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Chicken viperin inhibits Newcastle disease virus infection *in vitro*: A possible interaction with the viral matrix protein

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ABSTRACT

Viperin is an interferon-inducible protein that helps in protecting mammals against various virus infections. Viperin is a highly conserved member of the interferon-stimulated genes (ISG) family in many species. Viperin has been shown to play a pivotal role in the innate immunity of chicken; however, its role has not been explored in its antiviral potential. Newcastle disease virus (NDV) is the causative agent of an infectious disease in poultry. In the present study, we have shown the anti-NDV effect of chicken viperin (cViperin). The impact of cViperin upon NDV infection was investigated in chicken embryo fibroblast. The modeling of the cViperin protein was done using I-TASSER and ZDOCK is used to predict the possible interaction with the matrix protein of NDV. The interaction was further confirmed by co-immunoprecipitation assay using recombinant matrix protein of NDV with the recombinant cViperin. The recombinant NDV expressing cViperin showed reduced replication of the virus upon its growth kinetics. Our results suggest downregulation of NDV replication in the presence of cViperin. The study will be critical to elaborate our understanding of the chicken innate immune system which could help develop antiviral strategies against NDV infection.

1. Introduction

Interferons (IFN) provide a robust first line of defence against viral infection. Induction of IFN triggers a plethora of genes in the host which mediates the host immune response. IFN play a decisive role in response to infection by impeding various stages of viral replication [1]. Three interferon families have been identified in both mammalian and avian species namely type I, II and III [2,3]. The antiviral activity induced by IFN has made it a potential therapeutic tool. IFNs have shown to activate several host genes, referred to as IFN-stimulated genes (ISG) [4–7]. An ISG plays diverse roles, and important with regard to their antiviral activity, the complexity of their regulation and its targeting by virus-encoded modulators of IFN [8,9]. Classical ISGs like Myxovirus resistance 1 (Mx), Protein kinase R (PKR) and 2′-5′-oligoadenylate synthetase (OAS) and ZAP have been vastly studied in mammals and avian species [6,10–15]. Viperin, also known as Cig5 (cytomegalovirus inducible gene5) and RSAD2 (radical SAM domain-containing 2) is among one of the few ISGs exhibiting antiviral activity upon induction by both types I and II IFN [16]. It was first discovered as an inducible gene in fibroblasts upon cytomegalovirus infection and was named for **v**irus **i**nhibitory **p**rotein, **e**ndoplasmic **r**eticulum-associated, **i**nterferon-**i**nducible [16]. Viperin is highly conserved across various species and involved in regulating host innate immune response [16–18]. A comparison between chicken viperin to its orthologous genes in mammals and fish showed 70% sequence identity [19]. Viperin localises in the endoplasmic reticulum (ER) and later transported to the lipid-enriched compartment called lipid droplets (LDs) [20]. LDs consist of neutral lipids surrounded by phospholipids and associated proteins and are thought to be derived from ER when neutral lipids accumulate in the ER bilayer [21,22]. Viperin has been reported to exhibit antiviral activity against a broad spectrum of viruses, including human cytomegalovirus, hepatitis C virus, West Nile virus, dengue virus, Sindbis virus, influenza A virus, Sendai virus, vesicular stomatitis virus, HIV and classical swine fever virus [16,17,23–28]. Upregulation of chicken viperin has been shown upon IFN treatments, as well as upon pathogenic avian influenza and infectious bursal disease virus infection. Also, chicken viperin has proved to be up-regulated in response to lipopolysaccharide (LPS) suggesting its possible antimicrobial role along with antiviral potential [19]. Viperin is shown to upregulate in chicken splenocytes in response to TLR ligand such as poly (I:C) [19]. In chicken, the stimulator of IFN gene (STING) functions as an essential innate immune signaling molecule required for triggering dsDNA-mediated gene induction [29–31] and has been shown to inhibit viral replication [32].

The Newcastle disease (ND) is an extremely contagious disease in many species of domestic, exotic and wild birds [33]. The infection of

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ND causes a high death rate and substantial economic losses in the poultry industry. The causative agent, Newcastle disease virus (NDV) belongs to the genus *Avulavirus* under the family *Paramyxoviridae* [34–36]. NDV is a pleomorphic enveloped virus and contains a non-segmented, single-stranded, negative-sense RNA. Its genome size varies between 15,186, 15,192 and 15,198 nucleotides, which encodes for six major proteins, namely, nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and RNA dependent RNA polymerase (L) [37–41]. The N protein is the most abundant among all and forms the ribonucleoprotein complex along with P and L proteins [35]. The P protein plays a vital role in viral replication and transcription by stabilising the L protein [42]. The M protein is hydrophobic in nature, and its interaction with actin helps in virion assembly on the host cell membrane [43]. Besides, M protein helps the paramyxoviruses to bud off from the infected cells by interacting with the host cell membrane [44–46]. The HN protein is a surface glycoprotein that promotes the attachment of NDV to the sialic acid receptor present on the host cells [47]. The F protein is a surface glycoprotein present in the NDV envelope and mediates its fusion with the host cell membrane [48]. The interaction between homologous HN and F protein is vital for the fusion of virion to the cell surface [49]. The L protein is the largest protein encoded by the NDV genome and plays a crucial role in RNA replication [42]. The L protein also performs 5′ capping, methylation and poly A polymerase activity on the newly formed RNA [35,42]. Regular NDV outbreaks have been reported from different parts of the world [50–55]. The NDV strain B1 or LaSota and R2B widely used to vaccinate birds are effective in protecting against the disease [56]. In spite of the vaccinations, major vaccine failures are due to improper treatment, inadequate cold chain maintenance and unsound management practices [57,58]. Improving our knowledge of the innate immune response following viral infection is critical in the development of novel therapeutics. Therefore, it is crucial to examine the role of ISGs in detail. In the present study, we have analysed the modulation of NDV replication upon expression of cViperin in chicken embryo fibroblast (CEF). Furthermore, the recombinant NDV expressing cViperin showed reduced replication kinetics of NDV *in vitro*.

2. Materials and methods

2.1. Virus and cells

The NDV vaccine strain R2B used in this study was propagated in 9-days-old specific pathogen free (SPF) embryonated chicken eggs, and infected allantoic fluids were collected 48 h post-inoculation. Besides, the recombinant NDV expressing GFP (rNDV.GFP) available in the laboratory was used for the fluorescent-based and other comparative studies [59]. Chicken embryo fibroblast (CEF) was prepared using standard protocol [60] and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum at 37 °C in 5% CO_2. The chicken embryo fibroblast (DF-1) cells used in the colocalization experiments were obtained from ATCC (Manassas, VA, USA) and maintained in DMEM with 10% fetal calf serum. The presence of the virus in infected allantoic fluids was confirmed by hemagglutination assay (HA) using 1% chicken RBC and quantified by the plaque assay. For plaque assay, the CEF cells were infected with NDV following 80% confluent monolayer and maintained in DMEM with 0.8% methylcellulose post infection. Furthermore, the titration of NDV from infected cell culture supernatant was determined by TCID50 units/μl using the standard method [61]. The 293T cells grown in DMEM supplemented with 10% fetal calf serum at 37 °C were used for the immunoprecipitation assay.

