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Phage display technique identifies the interaction of severe acute respiratory syndrome coronavirus open reading frame 6 protein with nuclear pore complex interacting protein NPIPB3 in modulating Type I interferon antagonism

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IFN antagonism; NPIPB3; ORF6; phage display; SARS-CoV

Abstract Background/Purpose: Severe acute respiratory syndrome coronavirus (SARS-CoV) proteins including ORF6 inhibit Type I interferon (IFN) signaling.
Methods: This study identified SARS-CoV ORF6-interacting proteins using the phage displayed human lung cDNA libraries, and examined the association of ORF6–host factor interaction with Type I IFN antagonism. After the fifth round of biopanning with Escherichia coli-synthesized ORF6-His tagged protein, the relative binding affinity of phage clones to ORF6 was determined using direct enzyme-linked immunosorbent assay.
Results: The highest affinity clone to ORF6 displayed the C-terminal domain of NPIPB3 (nuclear pore complex interacting protein family, member B3; also named as phosphatidylinositol-3-kinase-related kinase SMG-1 isoform 1 homolog). The coinmunoprecipitation assay demonstrated the direct binding of ORF6 to the C-terminal domain of NPIPB3 in vitro. Confocal

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related deaths until December 26, 2014, according to the
identified in 941 laboratory-confirmed cases with 347
emerged from the Arabian Peninsula in 2012, was globally
respiratory syndrome coronavirus (MERS-CoV), which
26-december-2014-mers/en/). The CoV genome is a
ORF 8b, and ORF 9b).3 These accessory proteins could be
proteins (ORF 3a, ORF 3b, ORF6, ORF 7a, ORF 7b, ORF 8a,
nucleocapsid, membrane, and envelope), and accessory
replicase (ORF1a and ORF1ab), structural proteins (spike,
14 potential open reading frames (ORFs) and encodes
signaling.4,5
response, including inhibition of Type I IFN production and
and ORF6 proteins modulate the host innate immune
factors in Type I IFN antagonism. The C terminus of
further examining the association of SARS-CoV ORF6 and
infected cells6; ORF6 overexpression triggers the ER stress
of endoplasmic reticulum (ER) and Golgi apparatus in
transfected cells.7 The ORF6 protein is dispensable for
viral replication, but might have a specific function in viral infection. ORF3 and ORF 7a proteins activate JNK (c-Jun N-terminal kinase) and NF-κB (nuclear factor kappa B) signaling, upregulate interleukin-8 and
factor kappa B) signaling, upregulate interleukin-8 and
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and ORF6 proteins modulate the host innate immune response, including inhibition of Type I IFN production and signaling.4,5

The SARS-CoV ORF6 protein localizes in the membrane of endoplasmic reticulum (ER) and Golgi apparatus in infected cells6; ORF6 overexpression triggers the ER stress in transfected cells.7 The ORF6 protein is dispensable for viral replication in vitro and in vivo,8 but associates with viral escape from the innate immune system, particularly inhibition of Type I interferon (IFN) production and signaling pathways.7,9 ORF6 protein interacts with the C terminus of karyopherin alpha 2, leading to impeding the nuclear import of phosphorylated signal transducer and activator of transcription 1 (STAT1) in response to IFN-β. Thus, SARS-CoV ORF6 protein is an antagonist of Type I IFNs. In this study, we identified cellular ORF6-interacting factors using phage display human lung cDNA library, further examining the association of SARS-CoV ORF6 and host factors in Type I IFN antagonism. The C terminus of nuclear pore complex interacting protein NPIPB3 (nuclear

Introduction

The pandemic outbreak of severe acute respiratory syndrome coronavirus (SARS-CoV) arose from Guangdong Province of China in November 2002, and eventually infected > 8500 people worldwide.1,2 The Middle East respiratory syndrome coronavirus (MERS-CoV), which emerged from the Arabian Peninsula in 2012, was globally identified in 941 laboratory-confirmed cases with 347 related deaths until December 26, 2014, according to the World Health Organization (http://www.who.int/csr/don/26-december-2014-mers/en/). The CoV genome is a single-stranded, positive-sense RNA of ~30 kb; it contains 14 potential open reading frames (ORFs) and encodes replicase (ORF1a and ORF1ab), structural proteins (spike,
nucleocapsid, membrane, and envelope), and accessory proteins (ORF 3a, ORF 3b, ORF6, ORF 7a, ORF 7b, ORF 8a, ORF 8b, and ORF 9b).3 These accessory proteins could be dispensable for virus replication, but might have a specific function in viral infection. ORF3 and ORF 7a proteins activate JNK (c-Jun N-terminal kinase) and NF-κB (nuclear factor kappa B) signaling, upregulate interleukin-8 and
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Methods

