The host cellular protein Ndufaf4 interacts with the vesicular stomatitis virus M protein and affects viral propagation

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Research article

Keywords: VSV, Matrix Protein, Ndufaf4, Interaction

Posted Date: March 25th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-18819/v1>

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Abstract

Background: Vesicular stomatitis virus (VSV) is an archetypal member of Mononegavirales which causes important diseases in cattle, horses and pigs. The matrix protein (M) of VSV plays critical roles in the replication, assembly/budding and pathogenesis of VSV. To further investigate the role of M during viral growth, we used a two-hybrid system to screen for host factors that interact with the M protein.

Results: Here, NADH: ubiquinone oxidoreductase complex assembly factor 4 (Ndufaf4) was identified as an M-binding partner, and this interaction was confirmed by yeast cotransformation and GST pulldown assays. The globular domain of M was mapped and shown to be critical for the M-Ndufaf4 interaction. Two double mutations (E156A/H157A, D180A/E181A) in M impaired the M-Ndufaf4 interaction. Overexpression of Ndufaf4 inhibited VSV propagation, and knockdown of Ndufaf4 by short hairpin RNA (shRNA) markedly promoted VSV replication. Finally, we also demonstrate that the anti-VSV effect of Ndufaf4 is independent of activation of the type I IFN response. These results indicated that Ndufaf4 might exploit other mechanisms to affect VSV replication.

Conclusions: In summary, we identify Ndufaf4 as a potential target for the inhibition of VSV replication. These results provided further insight into the study of VSV pathogenesis.

Background

VSV is the pathogen of vesicular stomatitis (VS), an important disease in bovine, horses and swine. VS presents as widely erosive vesicles on the surfaces of the tongue, oral tissues, gums and feet and causes significant economic losses to the livestock industry[1]. VSV is a member of the Vesiculovirus genus, which belongs to the Rhabdoviridae family. The VSV genome is composed of a single strand of negative-polarity RNA that encodes five proteins: the nucleocapsid (N), phosphoprotein (P), matrix (M) protein, surface glycoprotein (G) and RNA-dependent RNA polymerase (L). N encapsidates the viral RNA to form the nucleocapsid (NC), which with the P and L proteins constitutes viral ribonucleoprotein (RNP) particles that are involved in genomic RNA replication and transcription [2]. The G protein of VSV is essential for virus attachment to specific receptors and entry into the cell.

The M protein of VSV is a multifunctional protein that plays a pivotal role in modulation of the host antiviral immune response[3, 4], suppression of host transcription/translation[5, 6], virion assembly/budding[7–10], and the induction of apoptosis[11, 12]. To perform numerous functions, viral proteins may need to interact with specific host proteins. Thus, further identification of host cellular protein(s) that interact with m is essential to better understand the roles of M in the replication and pathogenesis of VSV. In this study, we identified the host protein Ndufaf4 as a novel M-interacting partner that regulates the growth of VSV.

Ndufaf4 is an assembly factor of mitochondrial respiratory chain complex I that associates with assembly intermediates of the Q-module[13]. The mitochondrial respiratory chain has been shown to affect the propagation and pathogenesis of many viruses. For example, the hepatitis C virus (HCV) core

protein can increase reactive oxygen species (ROS) by directly interacting with mitochondria to inhibit mitochondrial electron transport, and the mitochondrial redox state and electron transport activity can be restored by reducing HCV replication[14]. The phosphoproteins of lyssaviruses, such as rabies virus (RABV) and Mokola (MOK) virus, interact with mitochondrial respiratory chain complex I to cause mitochondrial dysfunction and increase the generation of ROS and oxidative stress[15]. Ebermann et al. demonstrated that increasing the activity of mitochondrial respiratory chain complexes I and III inhibited coxsackievirus B3 (CVB3) replication[16]. Qu et al found that pharmacological inhibition of mitochondrial respiratory chain complex III potently restricted hepatitis E virus (HEV) replication[17]. However, no studies have investigated the role of the mitochondrial respiratory chain in the VSV life cycle. Here, we found that a subunit of mitochondrial respiratory chain complex I, Ndufaf4, is not only a VSV M-binding partner but also a cellular antiviral protein against VSV replication.

Results

The VSV M protein interacts with Ndufaf4

To identify potential cellular proteins that interact with the VSV M protein, yeast two-hybrid screening was performed using the VSV M protein as a bait against a normalized HeLa cDNA library. Putative positive clones were isolated and sequenced. Among these clones, Ndufaf4 was identified as a potential cellular partner of VSV M. The M-Ndufaf4 interaction was confirmed by yeast cotransformation assay. All the yeasts cotransformed with the indicated constructs grew well on SD/-2 medium, but only BD-M/AD-Ndufaf4 and BD-p53/AD-T (the positive control) grew on SD/-4 medium (Fig. 1A). Therefore, Ndufaf4 may be a binding partner of VSV M.