2.2. Cloning and amplification of cViperin

The peripheral blood mononuclear cells (PBMCs) were isolated from chicken whole blood by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, USA). Further, total RNA was extracted from PBMCs using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's protocol. The cDNA was synthesised using a SuperScript™III RT enzyme (Invitrogen, USA) and cViperin gene was amplified by gene-specific primers (forward: 5′ctagctagcatggactacaaagacgatgacgacaagatgctgctgggcgttc3′ and reverse: 5′cggaattcttaccagtccagaatc3′). The amplified cViperin gene product was cloned into a pcDNA3.1 mammalian expression vector (Invitrogen, USA). The positive clones containing cViperin gene were confirmed by restriction enzyme digestion and further by nucleotide sequencing. Similarly, the M gene of NDV was cloned into the pcDNA3.1 expression vector. Both the plasmids were constructed to have the FLAG and GFP at their C-terminus and named pcDNA.cViperin-FLAG and pcDNA.M-GFP, respectively.

2.3. Transfection and infection studies

The CEF cells were transfected with plasmid DNA expressing cViperin using Lipofectamine reagent (Invitrogen, USA) following the manufacturer's instructions to deduce the effects of cViperin on NDV replication. NDV with an MOI of 0.01 was used to infect the CEF at 24 h post cViperin transfection. The RNA isolated from the infected cells was used to check the expression of the host and NDV genes by semi-quantitative and real-time PCR. Also, the infected cell lysate was used to check the expression of the proteins by western blotting. The blots were developed using anti-NDV polyclonal antibody, anti-β-actin mAb (Invitrogen, USA) and anti-FLAG mAb (Thermo Fisher Scientific, USA) with ECL reagent (Thermo Fisher Scientific, USA). The HN protein-specific monoclonal antibody (a kind gift from Dr R Iorio, University of Massachusetts, Worcester, MA, USA) was used to confirm the presence of NDV in the lysates.

2.4. Three-dimensional protein structure prediction and docking studies

The amino acid sequence of cViperin gave the base to predict the structure and determine the physiological and biochemical properties of the protein. A ProtParam tool from the ExPASy bioinformatics resource portal was used to compute the physical and chemical features such as molecular weight, amino acid composition and molecular coefficient of the uncharacterized cViperin protein [62]. I-TASSER (Iterative Threading ASSEmbly Refinement), a computational server was used to predict and generate the 3-D structure of cViperin [63]. I-TASSER server initially retrieves a template from the PDB library using LOMETS, a meta-threading approach. The continuous fragments obtained from PDB prototypes were re-assembled into full-fledged models by MonteCarlo simulations. Of the obtained analysis, the lowest energy state proteins are picked using SPICKER [64]. The top five models were re-generated and guided by TM-align, an algorithm used to compare independent proteins with the help of their sequence-order [65]. As viperin is conserved over mammalian species [19], the structure that was closely related to the viperin protein was chosen using a superimposition by pyMOL based on the RMSD value [66]. The available crystal structure of *Mus musculus* viperin (PDB id 5VSL) is used to superimpose the predicted cViperin [67]. The quality of the selected model was further validated using PROCHECK [68], a tool that generates a Ramachandran plot and discusses the stereochemical aspects of the cViperin. VERIFY 3D [69] tool was also used for the validation of the predicted model, and the ProSA server [70] was used to calculate the native conformation. ZDOCK is a fast Fourier transform-based protein docking program used to dock the M protein of NDV with cViperin [71]. N-terminus and C-terminus of cViperin were truncated individually using pyMOL and checked for its interaction with M protein in ZDOCK to confirm the interaction between the mentioned proteins. The PDB sum server was used to find the interacting residues spanning the domain of M protein of NDV and cViperin [72].

Different viperin sequences from the NCBI protein repository were

collected to realize the identity and the conserved region among the different species. Clustal Omega was used to calculate the identity as well as the regions that are conserved among viperin protein among species. The phylogenetic tree was constructed using phylogeny.fr platform [73,74].

2.5. Co-immunoprecipitation assay

The 293T cells were transfected with pcDNA.cViperin-FLAG and pcDNA.M-GFP and harvested 48 h post transfection. The cell lysate was prepared using immunoprecipitation (IP) lysis buffer (Pierce, USA). The lysate was centrifuged at 12,000*g* for 10 min, and the supernatant was incubated with FLAG and GFP antibodies (Thermo, USA) overnight at 4 °C with rotation. The A/G magnetic beads (Pierce, USA) were washed twice with IP lysis/wash buffer and incubated with antigen sample/antibody mixture at room temperature for 1 h with constant shaking. Beads were separated after incubation, and unbound samples were removed. The sample bound beads were incubated with low-pH elution buffer for 10 min. Magnetic beads were separated, and the supernatant containing the target antigen was collected for further western blot analysis.

2.6. Cellular localisation study

DF-1 cells were grown on the coverslip and transfected with pcDNA.cViperin-FLAG and pcDNA3.1. The cells were fixed by 4% paraformaldehyde, washed and permeabilised with 0.1% Triton-X 100 post 48 h transfection. The fixed cells were rinsed with PBS and incubated in 5% blocking buffer (5% BSA in PBS) for 1 h at 37 °C. For viperin detection, cells were incubated with 1/100 dilution of mouse monoclonal anti-FLAG (Invitrogen, USA) followed by secondary incubation with 1/100 dilution of Alexa 594 conjugated goat anti-mouse IgG antibody (Invitrogen, USA). For lipid droplet staining, cells were incubated with 2 μM of BODIPY 493/503 (ThermoFisher Scientific, USA) for 20 min. After the final wash with PBS, cells were stained with 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI). After final washing, slides were mounted and examined under a 63X objective using a confocal microscope (Leica SP5, Germany).

2.7. Construction and recovery of recombinant NDV expressing chicken viperin

Reverse genetics approach is used to develop the NDV strain LaSota to express the cViperin gene (pNDV.cViperin). The genomic sequence of NDV was flanked by the T7 promoter and the hepatitis delta virus (HDV) ribozyme sequence. The restriction enzyme site of *AscI* was created in pNDV which was utilized to insert the cViperin gene in pNDV between the non-coding region of *P* and *M* gene of NDV. The ORF encoding the cViperin gene was engineered to contain NDV gene start and gene end sequence. PCR was done employing Ex Taq DNA polymerase (Takara, Japan) and PCR amplified fragment of the cViperin gene was cloned at the *AscI* site of pNDV.

