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Identification of cellular proteins interacting with influenza A virus PB1-F2 protein

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Summary. – The influenza A virus (IAV) protein PB1-F2, which is encoded by an alternative ORF of the PB1 polymerase gene, has been implicated as an important virulence factor and apoptosis inducer. However, the molecular mechanism of PB1-F2 function remains elusive. In this study, eight cellular proteins were identified as potential PB1-F2 interacting partners using the yeast two-hybrid system. Two positive candidate proteins, guanine nucleotide binding protein (G protein) beta polypeptide 2 (Gβ2) and macrophage migration inhibitory factor (MIF), were selected to be further characterized. The interaction of MIF and Gβ2 with PB1-F2 was confirmed by both GST pull-down and co-immunoprecipitation assays. Confocal laser microscopy showed that the interaction between PB1-F2 and the two cellular proteins occurred in the cytoplasm**.** The novel interactions between PB1-F2 and host proteins provide further pieces of evidence in the investigation of the pathogenic mechanism of IAV.

Keywords: influenza A virus; PB1-F2; yeast two-hybrid; protein-protein interaction

Introduction

Influenza A virus (IAV) is a pivotal pathogen of humans and many animals, with a high pandemic potential. The virus belongs to the family *Orthomyxoviridae* and possesses a negative strand RNA genome made up of eight gene segments that encode up to 11 viral proteins (Wise *et al*., 2009). PB1-F2 was fortuitously discovered in 2001 as the 11th IAV protein while searching for novel peptides recognized by CD8+ T lymphocytes during IAV infection (Chen *et al*., 2001). PB1-F2 is encoded by a second (+1) ORF in the PB1 gene and is translated from an AUG codon downstream of the PB1 start site. The PB1-F2 ORF is present in the majority of IAV isolates but absent in the influenza B virus genus (Zell *et al*., 2007). Most IAV strains encode a full-length PB1-F2, which is 87 or 90 amino acids (aa) long, but some isolates encode a C-terminally truncated PB1-F2 of varying lengths (Bruns *et al*., 2007). PB1-F2 localizes to mitochondria and its mitochondrial targeting sequence (MTS), which was predicted to form a positively charged amphipathic helix, is responsible for mitochondrial localization. However, PB1-F2 is also found in the cytoplasm and in the nucleus of infected cells (Gibbs *et al*., 2003; Yamada *et al*., 2004). It has also been demonstrated that PB1-F2 is a phosphoprotein that contains multiple PKC phosphorylation sites, and its function is regulated by PKC-mediated phosphorylation (Mitzner *et al*., 2009). In virus-infected monocytes, PB1-F2 is thought to initiate the intrinsic mitochondrial apoptosis pathway by the depolarization of mitochondrial membranes and release of cytochrome c (Chen *et al*., 2001). PB1-F2 may achieve this pro-apoptotic activity by interacting with the mitochondrial membrane-associated proteins adenine nucleotide translocator 3 (ANT3) and voltage dependent anion channel 1

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Abbreviations: ANT3 = adenine nucleotide translocator 3; Gβ2 = guanine nucleotide binding protein beta polypeptide 2; GR = glucocorticoid receptor; GREs = glucocorticoid response elements; IAV = influenza A virus; MAb = monoclonal antibody; MIF = macrophage migration inhibitory factor; MTS = mitochondrial targeting sequence; NTE = neuropathy target esterase; PKC = protein kinase C; PTX = pertussis toxin; VDAC1 = voltage dependent anion channel 1

(VDAC1) in specific immune cells (Zamarin *et al.*, 2005). Recently, PB1-F2 has been identified as an important virulence factor that enhances the severity of primary viral infection and secondary bacterial pneumonia in mice (Zamarin *et al.*, 2006; McAuley *et al.*, 2007). PB1-F2 modulates innate immune response to influenza virus infection by increasing both IFN-β expression in human respiratory epithelial cells and immune cell recruitment (McAuley *et al.*, 2007; Le Goffic *et al.*, 2010). Furthermore, a serine at the position 66 in PB1-F2 is associated with increased pathogenicity and mortality in mouse models by inhibiting the early IFN response *in vivo* (Conenello *et al.*, 2007, 2011). It has also been revealed that PB1-F2 increases influenza virus polymerase activity *in vitro* by interacting with the viral polymerase subunit PB1 (Mazuri *et al.*, 2008).