Construction and expression of recombinant ORF6 protein in Escherichia coli and human promonocyte HL-CZ cells

For generating bacterial and mammalian expression of recombinant ORF6 protein, the SARS-CoV ORF6 gene of the SARS-CoV TW1 strain genome (GenBank Accession No. AY291451) was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from genome RNA template and cloned into the pTriEx-4 Neo vector for the production of the recombinant ORF6 protein fused with an N-terminal His-tag. The primers included 5′-ATCGGATATC
TATGTTCATCCGT-3′ and 5′-ATGGGCGCGCTGAGAAT
CTAAGCTC-3′. The forward primer contained an EcoRI restriction site; the reverse primer included a NotI restriction site. The amplified RT-PCR product was cloned into the pTriEx-4 Neo vector (Novagen, Madison, Wisconsin, USA), resulting in construct named pTriEx-ORF6. For the production of E. coli-synthesized ORF6 protein, pTriEx-ORF6 was transformed into E. coli BL21 (DE3) cells. The induction expression and purification of E. coli-synthesized ORF6 protein were performed as described in our prior reports.10,11 Finally, the recombinant ORF6 protein was purified using immobilized-metal affinity chromatography as described in our previous study,12 and then analyzed by Western blotting with anti-His-tag monoclonal antibody and alkaline phosphatase-conjugated goat antimouse immunoglobulin G (IgG) antibodies. The immunoreactive band was developed with tetranitroblue tetrazolium/5-bromo-4-...
Biopanning of a phage display human cDNA library with SARS-CoV ORF6 protein

For identifying ORF6-interacting proteins, a human lung cDNA library (Novagen, Madison, Wisconsin, USA) was used to screen high-affinity phage clones to recombinant ORF6, as previously described. Briefly, biopanning of the phage display lung cDNA library was performed using ORF6-coated microplates. After five rounds of biopanning, ORF6-interacting phage clones were eluted with the soluble ORF6 protein. The ORF6-interacting phage clone was randomly picked up from individual plaques, amplified in E. coli, and then used for determining the binding affinity to the recombinant ORF6 protein using direct enzyme-linked immunosorbent assay. The nucleotide sequences of ORF6-interacting proteins displayed on the high-affinity phage clones were directly sequenced; their deduced amino acid sequences were analyzed using BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

Subcellular localization assays of STAT1

For testing the effect of ORF6 and NPIPB3 interaction on IFN-stimulated STAT1 activation, lysate from cells expressing single and both of ORF6 and NPIPB3 treated with IFN-β (Hoffmann-La Roche) for 1 hour, and then harvested. Cells were fixed by cold methanol, incubated with anti-STAT1 mAb (Cell Signaling Technology). The immune-reactive bands were probed using horseradish peroxidase-conjugated goat antimouse IgG antibodies, and then developed using enhanced chemiluminescence reagents.

Coimmunoprecipitation and colocalization assays

The nucleotide sequences of the C terminus (amino acid residues 936–1050) of NPIPB3 (Accesion Number Q92617) fused with the coat protein of ORF6-interacting phage clone 40 was amplified using PCR, and then cloned into bacterial expression vector pET32a for coimmunoprecipitation in vitro and mammalian expression vector pDsRed1-C (BD Biosciences Clontech) for colocalization assay. The two primer pairs were 5’-ATCGGATATCTCAACCTCCGCCTCTT-3’ and 5’-ATCGGAATTCGCTCAACCTCCGCCTCTT-3’ for pET32a cloning, as well as 5’-ATCGATGGGATCCGCTCAA-CCTCCGCCTCTT-3’ and 5’-ATCGGAATTCGCTCAACCTCCGCCTCTT-3’ for pDsRed1-C cloning, respectively. For coimmunoprecipitation assays, E. coli BL21 (DE3) cells were transformed with pET32a-NPIPB3; the E. coli-synthesized C-terminal domain of NPIPB3 protein was induced and purified using immobilized-metal affinity chromatography as described in our prior studies. The recombinant C-terminal domain of NPIPB3 with a thioredoxin (Trx) at the N terminus and a His-tag at the C terminus was mixed with the recombinant ORF6 protein with an N-terminal His-Tag; the mixture was incubated with the anti-Trx mAb overnight in a cool room, followed by addition of protein A-Sepharose beads for an additional 2 hours. After centrifugation (16,000 × g, 15 minutes), the immunoprecipitate was analyzed using Western blotting with anti-STAT1 (Tyr701), and anti-phosphotyrosine STAT1 (Tyr701) mAb (Cell Signaling Technology). The immune-reactive bands were probed using horseradish peroxidase-conjugated goat antimouse IgG antibodies, and then developed using enhanced chemiluminescence reagents.

