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Minireview

An insight into the PB1F2 protein and its multifunctional role in enhancing the pathogenicity of the influenza A viruses

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ABSTRACT

PB1F2 is the 11th protein of the influenza A virus. The protein has variable sizes with truncations either at the C- or N-terminal ends. The most recent example being the 2009 pandemic H1N1 virus which codes for only 11 amino-acids of the C-terminus. A review of the reports since the discovery of PB1F2 in 2001 suggests a multifunctional role for this protein that includes a proapoptotic function in immune cells and an ability to cause increased pathogenesis in animal models by dysregulating cytokines and inducing inflammation. It has also been suggested that PB1F2 regulates polymerase activity via co-localization with PB1 and causes enhanced secondary bacterial pneumonia. This review primarily focuses on understanding the proapoptotic ability of PB1F2, its sub-cellular localization and the mechanism through which it brings about apoptosis. We believe there is much more to learn about PB1F2, as many of its proposed functions are strain, host or cell-line specific.

Introduction

Influenza is an important and pathogenic viral disease infecting warm-blooded animals. This virus continuously evolves, thus causing the emergence of newer epidemics and pandemics in human swine, horses, dogs, cats, and other mammals. Aquatic birds are the natural reservoir for all the subtypes of influenza A virus and are most likely the definitive source of human pandemic influenza strains (Webster et al., 1992). Since 1500, there have been 14 or more influenza pandemics. In the past 133 years, during the so-called microbial era from 1876 to the present, there have been six pandemics. Those pandemics include the flu pandemics of 1889, 1918, 1957, 1968, and 1977, and the recent 2009 pandemic (Taubenberger et al., 2010). Compared to the pandemic influenza A strains, H5N1 viruses are more pathogenic and cause higher mortality, although they are not transmitted from human to human. Widespread and ongoing epizootic outbreaks of H5N1 viruses in Asia have increased the concern that this subtype may achieve human-to-human transmission and establish interspecies spread. Many transmission events involving H5N1 viruses to humans have been reported since 1997 (Lipatov et al., 2004). From 2003 until June 2012, six hundred and six cases of H5N1 human infection have been reported, of which 357 were fatal. To date, H5N1 has been reported in 15 countries. According to data available from up to June 2012, most cases of human infections with H5N1 virus have been reported in Indonesia and Egypt (WHO data, 2012).

Continuing evolution is most prominent in the surface glycoproteins of influenza viruses, but it also occurs in each of the eight gene segments of the virus. Among the eight segments, PB1 is of particular interest, as the PB1 gene is the only other segment that was exchanged in the pandemic viruses of 1957 and 1968 (Kawaoka et al., 1989). In addition, a novel PB1 gene was found in the 1998 swine reassortant viruses, implicating the gene's role in the pathogenesis of influenza (Karasin et al., 2000). Recent reconstruction of the 1918 virus has also confirmed that the viral polymerase is required for the pathogenicity of the recombinant 1918 virus in mice, and if it is replaced with the recent H1N1 polymerase genomic segment, the virus is attenuated in mice (Tumpey et al., 2005). Selection of the PB1 gene in the previous pandemic strains and its ability to enhance the pathogenicity and virulence of the influenza A viruses warrant further study of PB1 (Kawaoka et al., 1989; Zamarin et al., 2006; Chen et al., 2001). PB1 is encoded by segment 2 of the influenza viral genome and is a core component of the viral polymerase. The single mRNA transcribed from segment 2 encodes three proteins, PB1, PB1F2 and N40. PB1F2 is the second protein encoded by the +1 alternate open reading frame within the PB1 gene. The translation of the protein starts from the 4th initiation codon that is from nucleotide position 120 surpassing three other initiation codons of the PB1 gene (Chen et al., 2001). The fifth initiation codon of segment 2 is used to initiate translation of a third protein product

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of the PB1 gene, called PB1-N40. AUG5 of N40 is in frame with AUG1 of the PB1, and therefore, N40 is just a truncated form of PB1 that lacks the first 39 amino acids of the longer protein. N40 lacks the N-terminal region that is essential for the interaction of PB1 with PA (Wise et al., 2009).There is much to learn about the three translation products of PB1 mRNA, and it is believed that the three proteins are dependent on each other for their functions. Given the last outbreak scenario, wherein the emergence of the 2009 H1N1 influenza virus took public-health authorities by surprise, it is essential to gather in-depth knowledge about each of the viral proteins of the influenza A virus. There is a dearth of knowledge about and treatments for influenza. Thus, researchers need to develop effective vaccines and therapies, in addition to predicting newly emerging pandemic influenza viruses.

PB1F2 is a non-structural protein of the influenza A virus that was identified and characterized more than a decade ago by Chen et al. (2001). It was discovered in the A/PuertoRico/8/34 (H1N1) strain while screening for a antigenic peptides recognized by $CD8^+$ T lymphocytes (Chen et al., 2001). The PB1 gene has inefficient initiation of translation, as it does not possess a consensus Kozak sequence in that it does not have a purine nucleotide in the −3 position of the ORF. The fourth initiation codon is surrounded by an exact Kozak sequence and allows for the synthesis of PB1F2 (Košík et al., 2011). PB1F2 is absent in influenza B viruses. PB1F2 is exclusively expressed in infected cells with maximum expression at 5 h post-infection and is possibly not incorporated into the virion (Krumbholz et al., 2011). Many of the influenza A virus strains encode a full-length PB1F2 protein with 90 or 87 amino acids and a molecular weight of 10.5 kDa.