The molecular mechanisms underlying the functions of PB1-F2 are not fully understood. To further characterize the role of PB1-F2 in IAV infections, we searched for additional host cell factors that interact with PB1-F2 via yeast two-hybrid system. The physical interaction between PB1-F2 and candidate proteins was subsequently verified by GST pull-down and co-immunoprecipitation assays, and the subcellular localization of viral and cellular interacting partners was analyzed after co-transfection in cell culture. Knowledge of these binding factors could shed light on the pathogenic mechanism of IAV and provide important information to further clarify the biological function of PB1-F2.

Materials and Methods

Cells and reagents. The *Escherichia coli* strains DH5α and Rosetta (DE3) were used for the selection and amplification of DNA constructs and for the expression of recombinant proteins, respectively. HeLa and 293T cells were cultured in high-glucose DMEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/l L-glutamine, and 100 U/ml penicillin-streptomycin (Invitrogen) at 37°C with 5% CO_2. Restriction enzymes, anti-FLAG, and anti-HA monoclonal antibody (MAb) as well as goat anti-mouse HRP-conjugated secondary antibodies were purchased from New England Biolabs, Sigma, and Santa Cruz Biotechnology, respectively. A Matchmaker human leukocyte pretransformed cDNA library constructed in pACT2 was purchased from Clontech.

Plasmid constructs. The primers used to construct the recombinant plasmids are shown in Table 1. The full-length PB1-F2 ORF was amplified from a cDNA template that was synthesized from the RNA extract from A/Puerto Rico/8/34 (PR8)-infected allantoic fluid using the forward primer F1 and the reverse primer R1. The PCR product was digested with *Eco*RI and *Bam*HI and then ligated into the yeast expression vector pGBKT7 (Clontech) cleaved with the same restriction enzymes to generate pGBKT7-PB1-F2. To obtain GFP or GST epitope-tagged PB1-F2, the full-length ORF of PB1-F2 was amplified by PCR from pGBKT7-PB1-F2 with primer pairs F2/R2 and F3/R3 and inserted into pLEGFP-C1 and pGEX-6P-1, respectively. Plasmid pACT2-PB1-F2 was constructed by inserting the PB1-F2 gene into *Eco*RI/*Xho*I site of pACT2 vector. The full-length ORF of Gβ2 and MIF were amplified by PCR from the human leukocyte cDNA plasmid library with the primer pairs F5/R5 or F6/R6, respectively. The PCR products were digested with *Bam*HI and *Xho*I and cloned into the eukaryotic vector pcDNA3-FLAG or pcDNA3-HA, which were cleaved with the same restriction enzymes to generate FLAG-tagged Gβ2, HA-tagged and FLAG-tagged MIF. The Gβ2 and MIF genes were subcloned via *Hind*III and *Bam*HI restriction sites into pDsRed2-N1 for laser confocal microscopy. Plasmid construct accuracy was determined by sequence analysis.

Yeast two-hybrid screening. The human leukocyte cDNA library titer was determined after amplification and was approximately 1.0×10^8 CFU/ml. Matchmaker Gal4 Two-Hybrid System 3 was used to screen the library using PB1-F2 of IAV as the bait, according to the instructions provided by Yeast Protocols Handbook (Clontech). *Saccharomyces cerevisiae* strain AH109 was transformed simultaneously with the bait and the cDNA library plasmids using the polyethylene glycol/lithium acetate (PEG/LiAc)-mediated transformation method and was selected on minimal synthetic dropout (SD) medium lacking adenine, tryptophan, leucine, and histidine (denoted SD/-Leu/-Trp/-Ade/-His) and containing 1 mmol/l 3-aminotriazole. After the yeast was cultured for 5–7 days, colonies were restreaked onto SD plates containing 4 mg/ml 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) (Sigma) to check for MEL1 reporter gene expression (blue colonies). Blue colonies were screened four times for growth on the above-mentioned plates to rescue additional library plasmids and eliminate false positives. The final positive candidate plasmids were selected and determined after sequencing analysis. The sequences of positive clones were subsequently used for an advanced BLAST search against the GenBank database. To confirm the interaction specificity, retransformation assays were performed. Plasmid pGBKT7-PB1-F2 and isolated leukocyte cDNA library plasmids were co-transfected into the yeast strain AH109 to test for their ability to grow on SD/-Leu/-Trp/-Ade/-His plates. Protein interactions were also preliminarily identified by the LacZ filter lift assay to test β-galactosidase activity (Durfee *et al.*, 1993).

