned under the optimal regulatory sequence of pTriEx4 plasmid (Novagene) for efficient and strict translation initiation from the PB1 start codon. Since the cloning strategy of the gene for PB1 include complete PB1-F2 ORF, it was necessary to examine whether this serum did not contain in addition to the PB1 antibodies also antibodies specific for PB1-F2. As expected, the presence of anti-PB1-F2 antibodies in the anti-PB1 serum was excluded by ELISA and Western blot (data not shown). By DNA vaccination (DNAv) of the mice with pTriEx4 PB1 (a plasmid expressing full-length PB1 protein of the PR8 virus), it was possible to acquire an antiserum that was highly specific. Antiserum was excellent reagent in the immunofluorescence, Western blot, and ELISA allowing detection of PB1 protein in the biologically complex samples.

PB1-F2 increases the PB1 protein expression

It was known that PB1-F2 might affect the vRNA polymerase activity, PB1 accumulation and virus replication (Zamarin *et al.*, 2006; McAuley *et al.*, 2010b). We prepared cells transfected with pTriEx4 PB1-F2 plasmid. Later 24 hr p.t. these cells and nontransfeted cells were infected with PR8ΔPB1-F2. Comparison of these cells clearly showed that in cells expressing PB1-F2 the level of PB1 was much higher (Fig. 1). It is important to note that mutation in PB1-F2 start codon could lead to altered expression of the truncated form of PB1 protein called N40. Although N40 over-expression was not shown to alter viral replica-
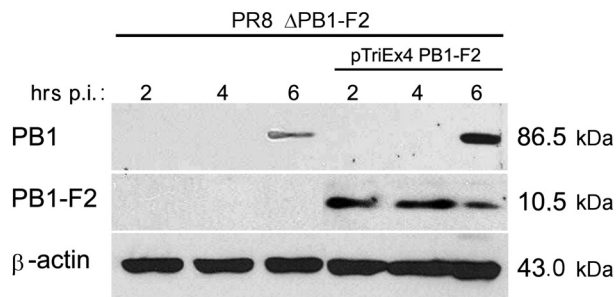
Fig. 1

Effect of the PB1-F2 presence or absence on expression level of the PB1 protein respectively

MDCK cells were infected with PR8ΔPB1-F2 or transfected with pTriEx4 PB1-F2 and 24 hours post transfection (hrs p.t.) cells were infected with PR8 ΔPB1-F2. Over expression of the PB1-F2 protein in cells infected with PR8ΔPB1-F2 virus dramatically increases PB1 protein level. As a loading control detection of the β-actin was used.
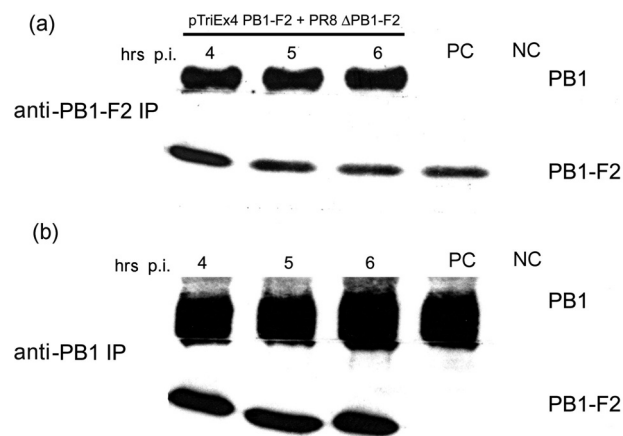

Fig. 2

Reciprocal co-immunoprecipitation of PB1 and PB1-F2

MDCK cells were transfected with pTriEx4 PB1-F2 and 24 hr p.t. infected with PR8ΔPB1-F2 and in the different time post infection samples were subjected to co-immunoprecipitation with anti-PB1-F2 (AG55) MAb and anti-PB1 serum. (a) In aliquots precipitated (IP) with AG55, PB1 was detected. Additionally PB1-F2 in the same sample was detected. (b) In aliquots precipitated with anti-PB1 serum both proteins were detected as well. As negative control for both cases neither transfected nor infected cells were used. As positive control a) pTriEx4 PB1-F2 transfected cells were used, b) PR8 infected cells were used.

tion (Wise *et al.*, 2009) it could complicate interpretation of results *in vivo* (McAuley *et al.*, 2010a) as well as overexpression of the PB1-F2 caused by transfection.