To further confirm the M-Ndufaf4 interaction, a GST pulldown assay was performed with purified GST-tagged M protein expressed in *E. coli* and Flag-tagged Ndufaf4 protein expressed in BSR-T7/5 cells. The presence of GST-M, but not GST alone, resulted in an interaction with Flag-Ndufaf4 (Fig. 1 B). Purified GST-Ndufaf4 protein expressed in *E. coli* and Flag-M expressed in BSR-T7/5 cells were also used in the pulldown assay. The results showed that GST-Ndufaf4, but not GST, interacted with Flag-M (Fig. 1C). Overall, these results indicated that M interacts with Ndufaf4.

Identification of critical amino acids in the M protein involved in the M-Ndufaf4 interaction

To determine the critical regions within the M protein that mediate its interaction with the Ndufaf4 protein, the carboxy-terminal globular domain and amino-terminal flexible domain of M were individually fused to the DNA-binding domain of GAL4, and the ability of the fusion proteins to interact with the Ndufaf4 protein was assessed using the Y2H system. Yeast transformed with BD-M/AD-Ndufaf4 and BD-M2/AD-Ndufaf4 grew well on SD/-2 and SD/-4 media; however, yeast transformed with BD-M1/AD-Ndufaf4 did not grow on SD/-4 medium (Fig. 2 A). The above results showed that the carboxy-terminal globular domain of M participates in the M-Ndufaf4 interaction.

To further identify the critical residues in the M protein involved in the M-Ndutfaf4 interaction, all amino acids at the surface of the globular domain of the M protein were chosen for mutagenesis analysis [18, 19]. As shown by the results, all cells expressing the mutants grew as well as cells expressing wild-type M on SD/-4 medium, except for cells expressing E156A, E156A/H157A, or D180A/E181A M (Fig. 2 B). These results indicate that the E156/H157 and D180/E181 amino acids in M are critical for the M-Ndutfaf4 interaction.

Ndutfaf4 inhibits VSV replication

To investigate the role of Ndutfaf4 in VSV propagation, HeLa cells were transfected with pCMV-Flag-Ndutfaf4 or pCMV-Flag-N empty vector and then infected with VSV at an MOI of 0.1. VSV G protein levels were detected by Western blotting, and the VSV titer was determined by TCID₅₀ assay at 4, 8 and 12 hours postinfection. As shown by the results, overexpression of Ndutfaf4 decreased expression of the VSV G protein at 12 hours postinfection and the VSV titer at 8 and 12 hours postinfection (Fig. 3 A and B). These results show that overexpression of Ndutfaf4 inhibits the growth of VSV.

To further analyze the effect of Ndutfaf4 knockdown on VSV replication, HeLa cells were transfected with plasmid to deliver short hairpin RNA (shRNA) targeting the Ndutfaf4 gene. Western blot analyses demonstrated that the specific shRNA efficiently decreased the expression level of Ndutfaf4 (Fig. 3 C). Furthermore, transfection of HeLa cells with the plasmid resulted in the efficient knockdown of Ndutfaf4 expression, which promoted expression of the G protein and the VSV titer (Fig. 3 D and E). Collectively, these results suggest that Ndutfaf4 is a cellular antiviral protein against VSV replication.

The anti-VSV effect of Ndutfaf4 is independent of the type I IFN response

To further explore the mechanism exploited by Ndutfaf4 to affect VSV replication, we detected the effect of Ndutfaf4 overexpression or knockdown on the type I IFN response. HeLa cells were transfected with Ndutfaf4-overexpression or Ndutfaf4-knockdown plasmid and then infected with VSV at an MOI of 5. The mRNA levels of IFN- β and the IFN-stimulated genes ISG56, IFITM3 and MX1 were detected by real-time RT-PCR at 4, 8, 12 hours post-infection. Compared with their transcription in the control group, neither the overexpression nor knockdown of Ndutfaf4 affected the transcription of these genes (Fig. 4 A and B). These results indicated that Ndutfaf4 inhibits VSV replication independent of the type I IFN response.