Expression and purification of GST fusion proteins. The recombinant prokaryotic expression vector, pGEX-6P-1-PB1-F2 or pGEX-6P-1-Gβ2, was transfected into competent *Escherichia coli* Rosetta (DE3) cells. Transformants were grown to an optical density (A_{600}) of 0.6 to 0.7, and the expression of different GST-fusion proteins (PB1-F2, Gβ2) was induced with 1 mmo/l IPTG for 8 hrs at 16°C. Total protein was extracted by sonicating cells in PBS containing 0.5% NP-40, 1 mmol/l dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% protease inhibitor cocktail, followed by centrifugation at 12,000×g for 15 min at 4°C. The supernatant was purified using Glutathione-Sepharose 4B beads according to the manufacturer's protocol (GE Healthcare

Life Sciences). Detection of the expression and purification of GST-fusion proteins was performed by Coomassie Blue staining and Western blot analysis.

GST pull-down assay. HA-MIF and FLAG-Gβ2 proteins were expressed in 293T cells using pcDNA3-MIF/Gβ2 plasmids in 10 cm plates that were 80% confluent. Cells were transiently transformed with the indicated plasmids using Lipofectamine2000 reagents (Invitrogen). After cultivation for 36 hrs, the cells were washed with PBS, resuspended in 0.5 ml lysis buffer (50 mmo/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 1 mmol/l EDTA, 1 % NP-40, 1 mmol/l DTT, 1 mmol/l PMSF and 1% protease inhibitor cocktail) and incubated on ice for 1 hr. Total cell extracts were clarified by centrifugation at 13,000 rpm for 10 min at 4°C. The total cell supernatant was incubated with GST-PB1-F2 protein bound to Glutathione-Sepharose beads. The binding reaction was performed at 4°C overnight, and the beads were subsequently washed four times with ice-cold lysis buffer. The beads were resuspended in 10 μl of 2×SDS sample buffer and resolved by SDS-PAGE, and the proteins were detected by Western blot analysis.

Co-immunoprecipitation assay. Immunoprecipitation assays were performed essentially as described previously (Guan Z *et al.*, 2010). Briefly, 293T cells were transiently transformed with the indicated plasmids using Lipofectamine2000 reagents (Invitrogen). After 24 hrs of cultivation, the cells were washed and resuspended in 0.5 ml lysis buffer (mentioned above). Equal amounts of cleared cell lysates were subjected to immunoprecipitation with anti-FLAG MAb M2-conjugated agarose. The reactions were performed overnight at 4°C, and then the beads were centrifuged at 3,000 rpm for 2 min and washed three times with lysis buffer. The antibody-protein complexes were then resolved by SDS-PAGE, and the GFP or FLAG-tagged proteins were identified by Western blot analysis with an anti-GFP/FLAG antibody.

Western blot analysis. Protein samples were resolved by SDS-PAGE on 10% polyacrylamide gels and transferred by electroblotting to PVDF membranes (Millipore). Membranes were probed with anti-HA antibody (Cell signaling technology) or anti-FLAG antibody (Sigma) to detect HA- and FLAG-epitope-tagged proteins and then probed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology) secondary antibodies diluted 1:5,000 in blocking solution. Membrane-bound antibodies were detected with the enhanced chemiluminescence (ECL) detection reagents (Thermo Scientific Pierce).

Laser confocal microscopy. HeLa cells were grown on slides up to 75% confluence and then transformed with EGFP-tagged PB1-F2 and/or indicated plasmid constructs using the Lipofectamine2000 reagent according to the manufacturer′s instructions. At 24 hrs post-transfection, cells were fixed with 3.7% formaldehyde and stained with DAPI (4′,6-diamidino-2-phenylindole, blue, Sigma). The co-localization of PB1-F2 and Gβ2 or MIF in HeLa cells was analyzed with a confocal laser scanning microscope (Olympus).