We confirmed direct interaction of PB1-F2 with PB1 by reciprocal co-immunoprecipitation without using of any cross-linking agents (Fig. 2). In contrast to Mazur *et al.* (2008) we believe that in the presence of PB1-F2 PB1 is not simply arrested in the nucleus in the late stage of the infection cycle (Mazur
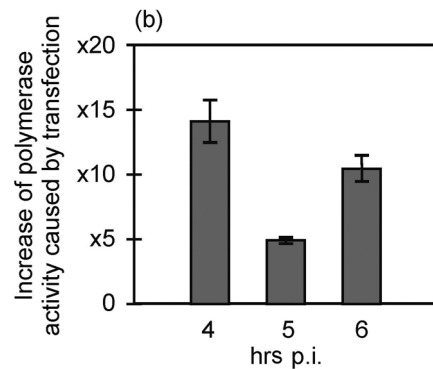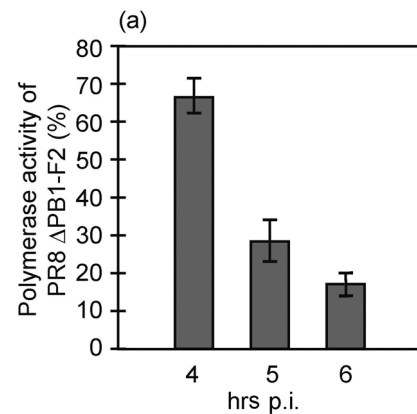

Fig. 3

Effect of PB1-F2 protein on vRNA catalytic activity

(a) In the absence of PB1-F2 protein in the cells infected with PR8 ΔPB1-F2 catalytic activity of vRNA polymerase rapidly decreases in the later stage of infection cycle. (b) MDCK cells were infected with PR8ΔPB1-F2 or transfected with pTriEx4 PB1-F2 and later (24 hrs p.t.) infected with PR8ΔPB1-F2. Detection of whole RNA from treated cells from different time is used to demonstrate differences in viral gene expression caused by over expression of the PB1-F2.

et al., 2008), but that PB1 is stabilized resulting in increase of viral polymerase in the cell and so increase viral polymerase activity. Notably it was shown that PB1 alone is able to catalyze mRNA synthesis in the absence of PB2 and PA subunits (Kobayashi *et al.*, 1996). Nevertheless, the differences in PB1 level in PR8ΔPB1-F2 infected cells and cells infected with PR8ΔPB1-F2 24 hrs p.t. with pTriEx4 PB1-F2 suggest that PB1-F2 plays an important role during the infection cycle.

PB1-F2 protein enhances vRNA polymerase activity

Next, we examined whether increased level indicating a stability of the PB1 protein could increase the catalytic activity of vRNA polymerase. Commonly used method for vRNA polymerase activity measurement is the construction

of a minireplicon with reporter gene. Unfortunately, the success of this approach depends on multiple steps such as the transcription initiation, mRNA stability, nuclear export, translation efficiency, folding, and degradation rate. When the reporter gene is luciferase or any other enzyme, the reaction conditions are important as well. We decided to use the most accurate and direct way to measure catalytic activity of the vRNA polymerase following total level of its product. Differences in the catalytic activity of vRNA polymerase in the presence or absence of PB1-F2 protein were measured in MDCK cells infected with PR8 (MOI 10PFU) or PR8ΔPB1-F2 (MOI 10 PFU) or transfected with pTriEx4 PB1-F2 and later 24 hrs p.t. infected with PR8ΔPB1-F2 (MOI 10 PFU) be QPCR. Non-structural NS gene obligatory expressed during infection was chosen for a quantification as suitable marker of vRNA production. All samples were normalized to β-actin RNA level. As the calibrator, expression of the NS gene during infection of MDCK cells with wild type PR8 virus was employed. At 4 hr p.i. the PR8ΔPB1-F2 catalytic activity of vRNA polymerase was decreased to 66% of activity of vRNA polymerase of the PR8 virus infection. At 5 and 6 hrs p.i. the decrease of catalytic activity went even more deep to 28% and 17% of vRNA polymerase catalytic activity during natural infection (Fig. 3a). Such decrease of vRNA polymerase catalytic activity in late stage of infection might lead to the insufficient vRNA production and viral