Discussion

Ndutfaf4, an assembly factor of mitochondrial respiratory chain complex I, is essential for normal mitochondrial function. Mutation of this gene is associated with a reduction in the mature form of complex I in muscle and causes Leigh syndrome and developmental delay[20]. However, the exact role of Ndutfaf4 in complex I biogenesis is not yet fully understood. In this study, Ndutfaf4, a component of mitochondrial respiratory chain complex I, was identified as a binding partner of the VSV M protein using the yeast two-hybrid system. To preclude possible autoactivation of Ndutfaf4 and confirm the M-Ndutfaf4 interaction, a yeast cotransformation assay was performed. This interaction was further demonstrated by

GST pulldown assay. The VSV M protein is composed of two domains, an amino-terminal flexible domain and a carboxy-terminal globular domain. The domains play different roles during viral replication and assembly. The flexible domain of M can interact with dynamin to promote viral assembly[8]. This domain is also involved in the viral budding process by recruiting cellular partners. The globular domain of M can inhibit the host antiviral response to promote viral replication by inhibiting host gene expression or impairing the type I IFN system[3]. To determine which domain of M participates in the M-Ndutf4 interaction, each domain of M was cloned into the PGK7 plasmid, and its ability to bind Ndutf4 was determined in the Y2H system. The results showed that the globular domain, but not the flexible domain, of the M protein interacted with Ndutf4. To further confirm the site of M that interacts with Ndutf4, exposed residues at the surface of the globular domain were selected for mutagenesis analysis. Residues E156/H157 and D180/E181 were demonstrated to be important for the M-Ndutf4 interaction. To test the role of the M-Ndutf4 interaction, we determined the effect of Ndutf4 silencing and overexpression on VSV replication. Overexpression of Ndutf4 reduced the VSV titer and G protein expression, whereas knockdown of Ndutf4 by shRNA dramatically promoted CSFV replication. These results demonstrated that Ndutf4 is a cellular factor that acts against VSV infection.

Mitochondrial proteins, such as mitochondrial antiviral signaling (MAVS), have been reported to be essential for the antiviral immunity response[21]. Furthermore, some studies provide evidence for the critical role of the mitochondrial respiratory chain in innate immunity. NLRX1, a regulator of mitochondrial antiviral immunity, was suggested to associate with UQCRC2, a subunit of mitochondrial respiratory chain complex III[22, 23]. Mitochondrial complex I deficiency triggers innate immune and inflammatory responses in the retina[24]. GRIM-19, a mitochondrial respiratory chain complex I protein, plays an important role in antibacterial immunity[25]. As type I IFN- β plays a critical role in the host response against VSV infection, we detected the effects of overexpression and knockdown of Ndutf4 on the I IFN response. The results showed that neither the overexpression nor the knockdown of Ndutf4 affected the type I IFN response during VSV infection.

Conclusion

In summary, our study indicate that Ndutf4 interacts with the VSV M protein and inhibits VSV replication. Although the precise mechanism of this effect needs to be further elucidated, the present study identifies Ndutf4 as a potential target for the inhibition of VSV replication.

Methods

Cells and virus

BSR-T7/5 and HEK293T cells were maintained at 37°C in DMEM medium, supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS; HyClone). Vesicular stomatitis virus serotype Indiana (VSV-IND) was propagated in HEK293T cells.

Construction of expression vectors

The Ndufaf4 gene was cloned into the pCMV-flag vector (catalog No. 635688; Clontech) to generate the pCMV-flag-Ndufaf4 plasmid. A eukaryotic Flag-M expression vector designated pT-Flag-M was constructed as previously described[26]. The Ndufaf4 and M ORFs were cloned at the C-terminus of GST in the pGEX-4T-1 vector to construct prokaryotic expression plasmids. The vector used to deliver short hairpin RNA (shRNA) targeting the Ndufaf4 gene was constructed using small interfering RNA sequences targeting the Ndufaf4 gene (1: 5'-GGGAAATCAGCAAGATGAAGCCTCGAGGCTTCATCTTGCTGATTTC-3', 2: 5'-GCACTAGTGATTTCGCGGTATCCTCGAGGATACCGCGAATCACTAGTGC-3'), and a negative control sequence (NC: 5'-ACGUGACACGUUCGGAGAA-3') was designed, synthesized and cloned into the pYr-Lvsh lentiviral vector.

Yeast two-hybrid assay

The VSV M gene was cloned into the pGBKT7 plasmid to construct the bait vector. The concentrated yeast strain AH109 transformed with pGBKT7-M was mated with the yeast strain Y187 containing a pretransformed HeLa cDNA library. Heterozygotes were selected on DDO/X/A plates. Blue colonies that grew on DDO/X/A were transferred to QDO/X/A plates. All putative positive prey plasmids were sequenced and analyzed. To eliminate false positive hits, the pGBKT7-M and prey plasmids were cotransformed into the AH109 yeast strain. AH109 yeast cotransformed with BD-Lam/AD-T and BD-p53/AD-T were used as negative and positive controls, respectively.

GST Pulldown Assay

GST pulldown assay was performed as previously described[26].

Western blot

Western blotting analysis was performed as previously described[27].