Table 1. Primers used in the study

Plasmid construct	Primer		
	Name	Sequence	Restriction site
pGBKT7-PB1-F2	F1	5′-CGGAATTCATGGGACAGGAACAGGATAC-**3′**	*Eco*RI
	R1	5′-CGGGATCCCTACTCGTGTTTGCTGAACAAC-3′	*Bam*HI
pLEGFP-PB1-F2	F2	5′-CCCAAGCTTTGATGGGACAGGAACAGGATAC-3′	*Hind*III
	R2	5′-CGGGATCCCTACTCGTGTTTGCTGAACAAC-3′	*Bam*HI
pGEX-6P-1-PB1-F2	F3	5′-CGGGATCCATGGGACAGGAACAGGATACA-3′	*Bam*HI
	R3	5′-CCGCTCGAGCTACTCGTGTTTGCTGAACAAC-3′	*Xho*I
pACT2-PB1-F2	F4	5′-CGGAATTCGAATGGGACAGGAACAGGATAC-3′	*Eco*RI
	R3	5′-CCGCTCGAGCTACTCGTGTTTGCTGAACAAC-3′	*Xho*I
pcDNA3-FLAG-Gβ2	F5	5′-CGGGATCCATGAGTGAGCTGGAGCAACTG-3′	*Bam*HI
	R5	5′-CCGCTCGAGTTAGTTCCAGATCTTGAGG-3′	*Xho*I
pcDNA3-HA-MIF	F6	5′-CGGGATCCATGCCGATGTTCATCGTAAAC-3′	*Bam*HI
	R6	5′-CCGCTCGAGCTAGGCGAAGGTGGAGTTG-3′	*Xho*I
pDsRed2-N1-MIF	F7	5′-CCCAAGCTTATGCCGATGTTCATCGTAAAC-3′	*Hind*III
	R7	5′-CGGGATCCCCGGCGAAGGTGGAGTTGTTC-3′	*Bam*HI
pDsRed2-N1-Gβ2	F8	5′-CCCAAGCTTATGAGTGAGCTGGAGCAACTG-3′	*Hind*III
	R8	5′-CGGGATCCCCGTTCCAGATCTTGAGG-3′	*Bam*HI

Results

Identification of cellular proteins interacting with PB1-F2

It is important to identify the cellular proteins that interact with PB1-F2 to elucidate the molecular mechanisms of PB1-F2 function. To identify novel proteins that interact with PB1-F2, we screened a commercially prepared human leukocyte cDNA library using the pGBKT7-PB1-F2 plasmid as bait via the yeast two-hybrid assay. Thirteen colonies grown on SD-Ade/-His/-Leu/-Trp plates were identified among the approximately 5.0×10^6 clones that were screened. Among them, ten clones were found to express the MEL1 reporter gene. The plasmids were rescued from these positive colonies, amplified in *E. coli*, sequenced and analyzed using BLAST. Sequence analysis of the plasmid DNA showed that sequences might correspond to eight independent cellular proteins, as shown in Table 1.

In the retransformation assays, growth on SD medium is supported only when the two hybrid proteins interact and induce transcription of the *his* reporter gene (Fig. 1a). The interaction was further confirmed with a β-galactosidase filter lift assay, (Fig. 1b). As positive control, yeast cells were co-transformed with pGBKT7-T antigen and pACT2-p53, and with pGBKT7-PB1-F2 and pACT2-PB1-F2, respectively. As negative control, the same yeast strain was co-transformed with pGBKT7-PB1-F2 and pACT2, and with pGBKT7-LaminC and pACT2-p53, respectively. Cells co-transformed with pGBKT7-PB1-F2 and any of the eight positive candidate plasmids grew and turned blue on SD/-Ade/-His/-Leu/-Trp/X-α-Gal agar medium, whereas the negative controls did not turn blue. Two of the candidate proteins encode Gβ2 and MIF, which were pursued for further evidence of binding.