Structure of PB1F2

Chen et al. described that the novel PB1F2 has a propensity to form an amphipathic helix extending from the 69th amino acid (Leucine) to the 83rd amino acid (Phenyalanine) of the protein (Chen et al., 2001). Detailed study of the protein revealed that PB1F2 consists of two independent structural domains consisting of two close short helices at the N-terminus and an extended helix at the C-terminus. Both helical domains are connected by a flexible and unstructured hinge region (Bruns et al., 2007). The PB1F2 molecule has an intrinsic strong propensity to form oligomeric structures, a characteristic that supports the recent observation that the molecule can form membrane pores in planar lipid bilayers (Bruns et al., 2007; Henklein et al., 2005). The major oligomerization domain is located in the C-terminal helix (Bruns et al., 2007). In another study, PB1F2 was determined to belong to a group of intrinsically disordered proteins that can switch their conformation from a random to α-helical or β-sheet secondary structure depending on the environment. PB1F2 has also been reported to permeabilize cellular membranes; however, this ability is dependent on the amino acid sequence of the influenza A virus strain. The PB1F2 protein has also been shown to oligomerize and form amyloid fibers in infected cells. Amyloid fiber formation in the infected cells could give insight into the pathogenicity of the virus and also into the relationship of the influenza virus with nervous system disorders (Chevalier et al., 2010). PB1F2 has also been reported to be a phosphoprotein, wherein its function is regulated by protein kinase C (PKC). PKC phosphorylation sites have been mapped to amino acid positions 27 and 35 of the PB1F2 protein (Mitzner et al., 2009).

Varying length of PB1F2 protein

Varying sizes of the PB1F2 protein have been reported in different subtypes of influenza A viruses. A comprehensive analysis of the varying lengths of the PB1F2 protein from 20th-century pandemics and H5N1 subtypes was previously conducted by our group (Pasricha et al., 2012). The 1918 H1N1 virus responsible for the Spanish flu harbored a complete PB1F2 protein (McAuley et al., 2007). However, since 1949, most H1N1 virus strains have an incomplete PB1F2 protein with truncation either at the N- or C-terminal end (Zell et al., 2007; Pasricha et al., 2012). The N-terminal end of the protein is preserved in human hosts, while the C-terminal end is retained in swine (Table 1). Interestingly, the recent 2009 pandemic H1N1 virus harbors only an 11 amino acid C-terminal-truncated protein, which is thought to be non-functional. This indicates that this protein is not essential for the fitness of the H1N1 strain. The H2N2 subtype responsible for the 1957 pandemic (Asian flu) infected close to 250,000 people. Analysis of the 83 available PB1F2 sequences belonging to the H2N2 subtype revealed that 98.8% of the strains harbored a full-length PB1F2 protein (Table 1) and more than half of its amino acids were conserved in the strains. The circulating strains of H2N2 were replaced by H3N2 strains in 1968, which emerged as new pandemic strain (Hong Kong flu). The majority of the strains (94.27%) isolated during this pandemic also harbored a full-length PB1F2 protein (Table 1). An analysis of the length of PB1F2 protein in H5N1 strains indicates that it is comparable to that of the H2N2 and H3N2 subtypes, wherein a complete protein is found in 96% of the strains, suggesting that PB1F2 is positively selected in these subtypes and is definitely essential for the virus.

Cellular and humoral response to the PB1F2 protein

The PB1F2 protein is recognized by the human immune system and therefore has the ability to elicit both humoral and cell-mediated immune responses. The discovery of PB1F2 was based on its ability to generate a robust CD8+T cell response specific for a well-defined peptide encoded by residues 62-70 (LSLRNPILV) of the protein (Chen et al., 2001; La Gruta et al., 2008). PB1F2-specific antibodies have been detected in the sera of

Table 1
PB1F2 variants present in IAV strains H1N1, H2N2, H3N2 and H5N1 from various hosts.

S. no.	Strains/host	No. of analyzed strains	101aa	90aa	87aa	N57aa[a]	C52aa[b]	Varied size
1	**H1N1**	**1530**	**–**	**176**	**42**	**1080**	**221**	**11**
	Human	1155	–	32	41	1073	6	3
	Swine	261	–	43	1	7	202	8
	Avian	112	–	99	–	–	13	–
	Environment	1	–	1	–	–	–	–
	Others	1	–	1	–	–	–	–
2	**H2N2**	**83**	**1**	**81**	**–**	**1**	–	–
	Human	54	1	52	–	1	–	–
	Swine	–	–	–	–	–	–	–
	Avian	22	–	22	–	–	–	–
	Environment	1	–	1	–	–	–	–
	Others	6	–	6	–	–	–	–
3	**H3N2**	**2566**	**26**	**2105**	**45**	**–**	**337**	**53**
	Human	2419	25	1979	44	–	324	47
	Swine	76	–	63	1	–	7	5
	Avian	68	1	60	–	–	6	1
	Environment	2	–	2	–	–	–	–
	Others	1	–	1	–	–	–	–
4	**H5N1**	**919**	**–**	**886**	**2**	**4**	**21**	**6**
	Human	220	–	211	–	2	4	3
	Swine	13	–	13	–	–	–	–
	Avian	666	–	643	2	1	17	3
	Environment	16	–	15	–	1	–	–
	Others	4	–	4	–	–	–	–

