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Antibodies to PB1-F2 protein are induced in response to influenza A virus infection

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Abstract

PB1-F2 is a small influenza A virus (IAV) protein encoded by an alternative (+1) reading frame of the PB1 gene. While dispensable for IAV replication in cultured cells, PB1-F2 has been implicated in IAV pathogenicity. To better understand PB1-F2 expression in vivo and its immunogenicity, we analyzed anti-PB1-F2 antibodies (Abs) in sera of mice infected intranasally (i.n.) with A/PR/8/34 (H1N1) virus and human acute and convalescent sera collected from the influenza H3N2 winter 2003-2004 epidemic. We explored a number of methods for detecting anti-PB1-F2 Abs, finding that PB1-F2-specific Abs could clearly be detected via immunoprecipitation or immunofluorescence assays using both immune mouse and human convalescent sera. Importantly, paired human sera exhibited similar increases in HI titers and PB1-F2-specific Abs. This study indicates that PB1-F2 is expressed in sufficient quantities in mice and humans infected with IAV to elicit an Ab response, supporting the biological relevance of this intriguing accessory protein.

Introduction

During a systematic search for peptides recognized by CD8⁺T lymphocytes and encoded by alternative positive-strand open reading frames (ORFs) of the influenza A virus (IAV) strain A/PR/8/34 (H1N1) (PR8), Chen et al. [1] reported the existence of a novel 87-aa protein representing the eleventh defined IAV gene product. Since this protein is encoded by the second (+1) ORF of the PB1 gene, it was designated PB1-F2. The PB1-F2 ORF is present in most IAVs isolates, with most strains 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 [2,3].

Without exception, human H1 isolates from 1918 to 1988 encode full-length PB1-F2. Isolates from 1988 to 1998 encode an ORF either for full-length or for C-terminally truncated PB1-F2. By contrast, all human H1 isolates obtained after 1998 encode an ORF for truncated PB1-F2 only, typically of 57 amino acids.

Several unusual features have been described for PR8 PB1-F2, including rapid degradation, tremendously variable levels of expression between infected cells, and significant localization to mitochondrial membranes [1]. A predicted amphipathic α -helical region in the C-terminal region of PB1-F2 has been identified as essential and sufficient for mitochondrial membrane localization [4,5].

PB1-F2 interacts with the mitochondrial permeability transition pore complex components ANT3 (adenine nucleotide translocator 3) and VDAC1 (voltage-dependent anion channel 1) and may thus play a role in the induction of mitochondria-mediated apoptosis [6]. The PB2 polymerase protein from both human and avian IAVs also localizes to mitochondria, where it may play a role in maintaining mitochondrial function during IAV infection [7]. Although PB1-F2 is not required for viral infectivity, it interacts directly with PB1, and the absence of PB1-F2 results in altered localization of PB1 and decreased polymerase activity [8]. Recent studies using mouse models support a role for PB1-F2 in pathogenicity and lethality [6,9]. PB1-F2 enhances inflammation during primary viral infection of mice and increases both the frequency and severity of secondary bacterial pneumonia [10,11].

The discovery of PB1-F2 was based on the ability of IAV infection to elicit a robust CD8⁺T cell response specific for a well-defined peptide encoded by residues 62-70. Although this clearly established that PB1-F2 is expressed *in vivo* during a natural IAV infection, PB1-F2 expression levels might be miniscule since the rapid degradation of PB1-F2 could enhance its immunogenicity for CD8⁺ T cells. In contrast to CD8⁺ T cells, the magnitude of Ab responses is based on steady-state levels of immunogen and this provides a better measure of viral gene expression *in vivo*.

In the present study, to gauge PB1-F2 expression in mice and humans, we have developed a number of assays for measuring anti-PB1-F2 Ab responses. Our findings clearly demonstrate the immunogenicity of PB1-F2, supporting its biological relevance in IAV infections.

Methods

Viruses and cells

The following IAVs were used: A/PR/8/34 (H1N1), A/Mississippi/1/85 (H3N2) and A/Wyoming/3/2003 (H3N2). The conditions for infection of embryonated hen eggs and purification of the viruses have been described [12].