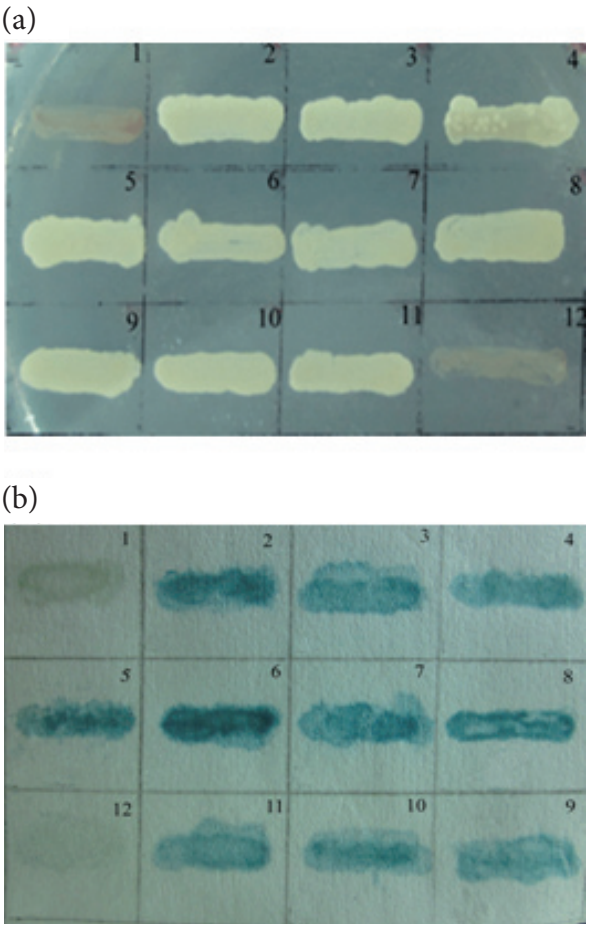

Fig. 1

Yeast two-hybrid screening

The yeast was co-transformed with pGBKT7-PB1-F2 (bait) and positive cDNA library plasmids (prey). (a) Growth of the yeast on selective medium. (b) Filter lift assay. Blue staining indicates a positive protein-protein interaction. Negative controls (1, 12), positive controls (2, 11), positive cDNA library plasmids (3–10).

Interaction of PB1-F2 with Gβ2 and MIF in vitro

To confirm the results of the yeast two-hybrid studies, GST pull-down assays were performed to verify protein interactions between PB1-F2 and Gβ2 or MIF *in vitro*. In this assay, PB1-F2 was fused to Glutathione S-transferase (GST) and was expressed by induction with 1 mmol IPTG in prokaryotic cells. The GST alone or the GST fusion protein, GST-PB1-F2, were immobilized on Glutathione-Sepharose beads, and the expression of the proteins was detected with Coomassie blue staining. Next, recombinant plasmids pcDNA3-FLAG-Gβ2 and pcDNA3-HA-MIF were transfected into 293T cells using the liposome method to produce FLAG-tagged Gβ2 and HA-tagged-MIF protein. Finally, the 293T cell lysates containing Gβ2 or MIF were incubated with GST fusion proteins immobilized on Glutathione-Sepharose beads, and the bound proteins were separated by SDS-PAGE and detected by Western blot analysis. The presence of FLAG-Gβ2 or HA-MIF demonstrated the interaction *in vitro*. No complex formation was observed when Gβ2/MIF was combined with a control GST protein (Fig. 2). The results demonstrate that PB1-F2 may interact with Gβ2 and MIF and are in agreement with the results obtained from the yeast two-hybrid system.

Interaction of PB1-F2 with Gβ2 and MIF in vivo

To further assess the interaction between MIF, Gβ2, and PB1-F2 *in vivo*, the plasmids that express GFP-PB1-F2, FLAG-Gβ2 or FLAG-MIF were co-transfected into 293T cells. The cell lysates were immunoprecipitated with the anti-FLAG M2-conjugated agarose and subsequently immunobloted with anti-GFP or anti-FLAG antibody. Consistent with the GST pull-down and yeast two-hybrid results, PB1-F2 specifically

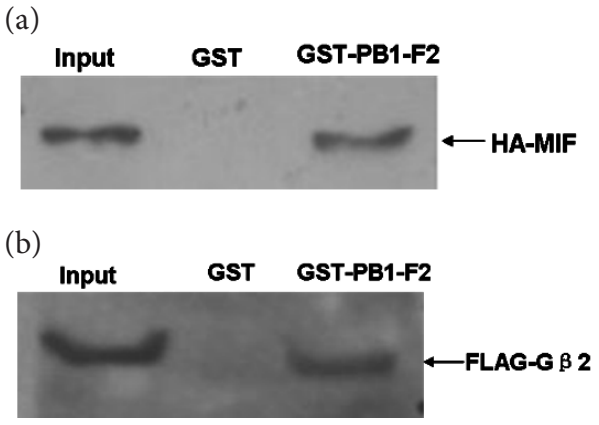

Fig. 2

Interaction of PB1-F2 with MIF or Gβ2 *in vitro*

GST pull-down assay for MIF (a) and Gβ2 (b) using Western blot analysis.