[a] Fifty-seven amino-acid fragment with C-terminal end truncated.
[b] Fifty-two amino-acid fragment with N-terminal truncation.

mice infected intranasally with A/PR/8/34 (H1N1) virus and in human acute and convalescent sera collected during the H3N2 2003–2004 epidemic. This result provides evidence that PB1F2 is recognized by the immune system and can elicit an adaptive immune response, although only for a short duration (Krejnusová et al., 2009). Khurana et al. (2009) also detected antibodies against PB1F2 in the sera of convalescent patients recovering from the H5N1 infection caused by A/Vietnam/1203/2004 in Vietnam.

Multifunctional role of PB1F2 protein

Five main functions of the PB1F2 protein have been found: (i) direct or indirect role in apoptosis of the immune cells via mitochondrial pathway, (ii) exacerbation of pathogenicity in animal models, (iii) modulator of innate immune response in host cells both *in vitro* and *in vivo* model, (iv) regulation of polymerase activity by co-localization with PB1 and (v) enhancement of secondary bacterial pneumonia (Fig. 1).

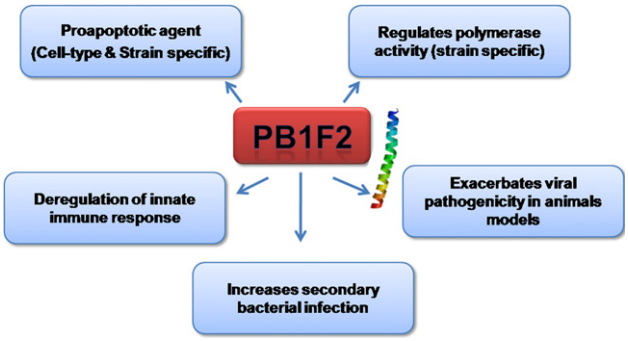

Fig. 1. Multifunctional role of PB1F2 protein.

Proapoptotic function of PB1F2

PB1F2 is a small protein that has an intrinsic property to localize to mitochondria and cause alterations in its morphology that ultimate dissipate the mitochondrial membrane potential, which induces apoptosis (Chen et al., 2001). Chen et al. (2001) demonstrated that the apoptosis was more pronounced in immune cells (macrophages), thus reducing the ability of the host to contribute to an immune response. Among the various apoptotic agents produced by influenza A viruses, PB1F2 is the only influenza viral protein that intrinsically localizes and interacts with the mitochondrial-dependent apoptotic pathway (Lowy, 2003).

Mechanism by which PB1F2 protein brings about apoptosis

The PB1F2 protein has a short mitochondrial targeting sequence (MTS, 65–87aa) at the C-terminus of the protein that is thought to be essential for inner mitochondrial membrane localization and subsequent apoptosis (Gibbs et al., 2003). Fine mapping and mutational analysis of the putative MTS of the PB1F2 protein revealed that the presence of a double leucine residue in the MTS is very crucial. Leucine forms a hydrophobic groove in the PB1F2 protein that, in turn, interacts with the TOM (transporter outer membrane) receptor. This interaction facilitates translocation of PB1F2 α helix through the IMM (inner mitochondrial membrane) and OMM (outer mitochondrial membrane), resulting in a loss of MMP (mitochondrial membrane potential) and the initiation of apoptosis (Gibbs et al., 2003) (Fig. 2). However, in another study, amino acid residues from