The recombinant pNDV.cViperin was transfected together with three accessory plasmids bearing the N, P and L genes of the NDV in HEp-2 cells using Lipofectamine reagent following the manufacturer's instructions (Invitrogen, USA). Before transfection, the HEp-2 cells were infected with recombinant vaccinia virus strain Ankara expressing T7 polymerase (a Kind gift from Dr Bernard Moss, NIH USA). The recovered rNDV.cViperin was further amplified and passaged in 9-day-old SPF chicken embryonated egg. RT-PCR was performed from genomic RNA of rescued rNDV.cViperin to check the integrity of viperin gene.

2.8. Pathogenicity test and growth kinetics of the recombinant NDV

The mean death time (MDT) in 9-day-old SPF embryonated chicken eggs and the intracerebral pathogenicity index (ICPI) in 1-day-old SPF chicks was performed to check the pathogenicity of recovered rNDV. The growth kinetics of rNDV.cViperin was performed by infecting CEF cell line with rNDV.cViperin at different time points. The CEF cells were infected with rNDV and rNDV.cViperin at an MOI of 0.01. The virus released was calculated by $TCID_{50}$ titer from the collected cell culture supernatant every 12 h post-infection.

2.9. Statistical analysis

The data presented in the results are the average of three independent experiments and was shown as the mean ± S.D. Results were statistically analysed using the *t*-test (Microsoft Excel). An asterisk means a significant difference (*), (**), (***) of $P < 0.05$; $P < 0.01$; $P < 0.001$ respectively.

3. Results

3.1. The cViperin expression is upregulated in CEF cells by NDV infection

The expression of cViperin was investigated in CEF cells following NDV infection. The viperin mRNA expression was continued to increase up to 60 h following viral infection (Fig. 1A). Similarly, NDV infection showed upregulation of its gene expression (N gene) from 24 h till 36 h post-infection. The expression of the viral gene was reduced marginally from 48 h till 60 h post infection (Fig. 1B). No expression of cViperin was observed in the basal level at a different time point in the mock treated CEF cells (Fig. S1).

3.2. Construction and expression of cViperin in the eukaryotic expression system

The cViperin was successfully amplified from isolated PBMC and cloned into the pcDNA3.1 expression vector (pcDNA.cViperin). The positive clone of pcDNA.cViperin showed a release of the 1065 bp band upon digestion with *Nhe*I and *Eco*RI (Fig. S2). The sequence analysis of pcDNA.cViperin confirmed the absence of any inadvertent mutation and right orientation of the cViperin into a eukaryotic expression system. The pcDNA.cViperin showed positive fluorescence with viperin antibody upon its transfection in the CEF cells (Fig. S3). The pcDNA.cViperin showed expression of viperin protein following its transfection in CEF cells. β-actin was used as an internal control to check the expression of the cellular protein. Also, western blot analysis with a polyclonal antibody against the cViperin protein (Thermo Scientific, USA) detected a band of molecular mass of ~40 kDa in cell lysate transfected with pcDNA.cViperin (Fig. 1C).

3.3. Inhibition of NDV upon cViperin expression in CEF cells

To determine the role of cViperin in inhibiting NDV infection, CEF cells were transfected with pcDNA.cViperin followed by NDV infection. NDV gene expression was quantified by semi-quantitative PCR considering β-actin as an internal control (Fig. 2A). Transfection of pcDNA.cViperin showed a reduction in NDV gene expression by five-fold (log_2 scale) as compared to mock-infected control in the q-PCR analysis (Fig. 2B). The finding of NDV reduction was further validated by western blot analysis using NDV HN specific antibody which displayed no visible NDV protein band upon cViperin expression (Fig. 2C). The release of NDV, post cViperin expression was analysed by infecting rNDV expressing GFP in CEF cells. Approximately 70% reduction in the GFP expression was observed upon overexpression of cViperin in CEF cells as compared to the mock-infected control (Fig. 2D). Furthermore, plaque assay showed 4.5 fold-reductions in plaques upon cViperin expression in NDV infected cells (Fig. 2E and F). Also, the HA titer showed a 50% reduction of NDV following cViperin expression in CEF cells (Fig. 2G).