Recombinant vaccinia virus VV-PB1-F2 expressing PB1-F2 in infected cells was generated as described [13]. Wild-type (wt) vaccinia virus CR19 and recombinant VV-PB1-F2 were grown in human osteosarcoma 143 TK⁻ cells in DMEM with 5% FBS. After 3 days of incubation at 37°C in a humidified atmosphere containing 5% CO₂, the infected cells were harvested. The cell sediment was resuspended in 10 mM Tris-HCl buffer, pH 9. The virus was released from cells after their disruption by three cycles of freezing and thawing and subsequent sonication. The titer of infectious vaccinia viruses, expressed as PFU/ml, was determined by plaque titration using 143 TK⁻ cells. For immunofluorescence analysis, MDCK cells were infected with the recombinant vaccinia virus. The sequence of sPB1-F2 derived from influenza virus A/PR8 (H1N1) was described previously [14]. Fusion protein PB1-F2-MBP was prepared using the pMAL-c2X.MBP Protein Fusion and Purification System (New England Biolabs).

Mouse and human sera

Mice were first infected i.n. with 1 LD₅₀ of A/PR8 (H1N1). To boost induction of PB1-F2-specific Abs, the surviving mice were infected i.n. 31 days later with 1/8 LD₅₀ of A/Mississippi/1/85 (H3N2). Immune serum was collected 21 days later. Immune mouse serum to the N-terminal part of PB1-F2 was prepared by intraperitoneal immunization with the N-terminal peptide of PB1-F2 (aa 3-13) conjugated to KLH [15]. Acute and convalescent human serum samples taken on the first days of illness and 3-5 weeks later, respectively, were obtained during the winter epidemics of 2003-2004, for which mostly influenza A viruses H3N2 were responsible. Mouse and human serum samples were stored at -70°C.

Monoclonal Abs (MAbs)

MAb AG55 specific for the N-terminal part of PB1-F2 was prepared by fusion of mouse myeloma cell line Sp2/0 with spleen cells from BALB/c mice immunized with fusion PB1-F2-MBP protein by standard hybridoma technique. MAb TW2.3 was used for detection of vaccinia virus infection [16].

Hemagglutination-inhibition (HI) test

Sera were treated with receptor-destroying enzyme from *Vibrio cholerae* (DENKA SEIKEN, Japan) for 16 h at 37°C prior to heat inactivation for 30 min at 56°C. HI titers were determined by a microtechnique and were expressed as reciprocals of the highest dilutions of sera causing inhibition of four hemagglutination units of the virus A/Wyoming/3/2003 (H3N2) used in the form of infectious allantoic fluid.

ELISA

Binding of serum Abs to sPB1-F2 and purified A/PR8 (H1N1) was determined by ELISA. sPB1-F2 and A/PR8 were adsorbed onto wells of microtiter plates, washed, and saturated with 1% of nonfat dry milk. Twofold dilutions of serum samples were added, and after incubation and washing, the binding of immunoglobulins to antigens was detected with rabbit anti-mouse or anti-human IgG conjugated to horseradish peroxidase and 1,2 phenylene diamine dihydrochloride at 492 nm.

Western blotting (WB)

PB1-F2-MBP fusion protein or sPB1-F2 was separated by 15% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. The proteins were electroblotted onto nitrocellulose membrane (Schleicher and Schuell; 0.45 µm) in 10 mM Tris-glycine buffer with 20% methanol. Blots were blocked overnight at 4°C in PBS containing 5% nonfat dry milk, washed twice with PBS and cut into strips. The strips with proteins were incubated individually with immune sera diluted 1/100 in PBS containing 2% nonfat dry milk for 2 h at RT and washed in PBS. After extensive washing, the strips were incubated for 2 h with peroxidase-conjugated

rabbit anti-mouse or anti-human IgG Abs diluted in 0.01% Triton X-100 in PBS (Dako, Germany) and washed with 0.01% Triton X-100 in PBS, and the immunoreactive bands were visualized by chemiluminescence (ECL Detection System Santa Cruz Biotechnology Inc., USA).

Immunoprecipitation analysis

Five-hundred ng of sPB1-F2 in 50 µl of PBS was incubated overnight with 10 µl of mouse or human serum. This mixture was then incubated for 1 h with protein G Sepharose. After extensive washing, sPB1-F2 was eluted from the beads by boiling in SDS-PAGE sample buffer, separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane. The presence of sPB1-F2 on blots was detected by chemiluminescence following incubation with MAb AG55 and peroxidase-conjugated rabbit anti-mouse or anti-human IgG Abs.

Immunofluorescence analysis

MDCK cell cultures on coverslips were infected with CR19 or VV-PB1-F2 viruses at an MOI of four for 6 h. The infected cells were then fixed with 2% paraformaldehyde for 15 min and subsequently permeabilized with 1% Triton X-100 in PBS for 1 min. After washing, the cells were blocked with 2% normal rabbit serum in PBS and incubated for 90 min at 4°C with mouse or human serum diluted 1/100 in PBS containing 1% normal rabbit serum. After washing with PBS, the cells were incubated for 90 min at 4°C with PBS containing 1% (v/v) fluorescein-conjugated rabbit anti-mouse or anti-human IgG, washed, and mounted in UltraCruz Mounting Medium. The fluorescence was observed using a Zeiss fluorescent microscope.