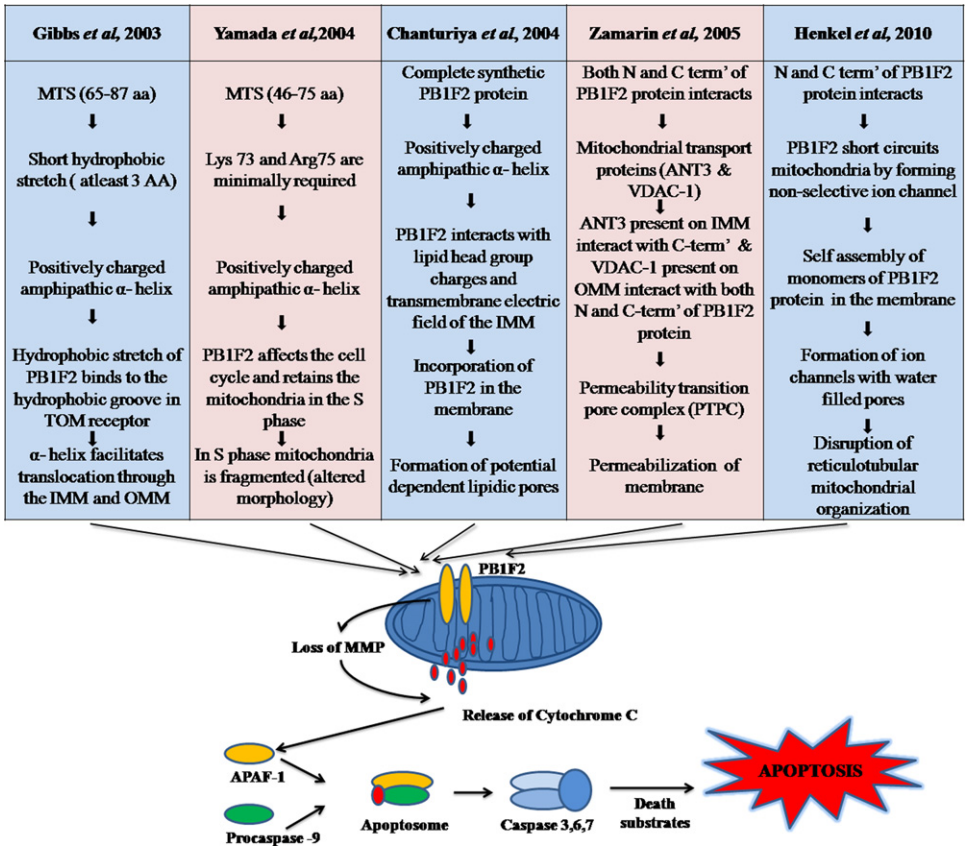

Fig. 2. Different mechanisms by which PB1F2 protein brings about depolarization of mitochondrial membrane.

46 to 75 were reported to be essential for apoptotic function, of which Lys73 and Arg75 were minimally required for a functional MTS (Yamada et al., 2004) (Fig. 2). The PB1F2 protein was shown to arrest the cell cycle in the 'S' or 'Synthesis' phase. In the S phase, the mitochondria are fragmented, hence altered morphology results in the loss of MMP and subsequent apoptosis (Yamada et al., 2004) (Fig. 2). Chanturiya et al. (2004) worked with synthetic PB1F2 proteins and described the protein's interaction with lipid head group charges and a transmembrane IMM electric field. This interaction leads to the incorporation of PB1F2 in the mitochondrial membrane and formation of potential-dependent lipidic pores. These pores depolarize the mitochondrial membrane, causing an increase in the conductance of the planar lipid bilayers, subsequent breakdown of the bilayers and finally apoptosis (Fig. 2). However, in another study, PB1F2 was shown to have the ability to form non-selective protein-mediated pores in planar lipid membranes. These non-selective water-filled protein pores are formed by the oligomerization or self-assembly of PB1F2 monomers in the mitochondrial membrane. These pores disrupt the reticulotubular mitochondrial organization and damage the inner membrane of the mitochondria and bring about apoptosis (Henkel et al.,2010). The C-terminal domain (amino acid residues Ile55-Lys85), which forms an α-helical structure, was found to be stable and was thought to be mainly involved in the formation of these protein channels. Despite the sequence variability between H1N1 and H5N1 virus, non-selective protein channels and similar apoptotic changes were observed in both (Henkel et al.,2010) (Fig. 2). In continuing to unravel the mechanism by which PB1F2 compromises the mitochondrial membrane and brings about apoptosis, Zamarin et al. (2005) demonstrated that PB1F2 indirectly interacts with the mitochondrial membrane through the mitochondrial transport proteins ANT3 (Adenine nucleotide translocator 3) and VDAC1 (voltage-dependent anion channel 1), present on the inner and outer mitochondrial membrane, respectively. The C-terminal domain of the PB1F2 protein interacts with ANT3, while both the N- and C-terminal regions are essential for its interaction with VDAC1. Both the mitochondrial membranes and PB1F2 form permeability transition pore complexes (PTPCs), which play a role in the permeabilization of the mitochondria and inducing cell death (Fig. 2) (Zamarin et al., 2005). Another study used an *in silico* approach to understand the interaction between the C-terminal end of the PB1F2 protein of a highly pathogenic influenza A H5N1 strain and the two mitochondrial proteins VDAC1 and ANT3. The study revealed that 22 amino acids of the VDAC1 protein make hydrophobic contacts with the 12 amino acids of the C-terminal end of the protein. Of these amino acids, Leu64, Arg75 and Val76 are the most important for mitochondrial targeting. In addition, 14 amino acids of ANT3 make hydrophobic contacts with 9 amino acids present on the C-terminal end of the PB1F2 protein (Danishuddin et al., 2010).