replication and might be responsible for the diminished virus pathogenicity and mortality in mice (Zamarin *et al.*, 2006). We were interested in the effect of PB1-F2 expression restoration by the transient transfection on vRNA polymerase catalytic catalytic activity. Viral RNA polymerase catalytic activity was about ten fold higher in the presence of PB1-F2 protein than in the absence (PR8 ΔPB1-F2) (Fig. 3b). Early after infection *de novo* synthesized cRNA seems to be degraded until sufficient amount of polymerase complex proteins and NP is produced to stabilize cRNA what in turn can serve as template for vRNA synthesis (Vreede *et al.*, 2004). We observed increase in RNA between 5 to 6 hrs p.i. which express changes in mRNA, cRNA and vRNA expression during the later stage of infection cycle (Dalton *et al.*, 2006). We assumed that for the increase in polymerase catalytic activity enhancement was responsible the quantity of enzyme, but not the properties of enzyme itself. Such explanation is supported by the PB1-F2 increase of PB1 protein.

PB1-F2 effect on the intracellular localization and expression of PB1 during infection cycle

Intracellular localization of PB1 and its relative amount changes in the presence and absence of the PB1-F2 were studied by confocal immunofluorescence microscopy. All the pictures were taken under the same condition and three independent
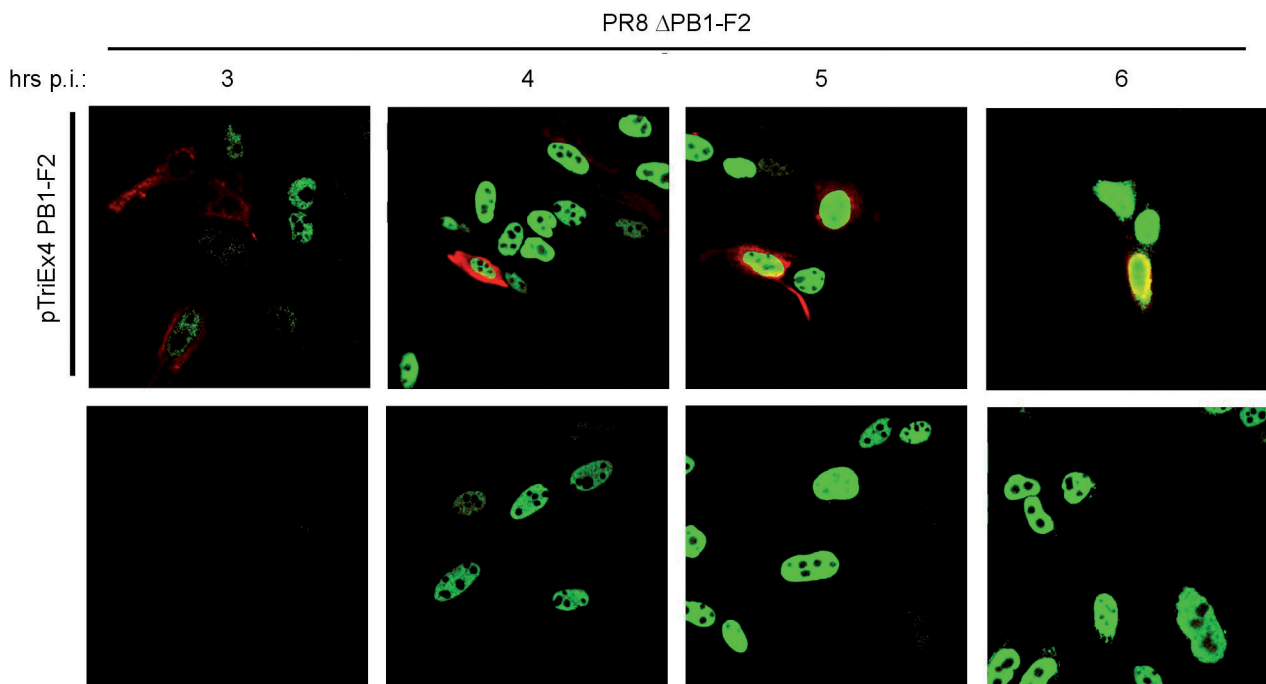

