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Molecular signatures of virulence in the PB1-F2 proteins of H5N1 influenza viruses

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Abstract

The PB1-F2 protein of influenza A viruses contributes to pathogenesis in animal models. Specific molecular signatures of virulence within PB1-F2 have been mapped for some functions. The 66S polymorphism may modulate interferon activity, and four C-terminal amino acids, 62L, 75R, 79R, and 82L, contribute to cytokine release and inflammatory responses in specific virus backgrounds. All available PB1-F2 sequences from H5N1 subtype influenza A viruses were analyzed. The majority (82.5%) of H5N1 sequences available in the Influenza Research Database code for PB1-F2 proteins with 4 or more of these virulence associated amino acids. Most of these are avian sequences from highly pathogenic strains isolated in Asia or Africa. The 66S polymorphism was uncommon (5.3% of sequences) but was found in association with the other 4 inflammatory amino acids in select highly pathogenic strains in Asia. These analyses suggest that if an H5N1 virus were to emerge as a pandemic strain, the PB1-F2 protein will be a contributor to pathogenesis. Research on the pathogenic effect of these signatures in an H5N1 background should be undertaken. Surveillance efforts should include sequencing of the PB1 gene segment and analysis for these molecular signatures to allow for the potential prioritization of resources during pandemic planning.

Keywords

influenza virus; PB1-F2; avian influenza; virulence; H5N1

1. Introduction

1.1 The need for molecular signatures of virulence

Influenza A viruses of the H5N1 subtype have emerged over the last 15 years as a serious threat to human health (Webby and Webster, 2003). A continuously evolving reservoir of highly pathogenic H5N1 viruses in domestic poultry has become a worrisome source of zoonotic infections of humans, causing severe respiratory disease with a strikingly high case

fatality rate (Van Kerkhove et al., 2011). In addition, H5N1 strains must be considered as an eminent threat to emerge in a form capable of causing a global pandemic (McCullers, 2008). The recent demonstration that such viruses can transmit via respiratory droplets with only a few mutations should be a clarion call for improved research into determinants of virulence (Russell et al., 2012). Only by understanding which viruses are capable of rapidly evolving to a point where they can both spread from human to human and cause severe disease will we be able to prioritize surveillance and pandemic preparation. The contribution of multiple viral factors to virulence in the human host is being explored by laboratories around the world. As we begin to understand the specific contributions of different variants of genes in particular backgrounds, we should be able to create a set of molecular signatures of virulence to use in surveillance of viruses in the animal reservoir. Identifying specific viral strains that possess many or all of these signatures would then allow prioritization of those viruses as targets for further surveillance and for antiviral and vaccine development.

1.2 The contribution of the PB1-F2 protein to virulence

PB1-F2 is a 90 amino acid influenza A virus accessory protein expressed from the +1 reading frame of the PB1 gene segment of some viruses (Chen et al., 2001). Its contribution to virulence is strain specific and poorly understood at present. Because the PB1-F2 protein contains a mitochondrial targeting sequence, can be shown to accumulate intracellularly in mitochondria, and can potentiate cell death in some cell lines (Chen et al., 2001), it was initially thought to act as a pro-apoptotic protein through interaction with specific membrane based proteins and formation of membrane pores (Chanturiya et al., 2004; Zamarin et al., 2005). Indeed, cytochrome c release occurs upon exposure of mitochondria to PB1-F2 and can be shown to be dependent on the apoptotic proteins BAX/BAK in several mammalian species, thus strengthening the theory (McAuley et al., 2010a). The ability of PB1-F2 to cause death of epithelial cells or immune cells through this mechanism would be predicted to contribute to virulence in vivo (Varga and Palese, 2011). However, cell death through apoptosis requires a specific set of amino acids in the C-terminal portion of the protein, and the molecular signature for this apoptotic phenotype is found only in a limited set of H1N1 strains that circulated in the early 20th century (McAuley et al., 2010a). Therefore, this effect on cell death may not be relevant to pandemic planning for the emergence of H5N1 viruses.