Cell type and virus strain-specific proapoptotic role of PB1F2

Chen et al. (2001) showed that apoptosis was more pronounced in immune cells than in epithelial cells. McAuley et al. (2010) studied PB1F2 proteins from pandemic strains from the 20th century (H1N1-1916, H2N2-1957; H3N2-1968), a HPAI virus of the H5N1 subtype, H1N1 PR8 and representative seasonal strains and demonstrated that only the laboratory strain A/Puerto Rico/8/34 (H1N1; PR8) had the ability to cause apoptosis in both epithelial and immune cell lines. Hence, it was deduced that the ability to cause cell death is virus-strain specific, and apoptosis is not a likely contributor to pathogenicity in humans. In the PR8 strains, cell death is achieved through the interaction of the PB1F2 protein with BAK/BAX and mediated through the release of cytochrome c from mitochondria (McAuley et al., 2010). In another study, the PB1F2 protein of an H5N1 strain (A/Hong Kong/156/1997) did not localize to mitochondria and also did not demonstrate proapoptotic potential (Chen et al., 2010). The researchers attributed this result to the lack of leucine-rich amino acids in the mitochondrial target sequence (MTS), which are essential for apoptosis (Gibbs et al., 2003). However, when they created recombinant PB1F2 H5N1 virus with double leucine mutations at positions 69 and 75, the virus localized to the mitochondria. This result emphasizes the importance of leucine in the MTS (Chen et al., 2010).

Contribution of the PB1F2 protein in exacerbating pathogenicity in animal models

The PB1F2 protein has been determined to exacerbate pathogenicity in mammalian and avian hosts. It has been hypothesized that PB1F2 increases pathogenicity in mice mainly by delaying the clearance of the virus by the host immune system. It is speculated that the delay is due to apoptosis of the antigen-presenting cells, such as macrophages and dendritic cells, which are responsible for clearance (Zamarin et al., 2006). PB1F2 has been shown to increase pathogenicity in recombinant Spanish flu virus (r1918) infected mice by elevating the cell death responses of the host (Kash et al., 2006). A PB1F2 protein with an N66S mutation contributes to the pathogenicity of the highly pathogenic H5N1 and 1918 Spanish flu H1N1 viruses. The above viruses cause increased morbidity and mortality in mice (Conenello et al., 2007). An N66S (arginine to serine) mutation is present in the C-terminal region of the PB1F2 protein. That position is part of the α-helical region and is in the MTS of the PB1F2 protein, which is proposed to be essential for the apoptotic function of the protein. This mutation was reported first in the H1N1 influenza A virus responsible for the 1918 Spanish flu and in the highly pathogenic H5N1 virus of the 1997 Hong Kong outbreak (Conenello et al., 2007). The exacerbated pathogenicity is mainly due to increased viral titers and elevated levels of IFNγ and TNFα in infected mouse lungs (Conenello et al., 2007). The N66S mutation contributed to the viral pathogenicity of the 1918 pandemic virus in mice and causes both primary viral as well as secondary bacterial infection (McAuley et al., 2007). Comparison of the *in vivo* kinetics of the PR8 influenza strain and its recombinant counterpart, rPR8, with PB1F2 from the 1918 influenza virus revealed that rPR8 resulted in a higher rate of viral production and cell death and hence, the virus strain was more pathogenic. Using mathematical equations and data from previous reports (McAuley et al., 2007; Mohler et al., 2005), it was suggested that the burst size of the rPR8 influenza virus-infected cells was much higher than that of its wild-type counterpart, which may mimic the effect of PB1F2 on the infection dynamics of the 1918 H1N1 virus (Smith et al., 2011).

The contribution of PB1F2 to enhancing the pathogenicity of the 1918 pandemic H1N1 virus is undoubted. In contrast, interestingly, this protein is present in a truncated form in the H1N1 2009 pandemic virus, which leads to a questioning of the functional utility of this protein in influenza viruses in human hosts. The PB1F2 protein of the H1N1 2009 pandemic differs from its recent ancestors by harboring a point mutation (C129A) in the PB1 gene that leads to the formation of a stop codon in place of serine. The result of which is the transcription of a truncated 11 amino acid protein. Apart from the stop codon at the 12th amino acid position, it harbors another two stop codons at positions 58 and 88 (Smith et al., 2009). To understand the contribution of PB1F2 to the recent 2009 H1N1 pandemic strain, Hai et al. (2010) generated recombinant viruses expressing full-length PB1F2 containing either an asparagine or serine at position 66 in the

background of the pandemic 2009 influenza strain A/California/04/2009. They performed both *in vitro* and *in vivo* infection assays and found increased replication of the N66S virus in A549 cells, while no significant changes were observed in mice and ferrets. The co-infection of mice with these viruses and *Streptococcus pneumoniae* resulted in insignificant changes in the mortality of BALB/c or DBA/2 mice. The only difference the researchers observed was in modulation of the immune system in the mice, wherein there was an increased proinflammatory response in the mice with the virus with the N66S mutation in the full-length PB1F2. The findings that full-length PB1F2 in pandemic strain A/California/04/2009 did not significantly alter both primary viral infection or the secondary bacterial infection with *S. pneumoniae* intrigued the authors, and they concluded that PB1F2 most likely enhances the virulence of the virus in as strain-specific manner (Hai et al., 2010). In continuing to unravel the mystery of the presence of the truncated PB1F2 in the 2009 pandemic strain, Chen et al. created a recombinant pandemic 2009 H1N1 virus strain with a complete PB1F2 fragment and found enhanced viral replication that resulted in high viral titers. The viral titer peaked unusually earlier than in the wild-type virus at 24 h. High viral titers caused the death of host cells, thus preventing newer viruses from continuing further replication. Chen et al. (2010) speculated this could be one of the many reasons why the pandemic H1N1 strain lacked full-length PB1F2 protein.