Fig. 4

Accelerated PB1 expression in presence of PB1-F2 during infection cycle

In the upper panel cells were transfected with pTriEx4 PB1-F2 and later (24 hrs p.t.) cells were infected with PR8ΔPB1-F2 (MOI 10 PFU). In the lower panel cells were infected with PR8ΔPB1-F2 (MOI 10 PFU). Cells were stained for presence of PB1 (green) and PB1-F2 (red) proteins.
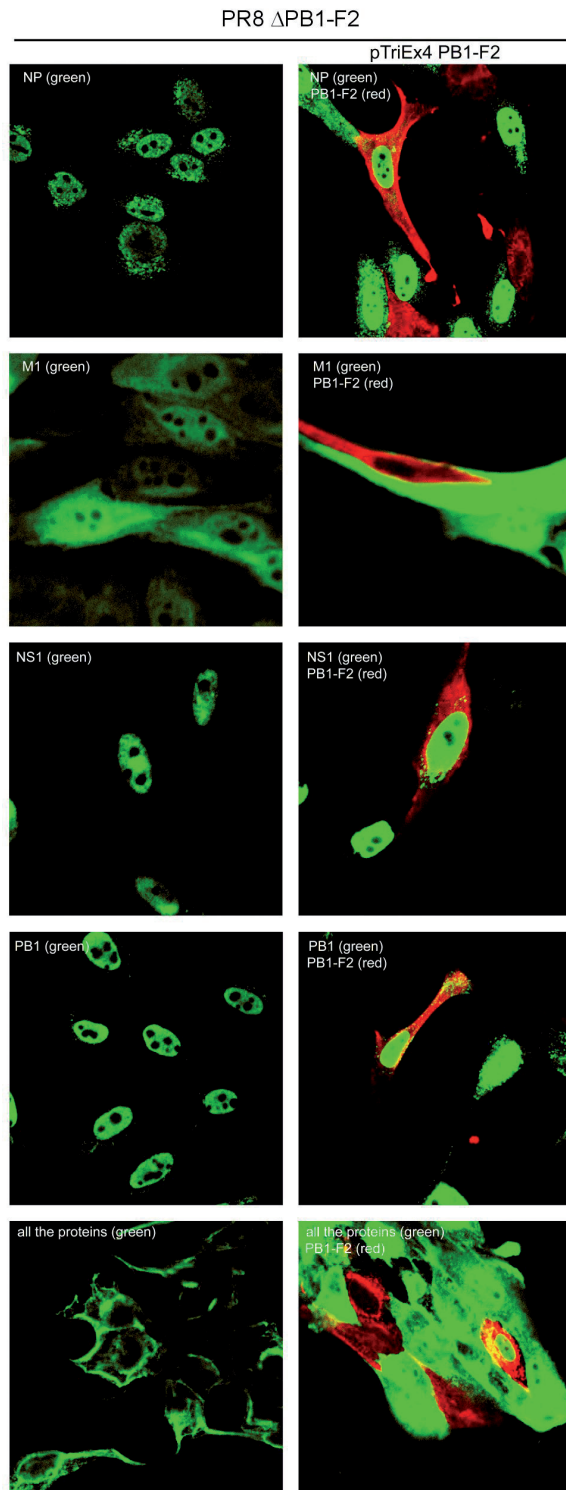
PR8 ΔPB1-F2