Results

ELISA detects anti-PB1-F2 Abs in mouse or human sera

ELISA is a rapid and inexpensive method for measuring Ab responses. Using a full-length synthetic version of PR8 PB1-F2 (sPB1-F2), we explored the ability of ELISA to detect anti-PB1-F2 responses. Mice were infected i.n. with A/PR8 (H1N1), and 31 days later, surviving mice were infected i.n. with A/Mississippi/1/85 (H3N2). Twenty-one days PI, immune serum was collected. Although PR8 infection was confirmed by detecting virus-specific Abs by ELISA with A/PR8 (H1N1), no specific PB1-F2 response was detected above background values obtained with preimmune sera (data not shown). This could not be attributed to the inability of PB1-F2 to bind to the ELISA plate in antigenic form, since it was easily detected by mouse MAbs or rabbit polyclonal Abs (pAbs) raised to PB1-F2 (data not shown).

We next used the ELISA to test paired human sera from the winter epidemics of 2003-2004 for the presence of anti-PB1-F2 Abs. For all paired sera, convalescent sera demonstrated a significant rise in HI Ab titers, confirming recent infection with influenza virus A/Wyoming/3/2003 (H3N2) (Table 1).

When the human sera were tested against sPB1-F2, ELISA titers varied, demonstrating only slight or no increase in convalescent sera (data not shown). There was no clear correlation between HI and anti-PB1-F2 Ab responses in the human convalescent sera tested. Given the broad exposure of the population to IAV, we cannot determine the extent to which PB1-F2 Abs detected in ELISA are specifically elicited by PB1-F2 as opposed to other immunogens. Resolution of this question in future studies will require samples from children experiencing their first IAV infection.

Immunoprecipitation reveals Abs recognizing PB1-F2 in mouse immune and human convalescent sera

Next, we examined detection of PB1-F2-specific Abs by WB using sPB1-F2 as antigen. Although polyclonal Abs and the MAb AG55 clearly detect sPB1-F2, we failed to detect Ab binding to sPB1-F2 with any of mouse immune (data not shown) or human convalescent sera (Fig. 1).

Since WB is largely limited to detecting Abs specific for linear epitopes, we next turned to immunoprecipitation, which is capable of detecting Abs specific for conformational determinants. sPB1-F2 was first incubated with mouse or human serum. Next, sPB1-F2 Ab complexes were collected by protein G Sepharose. After extensive washing, proteins were eluted from protein G Sepharose by boiling in SDS-PAGE sample buffer, separated by SDS-PAGE and electroblotted onto nitrocellulose membrane. Ab-bound PB1-F2 was detected by WB using the PB1-F2 specific MAb AG55. Normal mouse serum, immune mouse serum to the N-terminal part of PB1-F2, or MAb AG55 were used as controls.

Results of this analysis clearly showed that Abs binding to PB1-F2 were present in mouse immune and human convalescent sera (Fig. 2). PB1-F2-specific Abs were consistently found in sera from mice with two subsequent i.n. infections, and also in some sera following single i.n. infection. Immunoprecipitation of paired human sera showed a significant increase in the level of Abs binding to PB1-F2 in convalescent sera. Importantly, there was a good correlation between the increase in HI Ab titer in convalescent sera and in the amount of PB1-F2 precipitated in convalescent sera (Table 1; Fig. 2).

Immunofluorescence analysis of cells infected with recombinant vaccinia virus expressing PB1-F2 confirms the presence of PB1-F2-specific Abs in mouse immune and human convalescent sera

These findings indicate that infected mice and humans generate anti-PB1-F2 Abs that probably recognize conformational epitopes. To extend these findings, we examined the ability of sera to recognize PB1-F2 expressed in MDCK cells infected with a rVV expressing the PB1-F2 ORF. The conditions used for fixation and permeabilization favor the detection of Abs specific for conformational determinants. Controls established that MAb AG55 clearly stains cells infected with VV-PB1-F2 but not a control VV (CR19). We analyzed sera from three mice after primary infection and seven following secondary infection. All of these sera showed staining of cells infected with VV-PB1-F2 but not a control VV. Additional immunofluorescence analysis confirmed PB1-F2 antibodies in eight human convalescent sera and in two serum samples not listed in Table 1. Representative immunofluorescence results for two mouse and two human sera are shown in Fig. 3.