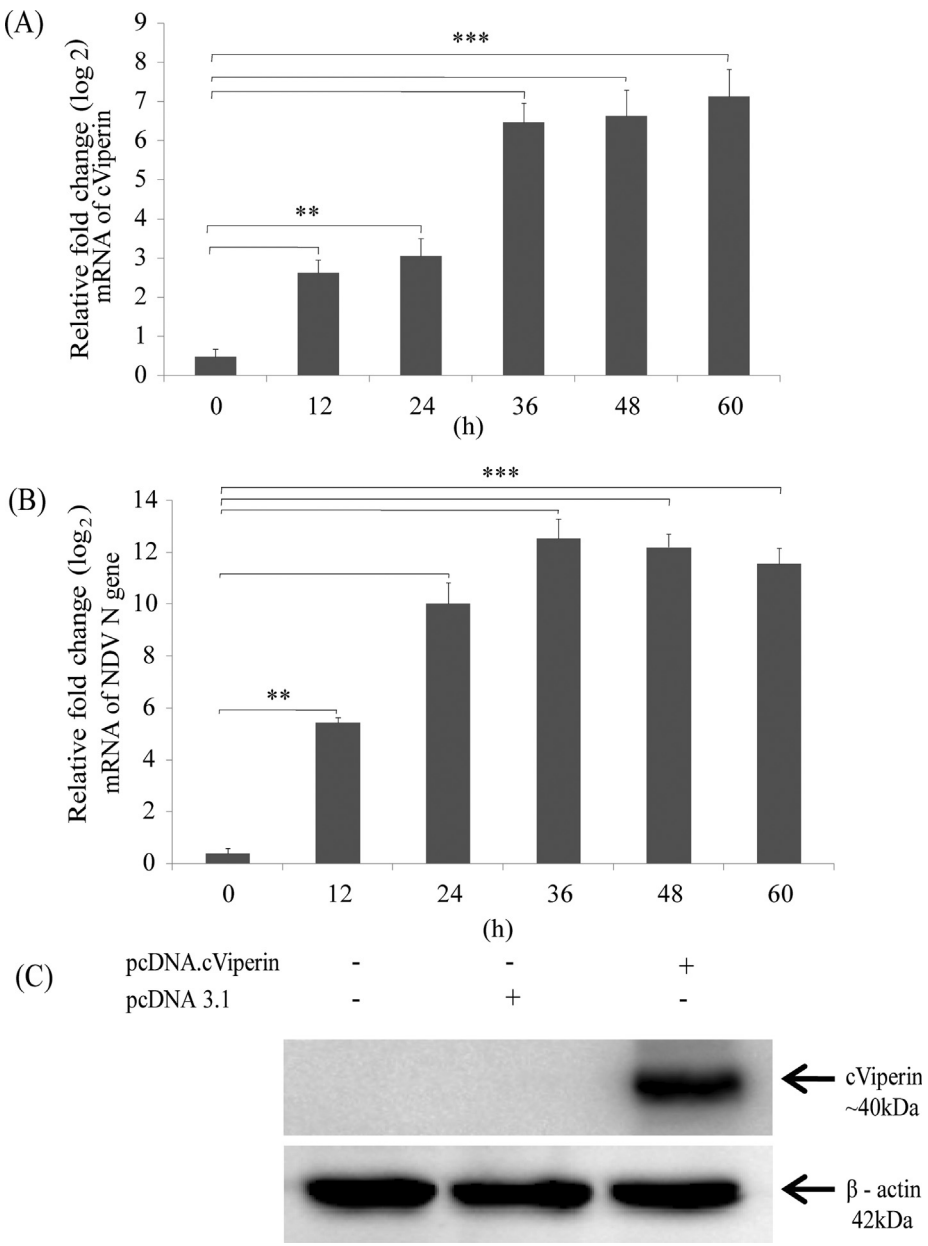

Fig. 1. Expression of cViperin upon NDV infection was analyzed by qRT-PCR (A) Expression of N gene of NDV was determined by the qRT-PCR. The CEF cells infected with NDV were collected after 0, 12, 24, 36, 48, 60 h post infections and analyzed for their relative mRNA levels (B). The data presented are the average of three independent experiments and was shown as the mean ± S.D. The mRNA determinations were performed by qRT-PCR, and mRNA levels were normalized to GAPDH mRNA and represented as mean fold expression with respect to control. The fold change in gene expression was calculated by $2^{-\Delta\Delta Ct}$ and the data were transformed in log base 2. The results were statistically analyzed using the *t*-test to derive the P value (Microsoft Excel). An asterisk indicates a significant difference (*), (**), (***) of $P < 0.05$; $P < 0.01$; $P < 0.001$ respectively. The cell lysates were prepared at 48 h post transfection and analyzed for cViperin protein by an immune blot. The β-actin was used as a loading control (C).

3.4. Physiological properties and protein structure prediction of cViperin

The molecular weight of the cViperin was predicted around 40 kDa with an isoelectric point (pI) of 6.69. Bioinformatics analysis showed the presence of 50% positively charged, 49% negatively charged and the rest non-polar amino acids in cViperin. Additionally, leucine was found to be the most abundant amino acid in cViperin. The molar extinction coefficient of cViperin was 1.265 when all cysteine residues form cystine while the value of 1.250 was calculated when all cysteine are reduced. The three-dimensional structure of cViperin was obtained using I-TASSER and validated by the PROCHECK server through the Ramachandran plot (Fig. 3A). Based on TM-align, the structure closest to viperin of *Mus musculus* had a confidence score of −1.38 and TM-align score of both the viperin was 0.73. Any TM-score in the range of (0.5, 1) is considered to be very similar in nature (structurally). The RMSD value was found to be 0.45.

The best model showed the presence of 213 residues (67.8%) in the most favourable regions, 67 residues (21.3%) in additionally allowed regions, 23 residues (7.3%) in generously allowed regions and 11 residues (3.5%) in disallowed regions (Fig. 3B).

3.5. cViperin-matrix protein docking

Docking studies between the M protein of NDV and cViperin were studied (Fig. 3C). The integrity of the complex model was done using PROCHECK. The Ramachandran plot of the M protein of NDV and cViperin complex showed 265 residues (91.7%) in the most favourable regions, 23 residues (8%) in additionally allowed regions, one residue (0.3%) in the generously allowed regions and no residues in the disallowed regions (Fig. 3D). The z-score obtained from ProSA server of the M protein of NDV and cViperin complex was −7.57. The interaction between individual A and B chain of M protein was also done with cViperin (Fig. 3E and G). PBDsum depicts the molecules that make up the protein and the interaction that is occurring schematically. The A and B chains of M protein of NDV showed interaction with cViperin, 27 residues in chain A of M protein and 19 residues of cViperin protein were involved in the interaction that includes 2 salt bridges, 5 hydrogen bonds and 237 non-bonded contacts. Similarly, 27 residues in chain B of