Fig. 5

Effect of PB1-F2 on expression of the other viral proteins

Cells were infected with PR8ΔPB1-F2 (MOI 10 PFU, left row) or transfected with pTriEx4 PB1-F2 and later (24 hrs p.t.) infected with PR8ΔPB1-F2 (MOI 10 PFU, right row). 7 hrs p.i. expression of NP, M1, NS1, PB1 (green) were detected in absence or presence PB1-F2 (red) respectively. Additionally the cells were also analyzed with antibodies induced by whole purified virus.

experiments were done with the same results. Transfection efficiency of pTriEx4 PB1-F2 in this study has reached up 30% to 50% in agreement with variable expression of PB1-F2 after natural infection (Chen *et al.*, 2001). Similarly to other groups (Zamarin *et al.*, 2005) we have observed that obvious effect of PB1-F2 expression on PB1 protein level was not only restricted to PB1-F2 positive cells, but also to nearly all the cells . This was common feature for all the fluorescence microscopy experiments in this study. Probable explanation for this could be PB1-F2 released from either transfected or infected damaged cells and successively affect surrounding cells, but detailed mechanism remains elusive. When the cells contained PB1-F2 protein, we were able to detect a significant amount of PB1 polymerase subunit exclusively in the nucleus as early as 3 hrs p.i., but only traces of the PB1 were visible in the cells infected with PR8ΔPB1-F2 virus. At 5 hrs p.i., a substantial difference in PB1 level protein was observed with partial co-localization of PB1-F2 in the nucleus. Finally, at 6 hrs p.i. an extensive co-localization of PB1-F2 with PB1 occurred within cytoplasm. At this time, the cytoplasm was already packed with PB1 protein (Fig. 4). In contrast to the Mazur and colleagues (Mazur *et al.*, 2008), who suggested that PB1 detained PB1-F2 in the nucleus, we suppose that PB1-F2 somehow directly or indirectly stabilized PB1 in the earlier stages of infection cycle, what could lead to increased vRNA polymerase catalytic activity, and prolonged nuclear localization in the later stages of infection cycle. There was no indication that PB1-F2 was present in the nucleus later during the infection cycle.

Increased expression of multiple viral proteins in the presence of PB1-F2

When vRNA polymerase catalytic activity was increased in the presence of PB1-F2, more mRNA should be produced and in turn the infected cells should contain not only more PB1 protein, but other viral proteins as well. To examine this hypothesis, the MDCK cells were infected with PR8 ΔPB1-F2 (MOI 10 PFU) or transfected with pTriEx4 PB1-F2 and later (24 hrs p.t.) cells were infected with the same MOI of PR8ΔPB1-F2. Expression of several viral proteins was compared at 7 hrs p.i. by the confocal immunofluorescence microscopy under the same conditions of exposure time, pinhole size atc. (Fig. 5). Expression of all analyzed proteins NP, M1, NS1, and PB1 was higher in the cells expressing PB1-F2. As NP and M1 did not directly interact with PB1-F2 (Mazur *et al.*, 2008), our results supported the following suggestion. PB1-F2 enhanced the expression of other viral proteins. For the relative quantification of the majority of viral proteins (e.g. hemagglutinin, neuraminidase), the immune mouse serum raised by immunization with the purified virus was used. In the lowest left panel (Fig. 5) is apparent that most of the detected viral proteins were concentrated to the space around the nucleus and intracellular membranes detecting the presence of hemagglutinin and neuraminidase in the Golgi

apparatus and cell surface. The lowest right panel showed the nucleus and cytoplasm packed by the viral proteins in cell transfected with pTriEx4 PB1-F2.