A second proposed mechanism for enhanced virulence is a potential contribution of PB1-F2 to replication efficiency. The rapidity of viral replication often correlates with virulence, thereby enabling a virus to outpace the immune responses designed to contain and control it. Deletion of PB1-F2 from the commonly utilized laboratory strain A/Puerto Rico/8/34 (H1N1) does not result in differences in viral titer in mouse lungs or in tissue culture (Mazur et al., 2008; McAuley et al., 2010b; Zamarin et al., 2006), although the rapidity of spread through cell monolayers and the clearance rate of the virus may be impacted (Mazur et al., 2008; McAuley et al., 2007; Zamarin et al., 2006). Expression of PB1-F2 proteins from highly pathogenic viruses such as H5N1 strains or the 1918 pandemic strain, however, does enhance replication in cell culture (Conenello et al., 2007; McAuley et al., 2007; McAuley et al., 2010b). This in vitro enhancement of replication does not extend to growth differences in mouse lungs when using chimeric viruses expressing the H5N1 PB1 gene segment, although pathogenicity is enhanced (Conenello et al., 2007; McAuley et al., 2010a;

McAuley et al., 2010b; Schmolke et al., 2011). Determining the precise mechanism for the potential effects on replication and subsequent changes to pathogenesis is complicated by potential interactions between the three proteins (PB1, PB1-F2, and N40) which can be expressed from the PB1 gene segment (Wise et al., 2009). It has been proposed that protein-protein interactions between PB1 and PB1-F2 affect replication through the sequestration of PB1 (Mazur et al., 2008), but differential regulation of one or both of these proteins through variances in expression of the third protein, N40, and potential protein-protein interactions involving this additional accessory protein make elucidation of the relative contribution of each difficult (Wise et al., 2009). Overall, the preponderance of evidence suggests that effects on replication are minor and may impact pathogenesis only in limited circumstances (McAuley et al., 2010b). No molecular signatures associated with these effects have been identified to date.

Modulation of the immune response appears to be the mechanism most relevant to human disease, and molecular signatures of pathogenicity have been identified for these phenotypes. A serine at position 66 of the PB1-F2 ORF (the 66S polymorphism) has been shown to impact virulence in highly pathogenic backgrounds (e.g., H5N1 and the 1918 strain) but not in the 2009 H1N1 background (Conenello et al., 2011; Conenello et al., 2007; Hai et al., 2010; Varga et al., 2012; Varga et al., 2011). The mechanism appears to be the inhibition of interferon induction through binding of PB1-F2 to the MAVS adaptor protein (Varga et al., 2012; Varga et al., 2011). The 66S polymorphism is found in the 1918 pandemic strain, and may have contributed to this virus' tremendous virulence in both single agent and secondary bacterial infection models (Conenello et al., 2007; McAuley et al., 2007). However, it appears to be a relatively rare polymorphism among sequenced isolates (Hai et al., 2010).

Independent of this polymorphism, the PB1-F2 protein from select strains is capable of promoting a pro-inflammatory environment characterized by cytokinemia and an increased influx of neutrophils and other inflammatory cells into the lungs and airways (McAuley et al., 2007; McAuley et al., 2010b). Although the precise mechanism is not yet clear, this phenotype maps to specific amino acids at 4 positions in the C-terminal portion of the protein; 62L, 75R, 79R, and 82L (Alymova et al., 2011). The 1918 pandemic strain possessed all four of these polymorphisms, which were subsequently lost in the H1N1 lineage when the protein became truncated around 1948 (McAuley et al., 2010a). The 1957 H2N2 and 1968 H3N2 strains also had all four inflammatory amino acids, but did not have the 66S polymorphism that was present in the 1918 strain. A series of mutations during the adaptation and evolution of viruses in the human H3N2 lineage resulted in the loss of all four inflammatory polymorphisms by the late 1980s, such that the PB1-F2 protein from these strains was no longer driving inflammation or supporting secondary bacterial infections (Alymova et al., 2011). Interestingly, the resulting changes conferred an anti-bacterial phenotype on these strains giving rise to viruses that were able to compete with bacteria in the airway through PB1-F2 expression (Alymova et al., 2011; Weeks-Gorospe et al., 2012). Presently in the swine reservoir, great variety in the PB1-F2 length and the expression of these polymorphisms exists, which results in a hierarchy of the viruses' ability to support secondary bacterial infections. A full complement of the inflammatory amino acids or the presence of the 66S polymorphism in certain backgrounds engenders strong