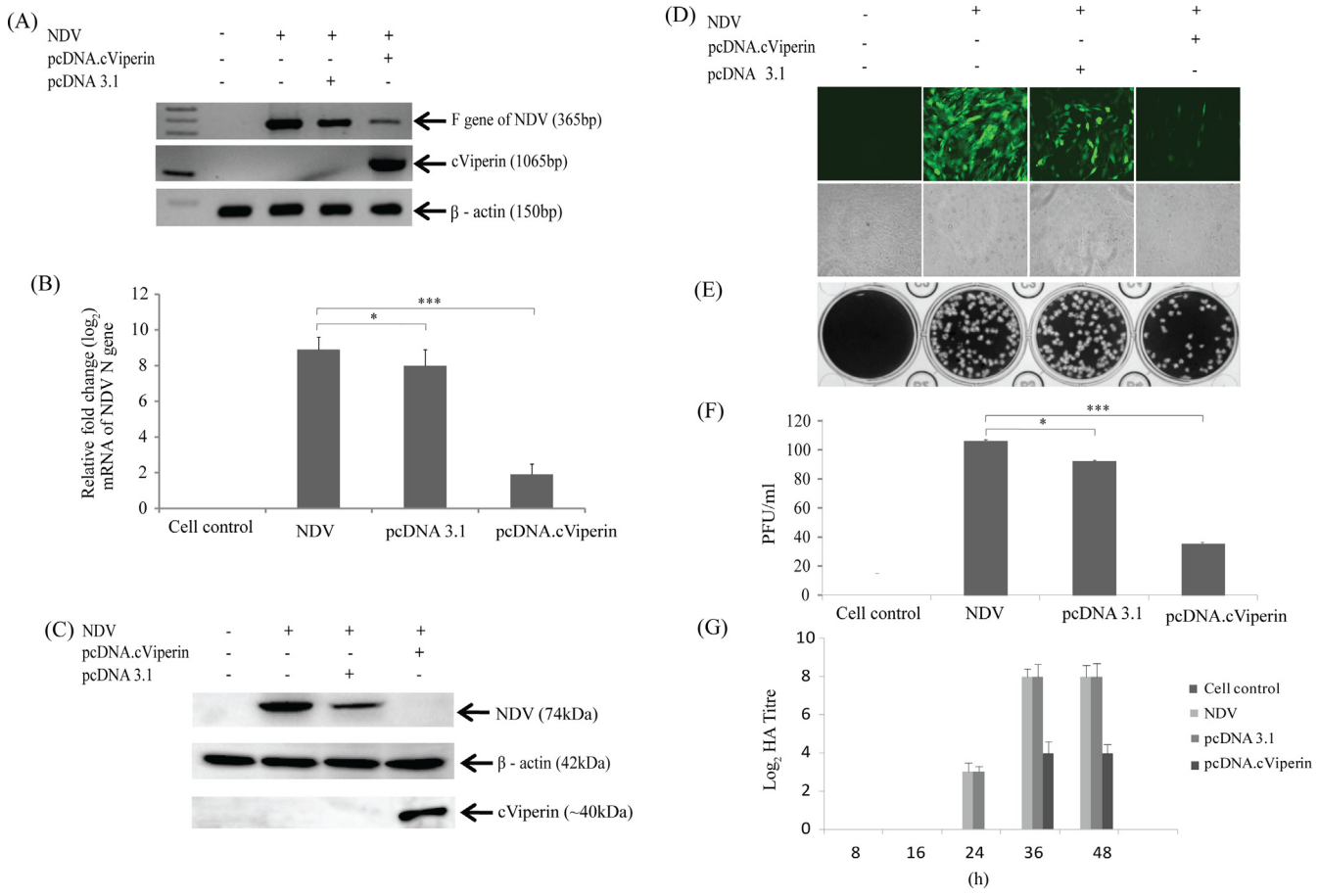

Fig. 2. Inhibition of NDV upon cViperin expression in CEF cells. The expression of NDV gene was analyzed by the semi-quantitative PCR in CEF cells transfected with pcDNA.cViperin (A). The mRNA expression of N gene of NDV was analyzed by the qRT-PCR, and mRNA levels were normalized to GAPDH mRNA and represented as mean fold expression with respect to control. The fold change in gene expression was calculated by $2^{-\Delta\Delta Ct}$ and the data were transformed in log base 2 (B). The CEF cells were transfected with pcDNA3.1 and pcDNA.cViperin, and cell lysates were analysed for NDV protein by western blot at 48 h post infection (C). The β-actin was used as a loading control. The CEF cells were transfected with pcDNA3.1 and pcDNA.cViperin followed by infection with recombinant NDV expressing GFP. The CEF cells were analysed for the GFP expression at 48 h post infection; lower panel indicates phase contrast microscopy (D). The plaque assays are showing the reduction in NDV titer upon expression of cViperin in CEF cells (E). The quantification of the NDV upon expression of cViperin by plaque assay in CEF cells (F). The results were expressed as mean ± SD from a representative experiment performed in triplicate. The CEF cells were transfected with pcDNA3.1 and pcDNA.cViperin followed by NDV infection. The virus titer was also determined by hemagglutination assay (HA) after 8, 16, 24, 36 and 48 h post-infection (G). Three independent experiments were performed and statistically analysed using the *t*-test to derive the P value (Microsoft Excel). An asterisk indicates a significant difference (*), (**), (***) of $P < 0.05$; $P < 0.01$; $P < 0.001$ respectively.

M protein and 28 residues of cViperin were involved in the interaction that includes 1 salt bridge, 6 hydrogen bonds and 264 non-bonded contacts (Fig. 3F and H). Different interaction of amino acids between chains of M protein and cViperin is shown in Table 1.

3.6. Interaction of NDV matrix protein with truncated N and C terminus of cViperin

Docking studies between the M protein of NDV and N terminus truncated cViperin were studied (Fig. 4A). The Ramachandran plot of the above complex showed 84.1% in the most favourable regions, 12.6% in additionally allowed regions, 2.2% in the generously allowed regions and 1.1% residues in the disallowed regions (Fig. 4B). The truncated N terminus of cViperin showed a significant interaction with the B chain of matrix protein. The 18 residues in chain B and 21 residues of cViperin protein were involved in the interaction, including 5 salt bridges, 4 hydrogen bonds and 441 non-bonded contacts. Furthermore, the truncated C terminus of cViperin was docked with the M protein of NDV (Fig. 4C). The Ramachandran plot of the above complex showed 86.14% in the most favourable regions, 12.14% in additionally allowed regions, 1.4% in the generously allowed regions and 0.5% residues in the disallowed regions (Fig. 4D). The truncated C terminus of cViperin exhibits an indicative interaction with A chain of matrix protein where 20 residues in chain A and 18 residues of cViperin are interacting including 4 salt bridges, 5 hydrogen bonds and 137 non-bonded contacts. The amino acid sequence of cViperin from 16 different species were analysed to compare the sequence identity and to determine the conserved regions using multiple sequence alignment by clustal omega. The sequence identity of cViperin with other species has been shown in Table S1, and a phylogenic tree was constructed. The phylogenic tree showed close clustering of cViperin with duck viperin gene with high boot strap value (Fig. S4).

3.7. Interaction study of cViperin with NDV M protein

The expression of M protein was confirmed by transfecting the plasmids bearing the gene in 293 T followed by its fluorescence microscopy (Fig. 5A). The M protein of NDV plays a crucial role in the assembly and budding of the viral particles. The interaction between cViperin and M protein of NDV was performed using co-IP assay by transfecting pcDNA.cViperin-FLAG and pcDNA.M-GFP. The GFP tagged M protein was detected in IP obtained with anti-FLAG antibody. Also,