Statistical analysis

Three independent experiments in each independent result were performed; all data are represented as mean ± standard deviation and statistically analyzed using SPSS software (version 10.1; SPSS Inc., Chicago, IL, USA) via one-way analysis of variance analysis by Scheffe’s test.

Results

Selection of SARS-CoV ORF6-interacting host facts using phage display library

SARS-CoV ORF6, cloned into the pTriEx-4 vector, was synthesized as a 17.5-kDa fusion protein with an N-terminal (FITC)-conjugated antimouse IgG antibodies, as described in our previous studies. A confocal image of stained cells was taken using Leica TCS SP2 AOBS laser scanning microscopy (Leica Microsystems, Heidelberg GmbH, Germany). In cells, red fluorescence indicated DsRed-NPIPB3 fusion protein, whereas green fluorescence was ORF6-His tag fusion protein. The colocalization of DsRed-NPIPB3 and ORF6 appeared orange to yellow in color.

Dual-luciferase reporter assay of IFN stimulated response element promoter

HL-CZ cells expressing single and both of ORF6 and NPIPB3, described above, were further cotransfected with pISRE-Luc cis-reporter (Stratagene) and control reporter pRLuc-C1, treated with IFN-β (Hoffmann-La Roche) for 4 hours, and then harvested. The activity of experimental firefly luciferase and control renilla luciferase in lysate was measured using dual Luciferase Reporter Assay System (Promega) and TROPIX TR-717 Luminometer (Applied Biosystems) described by Lin et al.
His-Tag in *E. coli* that was purified using immobilized-metal affinity chromatography (Figure 1). The recombinant ORF6 protein was used for the selection of its interacting cellular factors with phage-displayed human lung cDNA library. After the fifth round of biopanning, ORF6-specific binding phage clones eluted were selected from single phage plaques, amplified in *E. coli* for determining relative ORF6-binding affinities. Each phage clone was performed by direct binding enzyme-linked immunosorbent assay in ORF6-coated wells (Figure 2). Phage clone numbers 16, 26, and 40 with higher binding affinity to ORF6 were quantitated using plaque assays (*p* < 0.01), and we subsequently measured the binding specificity (Figure 3). Phage clones numbers 16, 26, and 40 bound to the recombinant ORF6 protein in a

![Figure 1](image1.png)

**Figure 1.** Expression and purification of *Escherichia coli*-synthesized SARS-CoV ORF6 protein. The ORF6 gene was amplified using PCR and cloned into pTriEx-4 Neo vector (A). ORF6-His tagged proteins were synthesized in transformed *E. coli* BL21 (DE3), purified using immobilized-metal affinity chromatography, separated using SDS-PAGE, and then examined using Western blotting with anti-His tag (B). ORF = open reading frame; PCR = polymerase chain reaction; SARS-CoV = severe acute respiratory syndrome coronavirus; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

![Figure 2](image2.png)

**Figure 2.** Biopanning of phage display a human lung cDNA library with ORF6-His tagged protein. After the fifth round of biopanning with ORF6, each phage clone was randomly picked up from individual plaques, amplified in *Escherichia coli*, and performed using direct binding ELISA with ORF6-coated plates and antiphage antibodies. * *p* < 0.05, by Scheffe’s test. ** *p* < 0.01, by Scheffe’s test. *** *p* < 0.001, by Scheffe’s test. ELISA = enzyme-linked immunosorbent assay; ORF = open reading frame.
ORF6 and NPIPB3 was further evaluated. The affinity to ORF6 protein (Figure 3), the interaction between NPIPB3 displayed on phage clone 40 had the highest binding. In the absence of ORF6 protein family, member B3), respectively. Because CCNL1 (cyclin L1), RBMXL2 (RNA binding motif protein, X-linked-like 2), and NPIPB3 (nuclear pore complex interacting protein family, member B3), respectively. Because NPIPB3 displayed on phage clone 40 had the highest binding affinity to ORF6 protein (Figure 3), the interaction between ORF6 and NPIPB3 was further evaluated.