support for bacterial pathogens. On the other hand, truncation of the PB1-F2 with loss of the C-terminal portion of the protein causes an intermediate phenotype, while expression of an anti-inflammatory PB1-F2 largely prevents secondary bacterial pneumonia (Weeks-Gorospe *et al.*, 2012). Linking these molecular signatures to their specific effects on pathogenicity could be used for surveillance of viruses in animal reservoirs and prioritization of pandemic planning efforts.

2 Methods

PB1-F2 sequences from H5N1 subtype influenza A viruses were analyzed to determine the distribution of molecular signatures of virulence. All data were obtained using the NIAID Influenza Research Database (IRD) online through the web site at <http://www.fludb.org> (Squires *et al.*, 2012), accessed on July 11, 2012. The IRD was queried to return all available sequences for the PB1-F2 protein of H5N1 strains. Additional search criteria were used to identify sequences isolated from particular host species (i.e., avian, human, and swine), and geographic areas (i.e., Africa, Asia, Europe, and North America). Sequences were aligned using the multiple sequence alignment (MSA) tool MUSCLE on the IRD website. Nucleotide distributions at amino acids sites 62, 66, 75, 79 and 82 of the PB1-F2 were analyzed using the “Analyze Sequence Variation (SNP)” and “Identify Point Mutations in Proteins” tools within the IRD website.

3 Results

3.1 Characteristics of PB1-F2 proteins from H5N1 strains

At the time of access of the IRD, 987 H5N1 subtype viruses had sufficient sequence information from the PB1 gene segment to predict the corresponding PB1-F2 amino acid sequences (Table 1). The majority of these viruses were isolated in Asia (77.0%), followed in frequency by Africa (16.3%), Europe (4.4%) and North America (2.3%). The host species of origin was predominantly avian (78.8%), followed by human (15.3%), and a minority were from swine (1.3%) or other sources (4.6%). The majority of the other sources (29/45) were environmental samples, presumed to be avian in origin.

Nearly all (97.3%) of the H5N1 isolates studied had a full length (87-90 amino acids) PB1-F2 (Figure 1). Relatively few isolates (2/987) had the truncation after amino acid 57 commonly seen in H1N1 and H3N2 subtype influenza A viruses circulating in humans and swine. Nineteen isolates had truncations that would prevent expression of the N-terminal portion of the protein, with transcription predicted to start at an upstream AUG. The start codons for these potential gene products (the 7th, 8th, and 9th AUGs in the +1 reading frame) are not located in strong Kozak translation initiation contexts (Wise *et al.*, 2009), and it is unknown whether initiation occurs, whether proteins are produced (Wise *et al.*, 2011), and whether the resulting peptides containing the inflammatory amino acids would have biologic activity similar to the intact full-length protein (McAuley *et al.*, 2010a).

3.2 Inflammatory signatures in H5N1 PB1-F2 sequences

Of the 973 isolates that encode the C-terminal portion of the protein inclusive of amino acids 62 to 82, the majority (80.0%) had 4 of 5 of the amino acids that have been associated with

virulence in the H3N2 background (Figure 2). Only 24 isolates (2.5%) had all 5 amino acids, and only 3 of these were from highly pathogenic viruses. The majority of these strains were sampled in North America from either birds or the environment and were low pathogenic viruses. The 66S polymorphism was found in only 5.3% of sequences (Table 2), exclusively in viruses with either 3 or 4 of the inflammatory signatures. The majority of viruses with the 66S polymorphism and 3 inflammatory amino acids (in all cases 62L, 79R, and 82L) were isolated in Cambodia in 2010-2011. When less than 4 of the inflammatory amino acids were present, 75R was the most likely to be absent (Table 2). Viruses with only 2 (0.6%), or less than 2 (0%) of the 5 amino acids were very rare among sequenced H5N1 isolates (Figure 2). The majority of viruses with four or more molecular signatures were of avian origin (Table 3) and were isolated in Asia (Table 4), in a distribution that reflected the overall sequence pool.