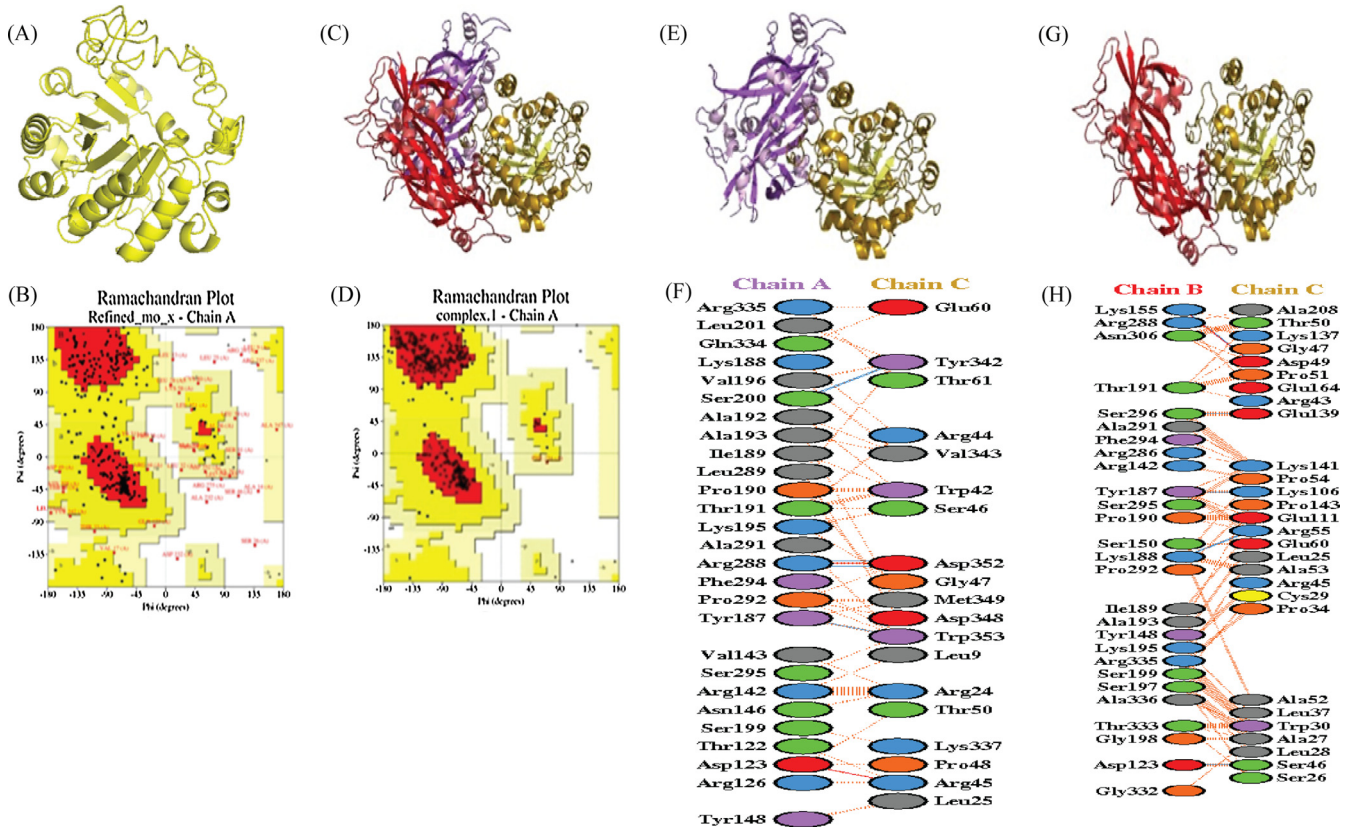

Fig. 3. The predicted protein structure of cViperin by I-TASSER (A) with its analysed Ramachandran plot (B). The interaction of M protein with cViperin (C) with its analysed Ramachandran plot (D). The interaction study between chain A of M protein (blue) and cViperin (gold) (E) and their interacting amino acids (F). The interaction study between chain B of M protein (red) and cViperin (gold) (G) and their interacting amino acids (H).

Table 1
Number of interface residues, interface area, salt bridges, H-bonds and non-bonded contacts present between interacting chains of M protein with complete chicken viperin protein. The N and C terminal truncated chicken viperin proteins were also analyzed for the parameters.

Chains	No. of interface residues	Interface area	No. of salt bridges	No. of H-bonds	No. of Non-bonded contacts
A & B	51:52	2450:2442	2	36	294
A & Viperin	27:19	1144:1249	2	5	237
B & Viperin	27:28	1409:1366	1	6	264
Matrix (chain A): Viperin (truncated C-terminus)	20:18	1174:1205	4	5	137
Matrix (chain B): Viperin (truncated N-terminus)	18:21	1034:963	5	4	441

FLAG-tagged cViperin could also be precipitated by GFP tagged M (Fig. 5B).

3.8. Cellular localisation study

The cViperin was examined for its association with LDs by immunofluorescence assay. Transfection study of cViperin showed its co-localization with the LDs (Fig. 6). The DF-1 cells stained with anti-viperin antibody showed red and lipid droplets stained with BODIPY dye as green fluorescence. The orange spots in the merged panel correspond to the overlap region.

3.9. Construction and recovery of recombinant NDVs expressing cViperin and GFP

Schematic representation of pNDV.cViperin was shown (Fig. 7A). The pNDV.cViperin was confirmed by *AscI* digestion, which showed the release of 1065 bp gene fragment (Fig. 7B). Additionally, the PCR amplification and nucleotide sequencing of the cViperin gene showed its correct orientation in the pNDV construct. The rNDV.cViperin was successfully recovered in HEp-2 cells following transfection of recombinant plasmid pNDV.cViperin. The recovered rNDV.cViperin showed a titer of 2^3 HA unit in the second passage, which increased to 2^7 HA unit following subsequent passage in the embryonated chicken egg. Confirmation of recovered recombinant virus was done by PCR using forward primer of pNDV vector and reverse primers of viperin as well as with gene-specific primers (Fig. 7C). Recovery of recombinant virus and expression of viperin was further confirmed by western blot using NDV-HN specific monoclonal antibody and viperin specific polyclonal antibody (Fig. 7D).

3.10. Characterisation of the recombinant NDV expressing cViperin

MDT and ICPI are tested to determine the virulence of rNDVs whether, it belongs to lentogenic, mesogenic or velogenic strain. The MDT values for rNDV, rNDV.cViperin and rNDV.GFP were found > 120 h. The ICPI values for rNDV, rNDV.cViperin and rNDV.GFP were recorded zero. To investigate the role of cViperin in the viral life cycle, replication analysis of rNDV and rNDV.cViperin was performed in CEF cell line. The genomic RNA analysis of rNDV.cViperin showed two fold