N-terminus of PB1-F2 alone increases the expression of PB1

Among IAV strains the ORF of PB1-F2 is highly conserved. Zell and colleagues (Zell *et al*., 2007) studied 2226 entries of second segment deposited in the Genebank and found that regardless of sequence origin, 87% of entries encoded for PB1-F2 polypeptide were greater than 78 aa. From 6720 aa sequences of the PB1-F2 accessible from Genebank database to date, 76.1% contain ORF that is 90 aa long and evolutionary selection seems to force to the C-terminal truncation and N-terminal preservation of PB1-F2, since only 19 of all entries possess ORF shorter than 50 aa. C-terminal region was shown to be responsible for the mitochondrial localization via basic amphipatic helix (Gibbs *et al*., 2003), change in mitochondrial morphology (Yamada *et al*., 2004), interaction with ANT3 protein (Zamarin *et al*., 2005), priming of the bacterial infection (McAuley *et al*., 2007) and major oligomerization domain (Bruns *et al*., 2007) Importance of N-terminal region of PB1-F2 is supported by the fact that in contrast to C-terminally truncated PB1-F2, N-terminally truncated

PB1-F2 is not known or extremely rare (Zell *et al.,* 2007). With respect to IAV pandemic potential (Perez-Padilla *et al*., 2009), we decided to study which portion of PB1-F2 is responsible for the observed PB1-F2 effect on viral gene expression. N-terminal portion (1–50 aa) as long as it naturally occurs between IAV strains and C-terminal portion (51–87 aa) of the PB1-F2 of the IAV PR8 were cloned and used for the confocal immunofluorescence microscopy. The same conditions for capturing images were applied. MDCK cells were either infected with PR8ΔPB1-F2 or transfected with plasmid expressing C-portion or N-portion of PB1-F2 protein and 24 hrs p.t. infected with PR8ΔPB1-F2. At 7 hrs p.i., the cells were stained for the presence of PB1, C-terminal, and N-terminal part of the PB1-F2 (Fig. 6). Expression of PB1 in the MDCK cells transfected with pTriEx4 C-terminal part of the PB1-F2 was equal to the mock transfected cells, but it was dramatically elevated in the MDCK cells, which contained N-terminal part of the PB1-F2. Thus, we provide evidence that N-terminal portion of the PB1-F2 was capable to elevate expression of the PB1 vRNA polymerase subunit directly and indirectly to force the expression of all viral proteins. This outcome could explain that the absence of PB1-F2 resulted in a reduced replication and spread of the virus (Mazur *et al*., 2008). In contrast to our findings, McAuley and colleagues (McAuley *et al*., 2010b) suggested that C-terminus of the PB1-F2
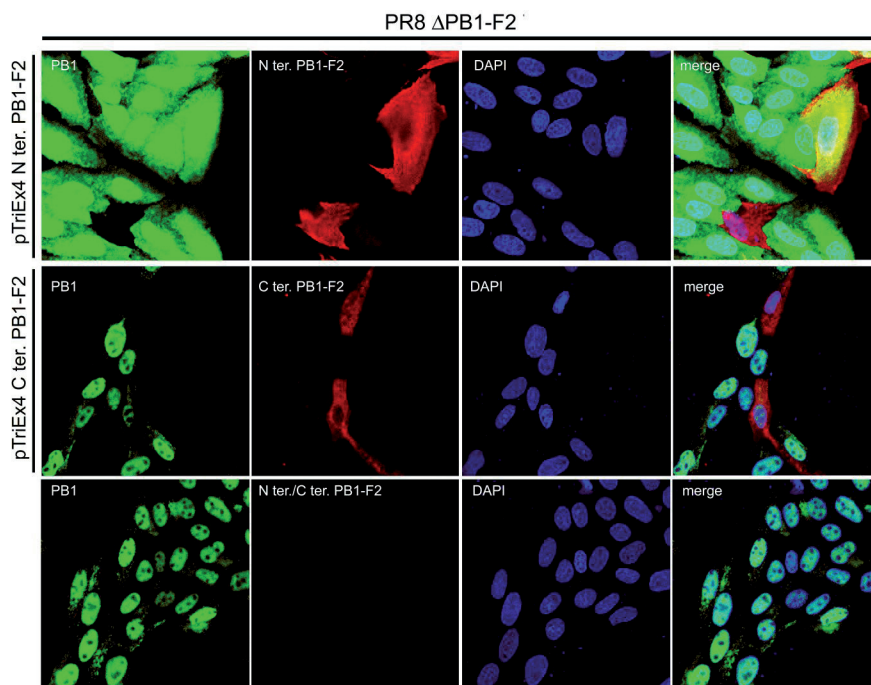