4. Discussion

The most important conclusion that can be drawn from these analyses is that H5N1 viruses in the avian reservoir are likely to express a full-length PB1-F2 protein containing signatures associated with a pro-inflammatory phenotype. This contrasts with findings in other subtypes where truncated proteins are common (Zell et al., 2007), and a broader diversity of molecular signatures of pathogenicity are present (Weeks-Gorospe et al., 2012). While around 96% of all avian PB1-F2 proteins, independent of subtype, are full-length, only 81% of sequenced human isolates and 75% of swine isolates are full-length (Zell et al., 2007). This full-length, pro-inflammatory genotype would be predicted to contribute to both primary viral virulence and the frequency of secondary bacterial infections if one of these strains emerged in humans as pandemic influenza. However, as the studies establishing these amino acids as signatures of virulence were done in an H3N2 background (Alymova et al., 2011), specific experiments probing the contribution in the H5N1 background are needed. The 66S polymorphism was relatively uncommon in the H5N1 isolates, particularly in highly pathogenic strains, but could be found in association with other inflammatory signatures in some strains including a cluster in Cambodia. This particular molecular signature of virulence thus remains a concern for pandemic planning with H5N1 viruses. The prevalence of viruses with all 5 molecular signatures was low, but was found in unexpected places including low pathogenic viruses in North America. Since H5 subtype viruses can convert to highly pathogenic viruses in nature with relatively few mutations (Kawaoka and Webster, 1989), these strains bear close surveillance.

The biggest caveat to these analyses is the significant selection bias in the sampling. Because we examined all PB1-F2 sequences in the IRD, this is a non-random sample. The distribution of viruses in the database reflects surveillance priorities other than those related to examining the PB1-F2 protein, and the specific viruses for which PB1 gene segment sequence information was available are also likely to have been selected non-randomly. For this reason, distribution frequencies may be biased towards more virulent strains and may include more strains from human cases. If PB1-F2 contributes to virulence, this bias may enrich the database for sequences with multiple molecular signatures of pathogenicity, artificially increasing the frequency with which these signatures are found relative to their true distribution in nature. Nevertheless, the analyses suggest that the PB1-F2 protein from

avian H5N1 viruses is a potential contributor to the virulence of any future pandemic strain to arise from this reservoir. Further research into the role of PB1-F2 in pathogenesis in humans is needed, the role of these signatures in different H5N1 backgrounds should be studied, and surveillance efforts should include sequencing of the PB1 gene segment so that viruses containing 4 or more molecular signatures of virulence in the PB1-F2 can be targeted.