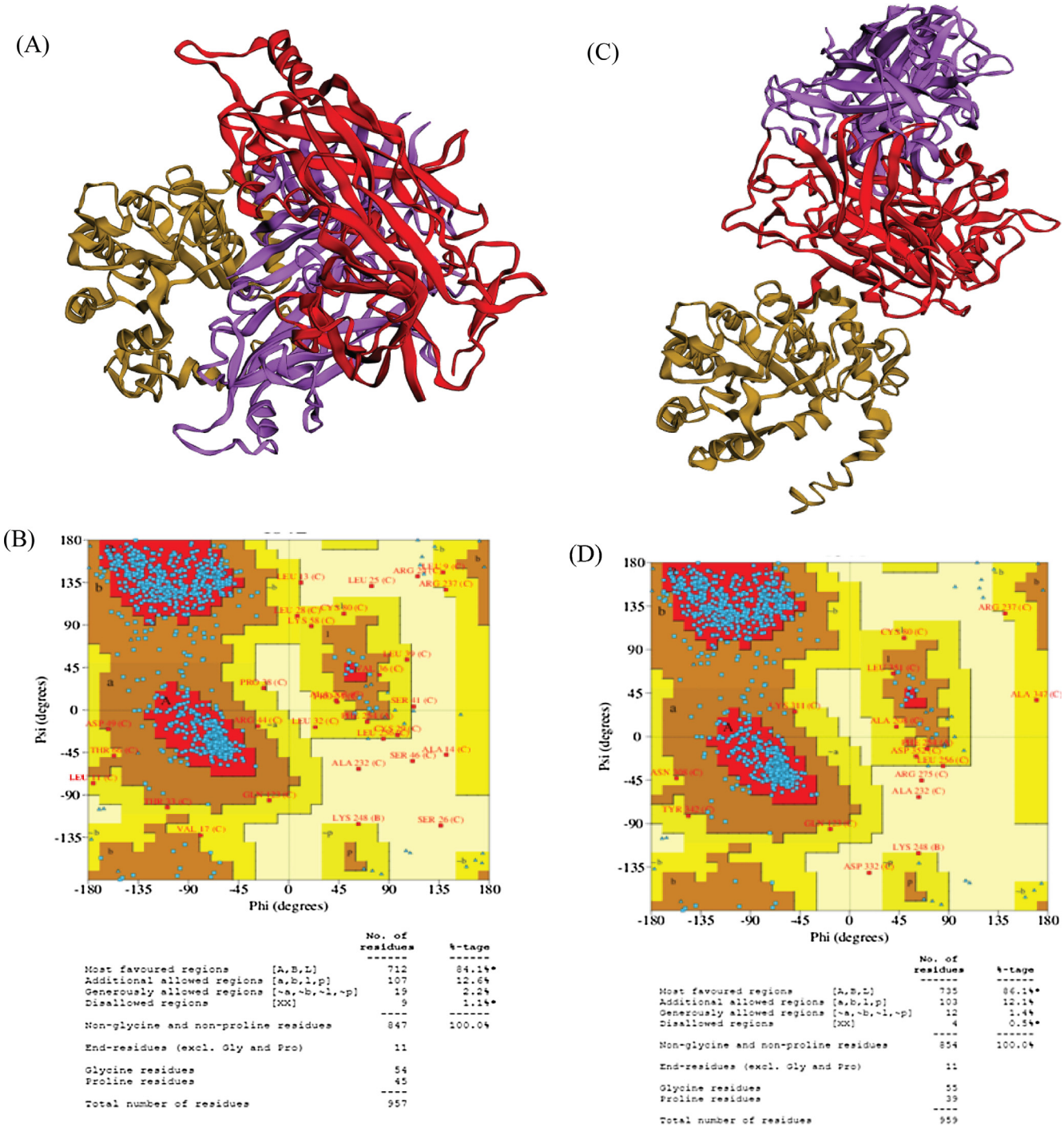

Fig. 4. Interaction studies between the M protein of NDV and truncated N terminus of cViperin were studied (A) with its analysed Ramachandran plot (B). Interaction studies between the M protein of NDV and C terminus truncated cViperin were studied (C) with its analysed Ramachandran plot (D).

reductions as compared to the rNDV at 72 h post infection (Fig. 8A). Similar findings were observed in their protein analysis where rNDV.cViperin showed lower protein concentration as compared to the rNDV (Fig. 8B). Moreover, the rNDV.cViperin showed a decrease in titer of more than one $\log_{10}$ as compared to parental rNDV and rNDV.GFP in all the time points in the growth kinetics study (Fig. 8C).

4. Discussion

ND is a highly virulent disease of poultry and has a severe impact on the economy of agriculture-based countries. Regardless of the availability of both live and inactivated vaccines, outbreaks have been reported due to the emergence of variant strains of NDV [58,75,76]. In developing countries, outbreaks of NDV are more evident due to the hot climatic conditions where the failure of vaccination is due to faulty storage and limitation in the transport facilities [77–79]. The innate immune system is an evolutionarily conserved system of defence against pathogens [80]. The IFNs are important cytokines released by the host immune system in response to diverse poultry pathogens [81]. The type I IFNs are released in response to viral infection and bind to the type I receptor, leading to the expression of other ISGs [82,83]. Upon infection, the host reacts by producing IFN-α/β which ultimately

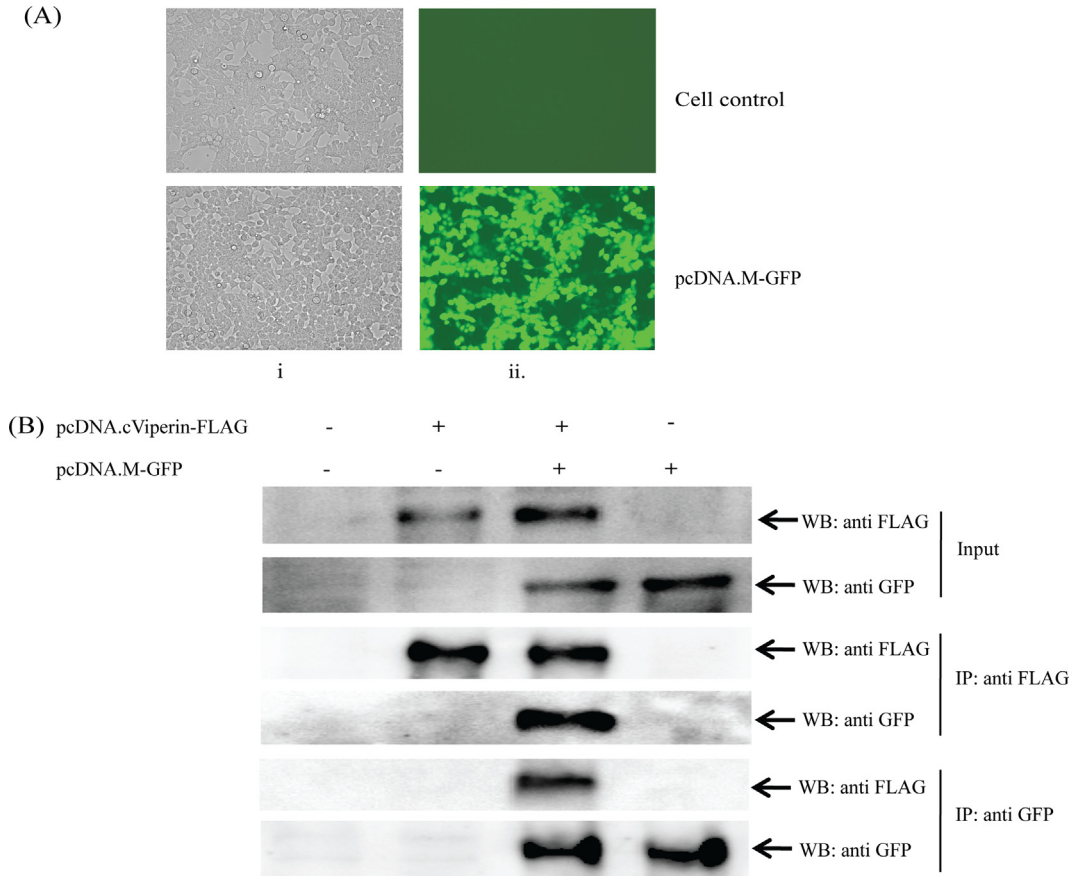

Fig. 5. The interaction of cViperin with the M protein of NDV was examined by co-immunoprecipitation assay (co-IP). The microscopic examination of pcDNA.M-GFP expression was shown in 293 T cells (A). The 293T cells plated in a 6-well plate were transfected with pcDNA.cViperin-FLAG and pcDNA.M-GFP. The cell lysates were immunoprecipitated by anti-flag and anti-GFP antibodies and subjected to western blot with rabbit anti-flag and anti-GFP antibodies at 48 h post transfection (B).