**In vitro and in vivo interaction of ORF6 with NPIPB3**

To test in vitro and in vivo interaction between ORF6 and NPIPB3, the C terminus (amino acid residues 936–1050) of NPIPB3 was cloned into bacterial expression vector pET32a for in vitro coimmunoprecipitation and mammalian expression vector pDsRed1-C for in vivo co-localization assay (Figures 4 and 5). In the coimmunoprecipitation assay, the ORF6-His tag protein reacted with the Trx-NPIPB3-His tag fusion protein for 4 hours in a cool room, and then the protein complex were coimmunoprecipitated using anti-Trx antibodies and protein A-Sepharose beads. The coimmunoprecipitants were analyzed using Western blotting with the anti-His tag antibodies (Figure 4). The Western blotting analysis of immunoprecipitates revealed that the ORF protein bound to the Trx-NPIPB3-His tag fusion protein, but not to Trx protein (Figure 4, lane 3 vs. lane 1). In the co-localization assay, single and both of DsRed-NPIPB3 and ORF6-His tag proteins were expressed in human promonocye HL-CZ cells. After immunofluorescence staining with anti-His tag and FITC-conjugated antimouse IgG antibodies, confocal microscopy revealed a very close colocalization of DsRed-NPIPB3 and ORF6-His tag protein appearing as orange to yellow fluorescent light (Figure 5P), but no colocalization between DsRed and ORF6-His tag protein, or DsRed-NPIPB3 and ORF6 tag protein (Figures 5D, 5H, and 5L). The results demonstrated SARS-CoV ORF6 directly interacting with the C terminus of NPIPB3 in vitro and in vivo.

**Attenuation effect of NPIPB3 overexpression on Type I IFN antagonistic activity of SARS-CoV ORF6**

To determine the role of NPIPB3 in Type I IFN antagonism of SRAS-CoV ORF6, the IFN-β induced responses of transfected cells with single or both of pDsRed-NPIPB3 and pTriEx-ORF6 were explored using ISRE (IFN stimulated response element) luciferase reporter, STAT1 subcellular localization, and Western blotting assays (Figures 6–8). Transient overexpression of NPIPB3 C terminus improved with 2.5-fold increase of the ISRE promoter activity in ORF6-expressing cells in response to IFN-β (Figure 6; p < 0.001). To examine the subcellular localization of STAT1, confocal imaging analysis indicated that the overexpression of NPIPB3 C terminus did not change the subcellular localization of STAT1 in vector control and ORF6-expressing cells [Figure 7A(4)–(6) and 7A(9)]. However, NPIPB3 C terminus overexpression significantly enhanced IFN-β-induced STAT1 nuclear translocation in ORF6-expressing cells [Figure 7B(10)–(12)]. The IFN-β treatment stimulated STAT1 nuclear translocation in vector control cells [Figures 7B(1)–(3)], but not in ORF6-expressing cells [Figure 7B(7)–(9)]. However, NPIPB3 C terminus overexpression significantly enhanced IFN-β-induced STAT1 nuclear translocation in ORF6-expressing cells [Figure 7B(10)–(12)]. In addition, Western blotting indicated NPIPB3 C terminus overexpression enhancing IFN-β-induced STAT1 phosphorylation at Tyr701 in ORF6-expressing cells (Figure 8). Results demonstrated that NPIPB3 C terminus overexpression reduced Type I IFN
The study demonstrated Type I IFN antagonism of SARS-CoV ORF6 through the inhibition of IFN-β-induced STAT1 phosphorylation and nuclear translocation in human promonocytes (Figures 6–8), in agreement with a previous report in that ORF6 blocked STAT1 nuclear translocation in response to Type I IFNs via disrupting the import complex formation by binding with karyopherin alpha 2. The C-terminal hydrophilic domain of ORF6 interacted with cellular karyopherins, but the N-terminal lipophilic part of ORF6 was also required for retaining cellular karyopherins at the ER/Golgi membrane, leading to impede nuclear import. Besides STAT1, SARS-CoV ORF6 also affected the activity of karyopherin-dependent transcription factors, including VDR, CREB1, SMAD4, p53, Epas1, and Oct3/4. Therefore, ORF6 plays the vital role in innate antiviral responses.

Biopanning of phage-displayed human lung cDNA libraries identified the binding interaction of ORF6 with its interacting host factors, including CCNL1, RBMXL2, and NPIPB3 (Figures 2 and 3). CCNL1 displayed on phage clone number 16, containing an arginine- and serine-rich domain and a cyclin domain was required for spliceosome assembly and regulated splicing. RBMXL2 displayed on phage clone number 26 was one of the heterogeneous nuclear
Figure 7. Effect of NPIPB3 overexpression on IFN-β-induced nuclear translocation of STAT1 in vector control and ORF6-expressing cells. (A) Mock and (B) IFN-β-treated cells transfected with single or both of pTriEx-ORF6 and pDsRed-NPIPB3 were fixed, and reacted with anti-STAT1 and FITC-conjugated antimouse immunoglobulin G antibodies. Finally, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes, imaging analyzed by confocal microscopy. FITC = fluorescein isothiocyanate; IFN = interferon; NPIPB3 = nuclear pore complex interacting protein family, member B3; ORF = open reading frame; STAT1 = signal transducer and activator of transcription.
pTriEx       +    +    -    -
pDsRed       +    +    -    -
pTriEx-ORF6  -    -    +    -
pDsRed-NPIPB3- +    +    +    +