Discussion

During IAV infections, Abs are generated to both structural (HA, NA, NP, NS2, M1, M2,) and non-structural proteins (NS1) [17,18]. Only Abs to HA can efficiently neutralize virus infectivity. Nevertheless, Abs to NS1, NS2 and M2 are practically useful because they can differentiate infected from vaccinated animals [18-21]. PB1-F2 is also a nonstructural protein, as there is less than one PB1-F2 molecule per virion (Russ and Yewdell, unpublished results).

The immunoprecipitation and immunofluorescence analyses described here provide clear evidence that PB1-F2-specific Abs are induced in response to IAV infection. Importantly, immunoprecipitation results revealed similar increases in binding to sPB1-F2 and in titers of HI Abs in paired human sera. As many of the serum donors were previously exposed to IAV infection, such increases indicate that Abs to PB1-F2 are of short duration. This is similar to

anti-M2 Ab responses, were Ab titers have been found to be very low in all patients at the onset of infection despite previous exposure to IAV [22-24].

We were unable to detect PB1-F2-specific Abs unambiguously by WB and ELISA. At this point, we cannot determine the extent to which this reflects the inability of anti-PB1-F2 Abs to interact with denatured PB1-F2 versus the obstruction/disruption of linear epitopes accompanying the binding of PB1-F2 to ELISA plates or nitrocellulose. Interpreting the antigenicity of PB1-F2 will be enhanced when further insights into its structure are available. It does appear clear, however, that antibodies with similar specificity as AG55, which was elicited by immunizing mice with a PB1-F2-containing fusion protein produced in bacteria, do not reach detectable levels in mouse or human sera.

While this manuscript was in preparation, Khurana et al. [25] reported that humans generate anti-PB1-F2 antibodies, as measured using a phage library expressing PB1-F2 sequences. Together with our findings, this firmly establishes the immunogenicity of PB1-F2 in humans and provides the first evidence for the biological relevance of PB1-F2 in human influenza. Inasmuch as antibody responses generally require robust expression of the immunogen, this implies a functional role for PB1-F2 during human infection.

PB1-F2 is reasonably well established as a pathogenicity gene in mouse models [9]. Interestingly, contemporary H1N1 strains, which express only a truncated PB1-F2 of 57 amino acids, are generally less pathogenic in humans than H3N2 or H2N2 strains that express full-length PB1-F2. Notably, the swine H1N1 that was recently introduced into the human population possesses a severely truncated PB1-F2 of 11 amino acids (GenBank PB1 sequences from 2009 H1N1 influenza outbreak including isolates from Mexico, USA, Canada and Korea) yet appears to be of moderate pathogenicity.

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Fig. 1. WB analysis of human paired sera for PB1-F2. Acute (**a**) and convalescent (**b**) human sera, MAb AG55, positive control (PC), normal mouse serum, negative control (NC)

Fig. 2.
Immunoprecipitation analysis of immune mouse sera and human paired sera for PB1-F2-specific Abs. Immune mouse sera (1-10), human paired sera (4-20, acute (a), convalescent (b)), immune mouse serum to PB1-F2, positive control (PC), normal mouse serum, negative control (NC)

Fig. 3. Immunofluorescence analysis of immune mouse sera (**e, f, g, h**) and human convalescent sera (**i, j, k, l**) for PB1-F2-specific Abs. MDCK cells infected with CR19 and detected with MAb TW2.3 (**a**), MAb AG55 (**c**), immune mouse sera (**e, g**), and human convalescent sera (**i, k**), respectively. MDCK cells infected with VV-PB1-F2 and detected with MAb TW2.3 (**b**), MAb AG55 (**d**), mouse immune sera (**f, h**), and human convalescent sera (**j, l**), respectively Table 1

Table 1
Increases in HI titer in paired human sera and IF analysis of human convalescent sera

Serum pair no.	Age	HI titer		Increase in HI titer	IF
		Acute serum	Convalescent serum		
1	102	10	40	4	
2	54	10	20	2	
3	53	10	20	2	
4	46	20	160	8	+
5	37	20	160	8	
6	33	10	80	8	
7	31	10	80	8	
8	30	10	160	16	+
9	27	40	80	2	+
10	24	20	160	8	
11	22	10	160	16	+
12	22	20	80	4	+
13	21	10	160	16	+
14	19	20	60	3	+
15	17	10	160	16	
16	15	10	80	8	
17	14	10	80	8	+
18	14	10	160	16	
19	11	10	160	16	
20	9	20	80	4	