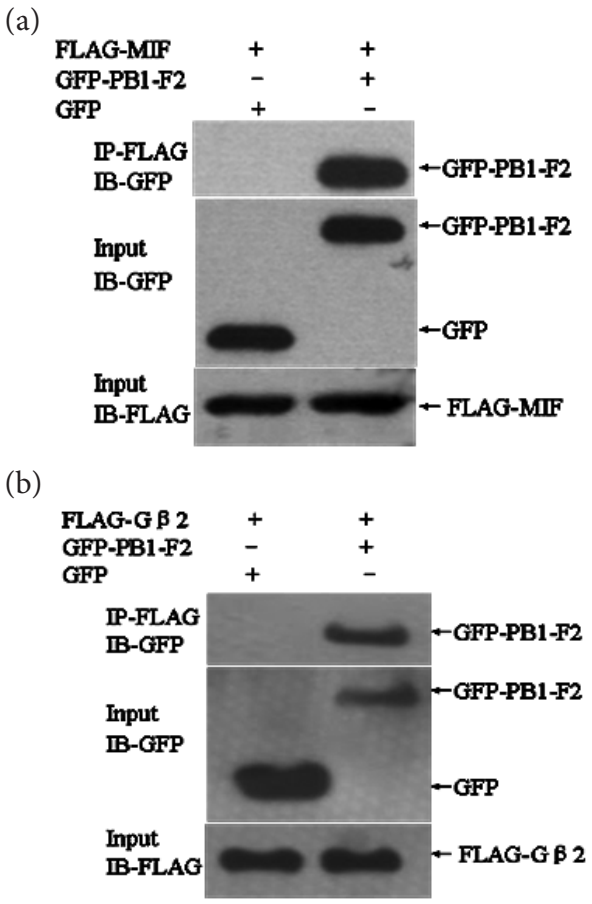

Fig. 3

Interaction of PB1-F2 with MIF or Gβ2 *in vivo*

Co-immunoprecipitation assay of MIF (a) and Gβ2 (b).

interacted with MIF (Fig. 3a) and Gβ2 (Fig. 3b). A reciprocal co-immunoprecipitation experiment also showed the physical interaction between PB1-F2 and Gβ2 or MIF (data not shown).

Co-localization of PB1-F2 with Gβ2 and MIF in HeLa cells

To determine whether MIF and Gβ2 co-localize with PB1-F2 in mammalian cells, laser confocal microscopy was performed. HeLa cells were transiently transformed with expression vectors encoding GFP-PB1-F2 and Red-MIF or Red-Gβ2, and the sub-cellular distribution of proteins was examined using confocal microscopy. Nuclei were visualized with DAPI. Our data indicated that PB1-F2 and MIF localized to similar parts of the cytoplasm, and the appearance of yellow color in the merged image represented co-localization of the two proteins (Fig. 4a). Similar to PB1-F2 and MIF, the co-localization of PB1-F2 and Gβ2 was also observed in the cytoplasm. In addition, PB1-F2 and Gβ2 formed both speckle-like structures within the cytoplasm (Fig. 4b). These data demonstrate that PB1-F2 binds to MIF and Gβ2 in human cells.

Discussion

IAV is one of the most common infectious pathogens that is a world-wide threat to human and animal health and is responsible for at least three pandemics in the last century. The pathogenic mechanism of IAV is complex because of its high genetic mutation or reassortment rate. PB1-F2 was recently discovered as an important virulence factor of IAV; however, the molecular mechanism of PB1-F2 pathogenicity and the dependence of viral growth on host cell factors are still poorly understood. More recently, it was demonstrated that PB1-F2 performed different functions depending on the cell type infected. In immune cells, PB1-F2's pro-apoptotic effect may be dominant, while its polymerase regulating activity may be a modulating event in epithelial cells (Mazur *et al.*, 2008). PB1-F2 localizes to mitochondria and promotes apoptosis by interacting with the mitochondrial membrane proteins ANT3 and VDAC1, but is also diffusely distributed in the cytoplasm and the nucleus, which suggests that PB1-F2 may have additional functions. Additionally, little is known about protein-protein interactions between PB1-F2 and host proteins. To gain knowledge about the virus-host interplay, we used a yeast two-hybrid screen to identify human host proteins that interact with PB1-F2 from a leukocyte cDNA library. Here, we successfully identified 8 proteins that interact with PB1-F2 proteins, including kinases, kinase related receptors, cell signaling related molecules, and other functional proteins. Of the putative candidates identified, Gβ2