p-Y701-STAT-1 0 min
p-Y701-STAT-1 15 min
p-Y701-STAT-1 30 min
β-Actin

Figure 8. Effect of NPIPB3 overexpression on IFN-β-induced STAT1 phosphorylation at Tyr701 in vector control and ORF6-expressing cells using Western blotting. Cells were transiently transfected with single or both of pTriEx-ORF6 and pDsRed-NPIPB3, and then treated with IFN-β. After 0 minutes, 15 minutes, and 30 minutes of treatment, the lysate was analyzed by Western blotting with antiphosphotyrosine STAT1 (Tyr701), and anti-β-actin antibodies. The immune complexes were visualized using horseradish peroxidase-conjugated goat antimouse immunoglobulin G antibodies and enhanced chemiluminescence. IFN = interferon; NPIPB3 = nuclear pore complex interacting protein family, member B3; ORF = open reading frame; STAT1 = signal transducer and activator of transcription.

ribonucleoproteins, suggested as a germ cell-specific splicing regulator.19 ORF6-interacting phage clone number 40 displayed the C-terminal domain of NPIPB3 (Figures 2 and 3). NPIPB3 had many alternative names such as nuclear pore complex-interacting protein-like 3, protein pps22-1, KIAA0220-like protein, nuclear pore complex-interacting protein B type, and PI-3-kinase-related kinase SMG-1 isof orm 1 homolog.20 NPIPB3 containing a transmembrane region at the N terminus was recognized as a membrane protein and served as an RNA splicing factor. NPIPB3 was upregulated in epithelial Caco-2 cells after exposure to probiotic Lactobacillus acidophilus L-92, linking with immune response, DNA binding, and protein synthesis.21 NPIPB3 was also identified to bind IFN-α promoter.22 In this study, overexpression of NPIPB3 C-terminal domain reduced the antagonistic activity of Type I IFN by SARS-CoV ORF6 protein (Figures 6–8). The diacidic cluster motif (residues 53–56) was found in the ORF6 protein, as the critical determinant of subcellular localization to vesicular structures.23 Interestingly, the C-terminal domain of NPIPB3 had several four positively charged residue (KRRR) repeats. Therefore, ionic interactions could be linked with the binding interaction between SARS-CoV ORF6 and NPIPB3 C-terminal domain. In addition, the interaction between SARS-CoV ORF6 and NPIPB3 C-terminal domain might influence the binding interaction between ORF6 and karyopherin alpha 2; thus, the C-terminal domain of NPIPB3 recovered the nuclear import carrier function of karyopherin alpha 2 in ORF6-expressing cells, correlating with STAT1 nuclear translocation after IFN-β treatment (Figure 7). In addition, overexpression of the NPIPB3 C-terminal domain reduced IFN-β-induced phosphorylation of STAT1 at Tyr701 (Figure 8, lane 2). Tyrosine-protein kinase JAK1 (Janus kinase 1) contained a putative phosphoinositide binding site; an interaction between JAK1 and PI-3-kinase was reported in interleukin-2 signaling pathway.24 Meanwhile, NPIPB3, also known as PI-3-kinase-related kinase SMG-1, showed functional and structural similarities to PI-3-kinase.25 Therefore, NPIPB3 overexpression might increase the interaction with JAK1, which influenced IFN-β-induced JAK/STAT signaling, resulting in the decrease of STAT1 phosphorylation at Tyr701.

In conclusion, SARS-CoV ORF6-interacting proteins including CCNL1, RBMXL2, NPIPB3, and karyopherin alpha 2 were involved in RNA splicing, nuclear pore complex formation, as well as nuclear export and import of some transcription and splicing factors. This study demonstrated SARS-CoV ORF6 inhibiting IFN-β-induced ISRE promoter, STAT1 nuclear translocation and phosphorylation. By contrast, the interaction of SARS-CoV ORF6 with the C-terminal domain of NPIPB3 in human promonocytes reduced the Type I IFN antagonism of ORF6.

Conflicts of interest
All authors have no conflicts of interest to declare.

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