REFERENCES

1. Alymova IV, Green AM, Van De Velde N, McAuley JL, Boyd KL, Ghoneim HE, et al. Immunopathogenic and antibacterial effects of H3N2 influenza A virus PB1-F2 map to amino acid residues 62, 75, 79, and 82. *J. Virol.* 2011; 85:12324–12333. [PubMed: 21937639]
2. Chanturiya AN, Basanez G, Schubert U, Henklein P, Yewdell JW, Zimmerberg J. PB1-F2, an influenza A virus-encoded proapoptotic mitochondrial protein, creates variably sized pores in planar lipid membranes. *J Virol.* 2004; 78:6304–6312. [PubMed: 15163724]
3. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, et al. A novel influenza A virus mitochondrial protein that induces cell death. *Nat. Med.* 2001; 7:1306–1312. [PubMed: 11726970]
4. Conenello GM, Tisoncik JR, Rosenzweig E, Varga ZT, Palese P, Katze MG. A single N66S mutation in the PB1-F2 protein of influenza A virus increases virulence by inhibiting the early interferon response in vivo. *J Virol.* 2011; 85:652–662. [PubMed: 21084483]
5. Conenello GM, Zamarin D, Perrone LA, Tumpey T, Palese P. A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. *PLoS. Pathog.* 2007; 3:1414–1421. [PubMed: 17922571]
6. Hai R, Schmolke M, Varga ZT, Manicassamy B, Wang TT, Belser JA, et al. PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models. *J Virol.* 2010; 84:4442–4450. [PubMed: 20181699]
7. Kawaoka Y, Webster RG. Interplay between carbohydrate in the stalk and the length of the connecting peptide determines the cleavability of influenza virus hemagglutinin. *J Virol.* 1989; 63:3296–3300. [PubMed: 2746732]
8. Mazur I, Anhlán D, Mitzner D, Wixler L, Schubert U, Ludwig S. The proapoptotic influenza A virus protein PB1-F2 regulates viral polymerase activity by interaction with the PB1 protein. *Cell Microbiol.* 2008; 10:1140–1152. [PubMed: 18182088]
9. McAuley JL, Chipuk JE, Boyd KL, Van De Velde N, Green DR, McCullers JA. PB1-F2 proteins from H5N1 and 20 century pandemic influenza viruses cause immunopathology. *PLoS. Pathog.* 2010a; 6:e1001014. [PubMed: 20661425]
10. McAuley JL, Hornung F, Boyd KL, Smith AM, McKeon R, Bennink J, et al. Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. *Cell Host. Microbe.* 2007; 2:240–249. [PubMed: 18005742]
11. McAuley JL, Zhang K, McCullers JA. The effects of influenza A virus PB1-F2 protein on polymerase activity are strain specific and do not impact pathogenesis. *J Virol.* 2010b; 84:558–564. [PubMed: 19828614]
12. McCullers JA. Preparing for the next influenza pandemic. *Pediatr. Infect Dis J.* 2008; 27:S57–S59. [PubMed: 18820579]
13. Russell CA, Fonville JM, Brown AE, Burke DF, Smith DL, James SL, et al. The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. *Science.* 2012; 336:1541–1547. [PubMed: 22723414]
14. Schmolke M, Manicassamy B, Pena L, Sutton T, Hai R, Varga ZT, et al. Differential contribution of PB1-F2 to the virulence of highly pathogenic H5N1 influenza A virus in mammalian and avian species. *PLoS. Pathog.* 2011; 7:e1002186. [PubMed: 21852950]
15. Squires RB, Noronha J, Hunt V, Garcia-Sastre A, Macken C, Baumgarth N, et al. Influenza Research Database: an integrated bioinformatics resource for influenza research and surveillance. *Influenza. Other Respi. Viruses.* 2012 doi: 10.1111/j.1750-2659.2011.00331.x.

16. Van Kerkhove MD, Mumford E, Mounts AW, Bresee J, Ly S, Bridges CB, et al. Highly pathogenic avian influenza (H5N1): pathways of exposure at the animal-human interface, a systematic review. *PLoS. ONE*. 2011; 6:e14582. [PubMed: 21283678]
17. Varga ZT, Grant A, Manicassamy B, Palese P. Influenza Virus Protein PB1-F2 Inhibits the Induction of Type I Interferon by Binding to MAVS and Decreasing Mitochondrial Membrane Potential. *J Virol*. 2012; 86:8359–8366. [PubMed: 22674996]
18. Varga ZT, Palese P. The influenza A virus protein PB1-F2: killing two birds with one stone? *Virulence*. 2011; 2:542–546. [PubMed: 21971186]
19. Varga ZT, Ramos I, Hai R, Schmolke M, Garcia-Sastre A, Fernandez-Sesma A, et al. The influenza virus protein PB1-F2 inhibits the induction of type i interferon at the level of the MAVS adaptor protein. *PLoS. Pathog*. 2011; 7:e1002067. [PubMed: 21695240]
20. Webby RJ, Webster RG. Are we ready for pandemic influenza? *Science*. 2003; 302:1519–1522. [PubMed: 14645836]
21. Weeks-Gorospe JN, Hurtig HR, Iverson AR, Schuneman MJ, Webby RJ, McCullers JA, et al. Naturally occurring swine influenza A virus PB1-F2 phenotypes that contribute to super-infection with Gram-positive respiratory pathogens. *J. Virol*. 2012; 86:9035–9043. [PubMed: 22674997]
22. Wise HM, Barbezange C, Jagger BW, Dalton RM, Gog JR, Curran MD, et al. Overlapping signals for translational regulation and packaging of influenza A virus segment 2. *Nucleic Acids Res*. 2011; 39:7775–7790. [PubMed: 21693560]
23. Wise HM, Foeglein A, Sun J, Dalton RM, Patel S, Howard W, et al. A complicated message: identification of a novel PB1-related protein translated from influenza A segment 2 mRNA. *J Virol*. 2009; 83:8021–8031. [PubMed: 19494001]
24. Zamarin D, Garcia-Sastre A, Xiao X, Wang R, Palese P. Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. *PLoS. Pathog*. 2005; 1:e4. [PubMed: 16201016]
25. Zamarin D, Ortigoza MB, Palese P. Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice. *J Virol*. 2006; 80:7976–7983. [PubMed: 16873254]
26. Zell R, Krumbholz A, Eitner A, Krieg R, Halbhuber KJ, Wutzler P. Prevalence of PB1-F2 of influenza A viruses. *J Gen. Virol*. 2007; 88:536–546. [PubMed: 17251572]