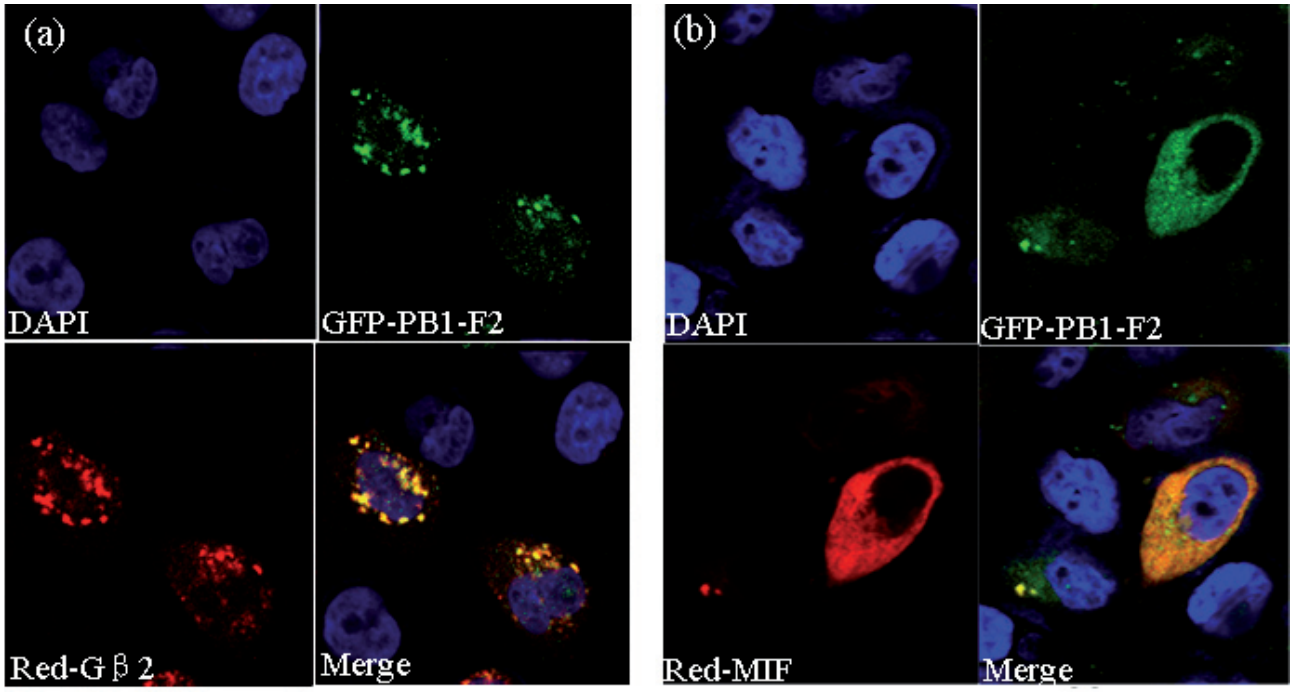

Fig. 4

Co-localization of tagged PB1-F2 and Gβ2 (a) or MIF (b) following their expression in HeLa cells

Confocal microscopy at 24 hrs post transfection. Red = Red-Gβ2 or Red-MIF, green = GFP-PB1-F2, blue = DAPI-stained cell nuclei, and merged images demonstrate colocalization of both proteins in yellow.

Table 2. The BLAST results of positive clones obtained from the leukocyte cDNA library using PB1-F2 of IAV as bait

Gene	GenBank Acc. No.
Homo sapiens SNF related kinase (SNRK)	NM_001100594
Homo sapiens PAS domain containing serine/threonine kinase (PASK)	NM_015148
Homo sapiens Rho guanine nucleotide exchange factor (GEF) 1 (ARHGEF1)	NM_199002
Homo sapiens UDP-glucose pyrophosphorylase 2 (UGP2)	NM_006759
Homo sapiens guanine nucleotide binding protein beta polypeptide 2 (GNB2)	NM_005273
Homo sapiens plasminogen activator, urokinase receptor (PLAUR)	NM_001005377
Homo sapiens phospholipid scramblase 1 (PLSCR1)	NM_021105
Homo sapiens macrophage migration inhibitory factor (MIF)	AF469046

and MIF were further investigated. To confirm the results of yeast two-hybrid studies, we conducted GST pull-down and co-immunoprecipitation assays between PB1-F2 and Gβ2 or MIF *in vitro* and *in vivo*, and the interaction of the two proteins was confirmed by double-direction binding assays (data of the reciprocal assays not shown). Furthermore, confocal microscopy also confirmed the interaction between PB1-F2 and Gβ2 or MIF in HeLa cells.