PB1F2 contributed to the pathogenicity of A/Vietnam/1203/04 (H5N1; VN1203) in mallard ducks. Three synonymous mutations inadvertently introduced into the PB1 gene caused substitutions at position 51, 56 and 87 of the PB1F2 gene of the reverse genetics-derived rgVN1203 virus, which reduced the lethality in mallard ducks (Marjuki et al., 2010). The potential of the PB1F2 protein (with and without the N66S mutation) to exacerbate viral pathogenicity was further explored and compared in both mammalian and avian hosts by Schmolke et al. (2011) HPAI (VN1203) virus with the PB1F2 protein harboring an N66S mutation, when inoculated into the mice, lead to increased replication of the virus, although a virus with a knocked-out PB1F2 gene presented minimal effects on the life cycle. In contrast, in ducks, HPAI virus with an N66S mutation played a minor role in pathogenesis, but infection with virus with a complete deletion of the PB1F2 ORF resulted in delayed onset of clinical symptoms and systemic spreading of the virus. Thus, PB1F2 is an important pathogenicity factor in ducks that is independent of sequence variations at position 66 and is hence conserved in avian influenza strains. In mammalian hosts, PB1F2 only impacts the pathogenicity when the protein contains particular amino acid motifs, such as the rare N66S polymorphism (Schmolke et al., 2011). The contribution of PB1F2 to the pathogenesis of primary viral infection with seasonal influenza virus (H1N1 A/USSR/90/77) was studied in ferret and macaque *ex-vivo* lung cultures by Meunier et al. (2012) The study revealed that PB1F2 modulated the initial host response but did not affect the pathogenesis of the H1N1 seasonal virus, thus questioning the importance of PB1F2 in enhancing pathogenicity in mammalian hosts.

In our study, we analyzed the presence of the N66S mutation in virus strains responsible for the 20th-century pandemics and the H5N1 subtype. The analysis revealed that this mutation was present in only 100(6.5%) of the total 1530 H1N1 viruses studied (Table 2). It was present in 91(81.25%) of the 112 H1N1 strains from avian hosts and in 7 (6.2%) viruses from swine hosts; however, none of the strains from humans harbored this mutation (our analysis did not include the H1N1 strains of the Spanish flu). As of October 2011, PB1F2 sequences of only 83 strains belonging to the H2N2 subtype were available in the global data base, (http://www.fludb.org) and among them, 24 (28.9%) of the strains carried the N66S mutation. Among those strains, 16 (72.2%) were present in the viruses derived from avian hosts (Table 2). In our comprehensive analysis of 2566 PB1F2 sequences from the H3N2 subtype strains, only 89 (3.96%) of the strains were positive for the N66S mutation. Majority of the strains (77.9%) which carried the N66S mutation were from avian hosts. Of importance, is the presence of N66S mutation in majority of the virus strains from avian hosts in H1N1, H2N2 and H3N2 subtypes. It would of interest to study the relevance and contribution of this mutation to the pathogenicity of the virus in these subtypes.

Table 2
Strains of the H1N1, H2N2, H3N2 and H5N1 subtypes with an N66S mutation in the PB1F2 protein from various hosts.

Subtype	Host	Total no. of strains	No. of strains harboring mutation (%)
H1N1		**1530**	**100 (6.5)**
	Human	1155	0
	Avian	112	91 (81.25)
	Swine	261	7 (2.68)
	Environment	1	1 (100)
	Others	1	1 (100)
H2N2		**83**	**24 (28.9)**
	Human	54	2 (3.7)
	Avian	22	16 (72.7)
	Environment	1	1 (100)
	Guinea pig	6	5 (83.3)
H3N2		**2566**	**89 (3.96)**
	Human	2419	30 (1.2)
	Avian	68	53 (77.9)
	Swine	76	3 (3.9)
	Environment	16	2 (12.5)
	Dog	1	1 (100)
H5N1		**919**	**35 (3.8%)**
	Human	220	6 (2.7)
	Avian	666	17 (2.5)
	Environment	16	12 (70.5)

In addition, the analysis of 919 PB1F2 sequences belonging to the H5N1 subtype revealed that only 35 (3.8%) of the strains harbored the N66S mutation. The mutation was present in 6 (2.7%) of the 220 H5N1 strains from human hosts, in 17 (2.5%) of the 660 strains from the avian hosts and in 12 (70%) of the 17 strains isolated from the environment, which are considered to have low pathogenicity. We found that this mutation is not present in any of the viral strains that were recently isolated from humans from in Indonesia, Vietnam or Egypt. Therefore, considering this mutation as a marker of high pathogenicity is questionable in the viruses from the H5N1 type. In searching for pathogenic markers in H5N1 viruses, we identified a mutation, N84S, which substituted the amino acid asparagine for serine at the 84th position of the C-terminus of the PB1F2 protein. This mutation was present in 86 (9.35%) of the 919 H5N1 strains. Although both the amino acids are polar, uncharged and hydrophilic, we hypothesize that this mutation might bring about a change in mRNA folding and the rate of protein translation, which may bring about changes in the way the protein functions. This mechanisms needs to be validated by both *in vivo* and *in vitro* experiments.