Figure 1. Truncated PB1-F2 Proteins of H5N1 Influenza Isolates

Of the total number of H5N1 sequences in the IRD (987), 27 isolates have truncated PB1-F2 proteins. These sequences were broken down into two categories: those that are missing all or part of the C-terminal region (left) or the N-terminal region (right). The isolates with truncated PB1-F2 proteins but with the C-terminal portion (a.a. 62-82) intact (13 isolates) were analyzed to determine which of the inflammatory signatures were present. The primary inflammatory signature grouping (62L-75R-79R-82L) is shown.

Figure 2. Inflammatory Signatures in the PB1-F2 Proteins of H5N1 Influenza Viruses

The 973 isolates that encode the C-terminal portion of the protein are categorized into the number (1-5) of inflammatory signatures (62L-66S-75R-79R-82L) present. Within each category, specific polymorphism groupings are shown along with the percentage of each grouping.

Table 1

Total number of analyzed PB1-F2 sequences of H5N1 lineage broken down by species origin and region of isolation.

	Avian	Human	Swine	Other [*]	TOTAL
Asia	566	149	13	32	760[‡]
Europe	42	0	0	1	43
N. America	11	0	0	12	23
Africa	159	2	0	0	161
TOTAL	778	151	13	45	987

^{*} Other Hosts: Environment (29), Ferret/Mink (7), Pika (6), Raccoon Dog (2), Feline (1)

[‡] Isolates with truncated PB1-F2 (14 total): Avian (12), Human (1), Other Host/Environment (1)

Table 2

Frequency of each inflammatory signature in the PB1-F2 proteins of H5N1 lineage influenza viruses.

Inflammatory Signature				
62L	66S	75R	79R	82L
99.8%	5.3%	90.0%	92.8%	96.4%
Total No. Isolates: 973				

Table 3

Number of H5N1 lineage influenza with inflammatory signatures at amino acid sites 62, 66, 75, 79 and 82 in the PB1-F2 stratified by host species.

Host	Number of Inflammatory Signatures					Total No. Isolates
	1	2	3	4	5	
Avian	0	6	138	610	12	766
Human	0	0	19	131	0	150
Swine	0	0	0	13	0	13
Other	0	0	7	25	12	44
Total No. Isolates	0	6	164	779	24	973

Table 4

Number of H5N1 lineage influenza with inflammatory signatures at amino acid sites 62, 66, 75, 79 and 82 in the PB1-F2 stratified by region of isolation.

Region	Number of Inflammatory Signatures					Total No. Isolates
	1	2	3	4	5	
Asia	0	6	125	613	2	746
Europe	0	0	3	40	0	43
N. America	0	0	0	2	21	23
Africa	0	0	36	124	1	161
Total No. Isolates	0	6	164	779	24	973