MIF was first identified in 1966 as a T-cell-derived cytokine that inhibits the macrophage migration *in vitro* (Bloom BR *et al.*, 1966; David *et al.*, 1966). Significant progress has been made on MIF since its discovery. It has been revealed that MIF is a pleiotropic cytokine with well-described roles in inflammation, immunity, cell proliferation, tumorigenesis, and angiogenesis (Mitchell *et al.*, 2002, 2004; Morand *et al.*, 2005; Liu *et al.*, 2008). MIF levels are elevated in the serum of animals and patients with infection or different inflammatory disorders, such as sepsis, Acute Respiratory Distress Syndrome, rheumatoid arthritis or viral infection (Donnelly *et al.*, 1997; Abed *et al.*, 2005). Recent studies have indicated that patients with viral infections, such as those caused by hepatitis B virus, West Nile virus, Dengue virus or HIV-1, have higher MIF plasma concentrations than control subjects (Zhang *et al.,* 2002; Regis *et al.*, 2010). It was reported that MIF plays a significant role in the response to Dengue virus infection and its pathogenesis (Assuncao-Miranda *et al.*, 2010). It also was demonstrated that HIV-1 infection triggers MIF release, and MIF secretion augments viral replication (Chen *et al.*, 2006). Upon the IAV infection, MIF is released

from primary human bronchiolar epithelial cells, and it may be involved in the inflammatory response by induction of pulmonary inflammatory cytokines and chemokines (Arndt *et al.*, 2002; Hou *et al.*, 2009).

Several proteins were identified as interacting partners of MIF. MIF interacts with the pro-apoptotic Bcl-2 family member Bim and inhibits Bim-mediated apoptosis (Liu *et al.*, 2008). MIF interacts with HBx and inhibits its apoptotic activity (Zhang *et al.*, 2006). It has been indicated that the interaction between metastasis suppressor NM23-H1 and MIF is critical for alleviation of MIF-mediated suppression of p53 activity (Jung *et al.*, 2008). Therefore, interactions with cellular proteins or viral proteins appear to be important for MIF function. In this study, we have demonstrated that IAV PB1-F2 and MIF physically interact, and this association may partially affect PB1-F2 function or viral pathogenesis.

The heterotrimeric guanine nucleotide-binding proteins (G proteins) are composed of three subunits designated as Gα, Gβ, and Gγ, each of which also has many isoforms (Zhu *et al.*, 2009). Humans and mice have 16 α, 5 β, and 12 γ subunit isoforms although not all combinations are formed. The Gβ-subunit of heterotrimeric G proteins has a seven times-repeated blade-like β propeller structure, called a WD repeat. It has been shown that the WD repeat is a new interaction motif, which forms the Gβ/Gγ complex that binds to and modulates the activity of effectors and regulators, including enzymes and several forms of potassium and calcium ion channels (Li *et al.*, 2005). An isoform of the G protein β-subunit, Gβ2, is widely expressed in the cytoplasm and the nucleus as well as at the cytoplasmic surface of the plasma membrane. It has been demonstrated that Gβ2 subunit interacts directly with neuropathy target esterase (NTE) and maintains the activity of NTE. Moreover, silencing the expression of Gβ2 or treatment with pertussis toxin (PTX) for an extended time down-regulates the activity of NTE without any change in protein level (Chen R *et al.* 2007). Gβ2 was associated with glucocorticoid receptor (GR)/glucocorticoid response elements (GREs) *in vivo* and suppressed activation function-2-directed transcriptional activity of the GR (Kino *et al.*, 2005). Furthermore, it has been confirmed that the Gβ2 protein is closely associated with microtubule assembly and that it may play a potential role in the regulation of cell proliferation and microtubule and mitotic spindle organization in mammalian cells (Wu *et al.*, 2001). Recently, Gβ2 was also found to regulate the mobility of mitofusin 1 on the surface of the mitochondrial membrane, and it affected mitochondrial fusion by interacting with mitofusin 1 (Zhang *et al.*, 2010). It has been reported that Gβ2 bound to Axin and inhibited Wnt-mediated reporter activity (Waragai *et al.*, 2006). Several recent studies have demonstrated the important role of G proteins in mediating signaling events through both CCR5 and CXCR4 that are necessary for productive HIV replication (Juno *et al.*, 2010). It is still important to understand the contribution of G protein signaling to viral replication and disease progression. In this study, we have identified Gβ2 as a novel binding partner of IAV PB1-F2 by several protein-protein interaction assays. The significance of the interaction between PB1-F2 and Gβ2 still require further investigation.

In conclusion, we have identified several cellular proteins that interact with the IAV PB1-F2, which may be associated with several aspects of viral replication. The novel interactions that were confirmed may also provide further evidence that PB1-F2 can interact with a number of other host proteins, which may influence a range of biological functions.

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