Real-Time RT-PCR

Total RNA was isolated from HeLa cells using TriPure reagent (catalog No. 11667165001; Roche). The RNA was quantified by NanoDrop and then used for cDNA synthesis using M-MLV reverse transcriptase (catalog No. 2641A; TaKaRa, Dalian, China) and oligo(dT) primers. The qRT-PCR assay was performed using a Premix Ex Taq kit (TaKaRa, Japan). The primer sequences used were as follows: Actin sense, 5'-TGACGTGGACATCCGCAAAG-3'; Actin antisense, 5'-CTGGAAGGTGGACAGCGAGG-3'; IFNB1 sense, 5'-TGTC AACATGACCAACAAGTGTC T-3'; IFNB1 antisense, 5'-GCAAGTTGTAGCTCATGGAAAGAG-3'; ISG56 sense, 5'-AACACCCACTTCTGTCTTACTGCAT-3'; ISG56 antisense, 5'-GATTTGGATCATTTGTGCCTTGT -3'; IFITM3 sense, 5'-GCTGATCTTCCAGGGCTA TGG -3'; IFITM3 antisense, 5'-GCGAGGAATGGAAGTTGGAGT-3'; MX1 sense, 5'-GGCCAGCAAGCGCATCT -3'; and MX1 antisense, 5'-TGGAGCATGAAGAACTGGATGA -3'.

Statistical analysis

The data were analyzed for statistical significance by using Student's t-test and one-way ANOVA.

Abbreviations

VSV: vesicular stomatitis virus; Ndufaf4: NADH: ubiquinone oxidoreductase complex assembly factor 4; shRNA: short hairpin RNA; HCV: Hepatitis C virus; ROS: reactive oxygen species; RABV: rabies virus; MOK: Mokola; CVB3: Coxsackievirus B3; HEV: Hepatitis E virus.

Declarations

Acknowledgments

We would like to thank many staff members who helped with this paper.

Authors' contributions

Wei Pan and Hongbing He conceived and designed the study. Wei Pan performed the experiments. Hongmei Wang wrote the manuscript. All authors read and approved the final version of the manuscript.

Funding

Financial support was provided by Taishan Scholar and Distinguished Experts (H.H.) (tspd20181207).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declared that there are no potential conflicts of interest in this study, authorship, and/or publication of this article.

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Figures

Figure 1

The VSV M protein interacts with Ndufaf4 (A) The M-Ndufaf4 interaction was examined using a Y2H system. All the yeasts cotransformed with the indicated constructs were streaked onto SD/-2 and SD/-4 plates. (B) GST-M pulldown assay. Purified GST and GST-M proteins were incubated with recombinant Flag-Ndufaf4 protein and detected by western blotting. (C) GST-Ndufaf4 pulldown assay. Purified GST and GST-Ndufaf4 proteins were incubated with recombinant Flag-Ndufaf4 protein.

Figure 2

Identification of critical amino acids in the M protein involved in the M-Nduf4 interaction (A)

Identification of the M protein domain that interacts with Nduf4. Y2H screens were performed to assess the interaction between the Nduf4 protein and the domains of the M protein. (B) Y2H screens were performed to confirm the interaction between mutant M proteins and Nduf4.

Figure 3

Ndutf4 affects VSV replication (A) HeLa cells were transfected with pCMV-Flag-Ndutf4 or pCMV-Flag-N empty vector and then infected with VSV at an MOI of 0.1. VSV G protein levels and the VSV titer at 4, 8 and 12 hours postinfection were detected by Western blotting and TCID₅₀ assay, respectively (B). (C) HeLa cells were transfected with plasmid to deliver short hairpin RNA (shRNA) targeting the Ndutf4 gene. At 48 h posttransfection, levels of the Ndutf4 protein were determined by Western blotting. (D) HeLa cells were transfected with plasmid to deliver control shRNA or shRNA targeting the Ndutf4 gene. At 48 hours posttransfection, the cells were infected with VSV at an MOI of 0.1. The VSV G protein level at 12 hours postinfection was detected by Western blotting, and the VSV titer at 4, 8, and 12 hours postinfection was detected by TCID₅₀ assay (E). The data are shown as the means ± SD (**p < 0.01; *p < 0.05;).

Figure 4

The anti-VSV effect of Nduf4 is independent of the type I IFN response (A) HeLa cells were transfected with pCMV-Flag-Nduf4 or pCMV-Flag-N empty vector and then infected with VSV at an MOI of 5. The mRNA levels of type I IFN pathway-related genes at 12 hours postinfection were detected by real-time RT-PCR. (B) HeLa cells were transfected with plasmid to deliver control shRNA or shRNA targeting the Nduf4 gene. At 48 hours posttransfection, the cells were infected with VSV at an MOI of 5. The mRNA levels of t IFN- β and the IFN-stimulated genes at 4, 8 and 12 hours postinfection were detected by real-time RT-PCR. The data are shown as the means $\pm$ SD.