PB1F2, a modulator of innate immune response

PB1F2 has been implicated in enhancing the lung inflammatory response in mouse models. PB1F2 proteins from pandemic strains from the 20th century, an HPAI virus of the H5N1 subtype, H1N1 PR8 and representative seasonal strains have been shown to cause inflammatory responses and increased cellularity of the

lungs. The inflammatory response includes the influx of T cells, dendritic cells, macrophages and neutrophils (McAuley et al., 2010). It was hypothesized that either PB1F2 is directly recognized by the pattern recognition receptors of the innate immune system or it just serves as a chemoattractant (McAuley et al., 2007). The proinflammatory role of PB1F2 is also thought to be due to the C-terminal end of the PB1F2 protein (McAuley et al., 2010).

PB1F2 with the N66S mutation has also been associated with a delay in induction of the IFN response, which causes delayed innate immune response (Conenello et al., 2007, 2011; Varga et al., 2011). IFN antagonist activity was demonstrated with the PR8 H1N1 strain with both in *in vitro* and *in vivo* experiments. PB1F2 interferes with the RIG-I/MAVs protein complex and thus inhibits the activation of the IFN regulatory factor 3, which is a downstream molecule of the RIG-I/MAVs protein complex (Dudek et al., 2011). In contrast, another study found that wild-type WSN influenza A virus with full length PB1F2 protein exacerbated the expression of IFN-β through the activation of the NF-kB pathway (Le Goffic et al., 2010).This particular hypothesis was further confirmed in an *in vivo* study wherein the PB1F2 protein significantly increased the inflammatory response by recruiting massive numbers of leukocytes into the air space of mice lungs (Le Goffic et al., 2011). Increased IFNγ levels in the mice repressed the innate immune response of the host against both the virus and invading bacteria, which in turn led an opportunistic infection (Le Goffic et al., 2011). L62, R75, R79, and L82 are the amino acid residues that constitute the proinflammatory domain of the PB1F2 protein of the H3N2 virus A/Hong Kong/1/1968 virus. These residues were identified as being responsible for causing a significant increase in morbidity and mortality in mice and also better-supported secondary bacterial infection (Alymova et al., 2011).

Co-localization of PB1F2 with PB1 regulates polymerase activity

Mazur et al. (2008) described a novel additional function for PB1F2 in epithelial cells. They demonstrated that this protein indirectly regulates polymerase activity through its interaction with PB1. They also demonstrated that a lack of PB1F2 during infection resulted in the altered localization of PB1 and, consequently, decreased viral polymerase activity, which in turn produced a smaller plaque phenotype (Mazur et al., 2008). The authors examined the PR8 strain and suggested that the co-localization of PB1F2 with PB1 most likely leads to retention of PB1 in the nucleus in the late phase of replication, which in turn leads to enhanced polymerase activity. They suggested that the C-terminal end of the PB1F2 protein is required for the co-localization of the protein with PB1, which was confirmed by McAuley et al. (2010). McAuley et al. (2010) extended the work of Mazur et al. (2008) and carried out studies on other strains, including A/Brevig Mission/1/18 (H1N1; 1918 PB1F2), A/Beijing/11/56(H1N1; Beij PB1F2), which has a truncated C-terminal end, and A/Vietnam/1203/04 (H5N1) and A/Wuhan/359/95. (H3N2). Košík et al. (2011) also demonstrated that PB1F2 co-localizes with PB1 and enhances the polymerase activity. However, they provided evidence that the N-terminal portion of the PB1F2 was responsible for this effect, which is in contrast to results of the aforementioned researchers. Košík et al. (2011) also suggested that apart from the increase in PB1 expression, there was also an increase in the expression of other viral protein, such as NP, M1, and NS1, in the presence of the PB1F2 protein.

Role of PB1F2 in enhancing secondary bacterial infection

The major cause of deaths in the patients infected with IAV is not virus infection *per se* but secondary bacterial infections (McCullers, 2006). The major pathogens responsible for the secondary infections are *S. pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae* (Sethi, 2002). IAV infection causes loss of ciliated mucus-secreting epithelial cells that line the lungs. This results in decreased mucous production and impaired clearance of microorganisms, as mucous is among the agents responsible for the first line of non-specific immune defense against any foreign body. Due to this, an opportunistic bacteria can reside longer in the lungs and cause secondary bacterial infection. PB1F2 also has been implicated in enhancing secondary pulmonary infection. It has been shown that PB1F2 causes cell death specifically in the alveolar macrophages present in the lungs. This elimination causes an increase in virulence by reducing antigen presentation and initial viral clearance and also by preventing crosstalk between the alveolar macrophages and CD4+ Th cells. In the absence of the alveolar macrophages, the antigen presentation to the CD4+ Th cells is hampered, and consequently, its clonal expansion and its downstream effector functions, such as cytotoxic T lymphocyte (CTL) activation, antibody production and inflammation, are impaired (Coleman, 2007). McAuley et al. (2007) demonstrated in mice that PB1F2 exacerbates inflammation during primary viral infection and also has the ability to increase the frequency and severity of the secondary bacterial infection. The observed cellular reaction in their experiments included increased inflammatory infiltration, epithelial cell hypertrophy, necrosis and fibrin deposition. They attributed this effect to the C-terminal region of the PB1F2 protein (McAuley et al., 2007).