Fig. 6
Identification of PB1-F2 domain responsible for increased expression of the PB1 protein of the IAV
MDCK cells were either mock transfected or transfected with pTriEx4 C-terminal part of the PB1-F2 or pTriEx4 N-terminal part of the PB1-F2. 24 hrs p.t. all kind of cells were infected with PR8 ΔPB1-F2. 7 hrs p.i. cells were probed with antibodies specific for PB1, PB1-F2. Relative amount of PB1 protein under this condition was then observed. Dapi staining was used for nucleus determination.

interacted with PB1 with reference to Mazur (Mazur *et al.*, 2008) who did not address this question. Similarly as in the previous experiments, observed elevated expression of PB1 was not restricted only to N-terminal part of PB1-F2 positive cells. On the other hand cells transfected with plasmid expressing C part of the PB1-F2 did not present such phenotype what prove that observed effects are not caused by transfection agens. Our results indicate the significance even for C-terminally truncated PB1-F2 protein in the pathogenicity of the IAV.

References

Bruns K, Studtrucker N, Sharma A, Fossen T, Mitzner D, Eissmann A, Tessmer U, Röder R, Henklein P, Wray V, Schubert U (2007): Structural characterization and oligomerization of PB1-F2, a proapoptotic influenza A virus protein. J. Biol. Chem. 282, 353–363. doi:10.1074/jbc.M606494200

Chen GW, Yang CC, Tsao KC, Huang CG, Lee LA, Yang WZ, Huang YL, Lin TY, Shih SR (2004): Influenza A virus PB1-F2 gene in recent Taiwanese isolates. Emerg. Infect. Dis. 10, 630–636.

Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O´Neill R, Schickli J, Palese P, Henklein P, Bennink JR, Yewdell JW (2001): A novel influenza A virus mitochondrial protein that induces cell death. Nat. Med. 7, 1306–1312. doi:10.1038/nm1201-1306

Coleman JR (2007): The PB1-F2 protein of Influenza A virus: increasing pathogenicity by disrupting alveolar macrophages. Virol. J. 4, 9. doi:10.1186/1743-422X-4-9

Dalton RM, Mullin AE, Amorim MJ, Medcalf E, Tiley LS, Digard P (2006): Temperature sensitive influenza A virus genome replication results from low thermal stability of polymerase-cRNA complexes. Virol. J. 3, 58. doi:10.1186/1743-422X-3-58

de Wit E, Kawaoka Y, de Jong MD, Fouchier RA (2008): Pathogenicity of highly pathogenic avian influenza virus in mammals. Vaccine 26, D54–58. doi:10.1016/j.vaccine.2008.07.072

Gibbs JS, Malide D, Hornung F, Bennink JR, Yewdell JW (2003): The influenza A virus PB1-F2 protein targets the inner mitochondrial membrane via a predicted basic amphipathic helix that disrupts mitochondrial function. J. Virol. 77, 7214–7224. doi:10.1128/JVI.77.13.7214-7224.2003

Henkel M, Mitzner D, Henklein P, Meyer-Almes FJ, Moroni A, Difrancesco ML, Henkes LM, Kreim M, Kast SM, Schubert U, Thiel G (2010): The proapoptotic influenza A virus protein PB1-F2 forms a nonselective ion channel. PLoS One 5, e11112. doi:10.1371/journal.pone.0011112. doi:10.1371/journal.pone.0011112