Summary and conclusions

PB1F2 is a non-structural accessory protein of influenza A viruses. Full-length PB1F2 protein is 87 to 101 amino acids long and encoded by an alternate +1 open reading frame within the PB1 gene. Translation of PB1F2 mRNA is mediated by leaky ribosomal scanning. The viral mRNA has a short half-life and is expressed early and transiently in infected cells. The PB1F2 protein is predicted to form a positively charged amphipathic helix and has the intrinsic property to form oligomeric structures. Varying lengths of PB1F2 have been reported in different hosts and subtypes of influenza A viruses. A full-length PB1F2 protein is present in all the 20th-century pandemics strains. However, in the recent 2009 H1N1 pandemic, the PB1F2 protein is non-functional due to a C-terminal truncation. Hence, the significance of the protein in pandemic strains is unclear. PB1F2 protein is expressed by almost all the avian influenza A strains; however, strains from human and swine hosts have forms with premature truncations at either the C- or N-terminal ends. It is worth noting that most of the virus strains isolated from avian hosts and belonging to H1N1, H2N2, H3N2 and H5N1 subtypes harbored complete PB1F2 protein. This suggests that PB1F2 is positively selected in these subtypes and still has not lost its function which is contrast to the viruses from mammalian hosts.

Since the time PB1F2 was first described by Chen et al. (2001), many researchers have attempted to unravel its function, utility in the life cycle and contribution towards exacerbating viral pathogenicity. Despite many attempts, the function of the PB1F2 protein in the life cycle of influenza virus remains unclear, as does its precise role in virulence. Studies have shown that PB1F2 has proapoptotic potential whereby it initiates apoptosis through the intrinsic mitochondrial pathway, apparently by depolarizing the MMP followed by cytochrome c release. However, the exact mechanism by which PB1F2 causes loss of the MMP is not yet resolved. Here, we have discussed many reports that have described mechanisms involved in the depolarization of the mitochondrial membrane. These include

direct interaction of the protein with the mitochondrial membrane or indirect modulation of the mitochondrial transport proteins, such as ANT3 and VDAC1. Apart from the unresolved mechanism involved in bringing about apoptosis, there is also ambiguity about the proapoptotic ability of the protein in many influenza A strains. It has been reported that the apoptotic function of PB1F2 is viral strain- and cell-type-specific. It would be very interesting to study the apoptotic potential of the PB1F2 protein in various influenza A stains. Further studies are also warranted to determine the mechanism of the interaction of the PB1F2 protein with various molecules implicated in apoptosis.

Apart from inducing apoptosis, PB1F2 has been implicated in possessing proinflammatory properties and the ability to exacerbate viral pathogenicity. PB1F2 has been found to be pathogenic in mice, swine and ducks, causing cellularity of the lungs and the influx of white blood cells, including T cells, dendritic cells, macrophages and neutrophils. There is ambiguity about the antagonistic or agonistic role of PB1F2 in regulating the IFN response. PB1F2 can also enhance viral polymerase activity. PB1F2 facilitates secondary infection with *S. pneumonia*, highlighting the complex contribution of PB1F2 in virulence. Interestingly, very recently, it has been hypothesized that the two functions of the PB1F2 protein, apoptosis and MAVS-dependent IFN antagonism, converge at the level of the MAVS protein. This crosstalk between the two cellular processes in the mitochondria may improve our understanding of the PB1F2 protein, which is definitely lacking in this present context (Varga and Palese, 2011).

Currently most of the research on influenza is carried out in genetically engineered viruses. In order to generate viruses lacking PB1F2 protein, its initiation codon is usually ablated. It has been recently shown that this ablation causes an increase in the expression level of the downstream protein N40. The over expression of N40 protein in turn has detrimental effect on the replication of the virus both *in vitro* and *in vivo* experiments (Tauber et al., 2012). Therefore, researcher should take utmost care in carrying out studies and interpreting the existing data on genetically engineered viruses deficient in PB1F2 protein. Key questions that are still unanswered regarding the PB1F2 protein are (i) the functional utility in different host, (ii) the effect of C- and N-terminal truncations, (iii) the apoptotic ability in various strains, (iv) the precise mechanism for inducing apoptosis, (v) the pathogenic ability in a greater range of hosts, (vi) the antagonistic or agonistic role in modulating the innate immune response and (vii) the correlation between sequence variability and virulence. Thus, we believe the precise function of the PB1F2 protein remains unclear, and there remains much to learn in this particular area. However, research on this particular protein indicates that the PB1F2 protein has strain-specific functions that could vary in different hosts. The effects of the protein on the host might range from being minimal to deleterious, either by increasing the number and level of expression of activated genes linked to cell death or intensifying the activity of genes causing uncontrolled inflammation or deregulating the innate immune responses of the host.

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