Kobayashi M, Toyoda T, Ishihama A (1996): Influenza virus PB1 protein is the minimal and essential subunit of RNA polymerase. Arch. Virol. 141, 525–539. doi:10.1007/BF01718315

Krejnusová I, Gocníková H, Bystrická M, Blaskovicová H, Poláková K, Yewdell J, Bennink J, Russ G (2009): Antibodies to PB1-F2 protein are induced in response to influenza A virus infection. Arch. Virol. 154, 1599–1604. doi:10.1007/s00705-009-0479-5

Mazur I, Anhlan D, Mitzner D, Wixler L, Schubert U, Ludwig S (2008): The proapoptotic influenza A virus protein PB1-F2 regulates viral polymerase activity by interaction with the PB1 protein. Cell. Microbiol. 10, 1140–1152. doi:10.1111/j.1462-5822.2008.01116.x

McAuley JL, Hornung F, Boyd KL, Smith AM, McKeon R, Bennink J, Yewdell JW, McCullers JA (2007): Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. Cell Host Microbe 2, 240–249. doi:10.1016/j.chom.2007.09.001

McAuley JL, Chipuk JE, Boyd KL, Van De Velde N, Green DR, McCullers JA (2010a): PB1-F2 proteins from H5N1 and 20 century pandemic influenza viruses cause immunopathology. PLoS Pathog. 6, e1001014. doi:10.1371/journal.ppat.1001014

McAuley JL, Zhang K, McCullers JA (2010b): The effects of influenza A virus PB1-F2 protein on polymerase activity are strain specific and do not impact pathogenesis. J. Virol. 84, 558-564. doi:10.1128/JVI.01785-09

Pančuchárová H, Russ G (2006): PB1-F2 gene in influenza a viruses of different hemagglutinin subtype. Acta Virol. 50, 269–272.

Perez-Padilla R, de la Rosa-Zamboni D, Ponce de Leon S, Hernandez M, Quiñones-Falconi F, Bautista E, Ramirez-Venegas A, Rojas-Serrano J, Ormsby CE, Corrales A, Higuera A, Mondragon E, Cordova-Villalobos JA, INER Working Group on Influenza (2009): Pneumonia and respiratory failure from swine-origin influenza A (H1N1) in Mexico. N. Engl. J. Med. 361, 680–689. doi:10.1056/NEJMoa0904252

Varečková E, Betáková T, Mucha V, Soláriková L, Kostolanský F, Waris M, Russ G (1995): Preparation of monoclonal antibodies for the diagnosis of influenza A infection using different immunization protocols. J. Immunol. Methods 180, 107–116. doi:10.1016/0022-1759(94)00307-I

Vreede FT, Jung TE, Brownlee GG (2004): Model suggesting that replication of influenza virus is regulated by stabilization of replicative intermediates. J. Virol. 78, 9568–9572. doi:10.1128/JVI.78.17.9568-9572.2004

Wise HM, Foeglein A, Sun J, Dalton RM, Patel S, Howard W, Anderson EC, Barclay WS, Digard P (2009): A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. Virology 83, 8021–8031. doi:10.1128/JVI.00826-09 doi:10.1128/JVI.00826-09

Yamada H, Chounan R, Higashi Y, Kurihara N, Kido H (2004): Mitochondrial targeting sequence of the influenza A virus PB1-F2 protein and its function in mitochondria. FEBS Lett. 578, 331–336. doi:10.1016/j.febslet.2004.11.017 doi:10.1016/j.febslet.2004.11.017

Zamarin D, García-Sastre A, Xiao X, Wang R, Palese P (2005): Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. PLoS Pathog. 1, e4. doi:10.1371/journal.ppat.0010004

Zamarin D, Ortigoza MB, Palese P (2006): Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice. J. Virol. 80, 7976–7983. doi:10.1128/JVI.00415-06

Zell R, Krumbholz A, Eitner A, Krieg R, Halbhuber KJ, Wutzler P (2007): Prevalence of PB1-F2 of influenza A viruses. J. Gen. Virol. 88, 536–546. doi:10.1099/vir.0.82378-0