triggers several ISG. Recently type III IFNs (IFN-λ1, IFN-λ2 and IFN-λ3) have been recognised as antiviral cytokines and inducer of ISGs [84–86]. Receptors of type I IFN are ubiquitous, whereas receptors of type III IFN are confined to the mucosal epithelium [87,88]. Antiviral role of type III IFN has been shown against porcine epidemic diarrhoea virus (PEDV) [89]. In poultry, the majority of ISGs remain uncharacterized and their antiviral functions need to be explored as antiviral therapy can be efficaciously supplemented to control NDV and other poultry viral infections. The antiviral activities of ISGs such as IFIT family proteins [90], OASL [91] and Mx [92–96] have been explored. Viperin is also recognised as one such ISGs, which is induced by IFNs (α, β, γ and λ), by a B-form of DNA, the dsRNA, lipopolysaccharides (LPS) and also upon virus infection [97–101]. It has been reported that the viperin upregulates upon NDV infection in the different tissues of duck [102]. However, the effect of cViperin on NDV replication has not been reported. Our results reveal that the infection of CEF by NDV led to the induction of cViperin with maximal expression at 60 h post-infection. Elseways, overexpression of cViperin significantly reduced the viral protein level. Our result demonstrates that the cViperin effectively suppresses NDV proliferation both in the viral RNA and protein level. Reduced virus release in cViperin overexpressed cells also suggests the reduced propagation of NDV. It has been reported that the viperin localises to the cytoplasmic face of the endoplasmic reticulum (ER) and lipid droplets [103]. NDV is a pleomorphic enveloped virus and budding of mature virions from the host cell membrane is crucial to complete its life cycle [104–106]. Additionally, the M protein of NDV is targeted towards the cell membrane and is involved in the budding of mature virions from the cell surface. These findings led us to explore the cross talk between M protein of the NDV and viperin.

A comparison between chicken viperin and mouse viperin showed 88% total amino acid identity. The radical SAM enzyme signature CxxxCxxC conserved motif was found in viperin of both the organisms [19]. The predicted structure of cViperin using bioinformatics tools showed a high confidence score. The study could have been more accurate provided the presence of more related proteins of radical SAM family in I-TASSER's dataset. The small yet sufficient dataset yielded four proteins out of which the best was picked and refined. In our study, the changes in conformation of a few amino acids were made using 3D refine server without altering their native conformation as reported during the modeling of other proteins [107]. This could have changed the amino acid residues into their favourable state without reducing their number in disallowed regions. ProSA analysis was done to understand the native structure, and the z-score turned out to be in the range of native conformations reassured to use the structure for further analysis. The z-score of −7.57 obtained from ProSA server suggests the native conformation of cViperin as reported for other proteins [70]. In earlier studies, it has been presented that human viperin is composed of three distinct domains, an N-terminal domain, with variable length and sequence among different species [98]. Amphipathic α helix present in the N-terminal is responsible for its association with the cytosolic face of the ER and lipid droplets [108]. The central domain contains three cysteine residues organized in CxxxCxxC motif, responsible for binding with iron-sulfur clusters [109,110] and the C-terminal domain a highly conserved between the species might perform the role in protein-protein interaction [98].

Our docking results suggest that cViperin interacts with the chains A and B of M protein, which could inhibit NDV budding. It was equally

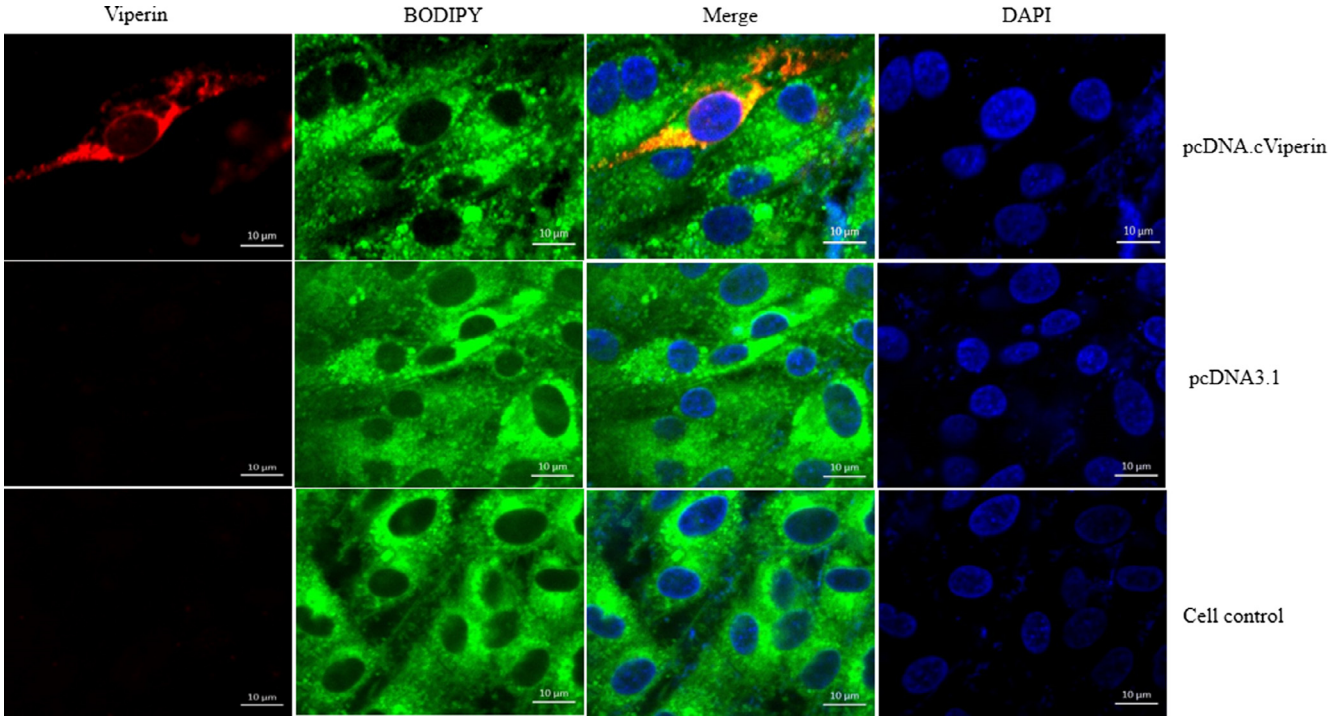

Fig. 6. Cellular localization of viperin and lipid droplets. The DF-1 cells were seeded over coverslip, and the cells have been transiently transfected with cViperin. The cells were analysed by immunofluorescence for localization to lipid droplets using BODIPY lipid droplet marker and mouse anti-FLAG antibody for viperin expression after 48 h post transfection. Representative images represent DF-1 cells stained with anti-viperin (red channel) and lipid droplets (green channel). Merge panel shows the co-localization of viperin and lipid droplets. Cell nuclei were stained with DAPI (blue channel). Samples were visualised under confocal microscopy with a magnification of 63X. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

important to note that both the N-terminal and C-terminal of cViperin are involved in the interaction. Further, the compatibility of the structure was checked using VERIFY 3D, which showed that 88.67% of the residues had an average 3D-1D score ≥0.2 [69]. This interaction resembles the ball-and-socket setup wherein the matrix protein plays the role of the socket. This renders the utilisation of the major pocket areas of the matrix protein for both ligands and proteins which, perhaps, inhibits its interaction with other viral proteins during assembly

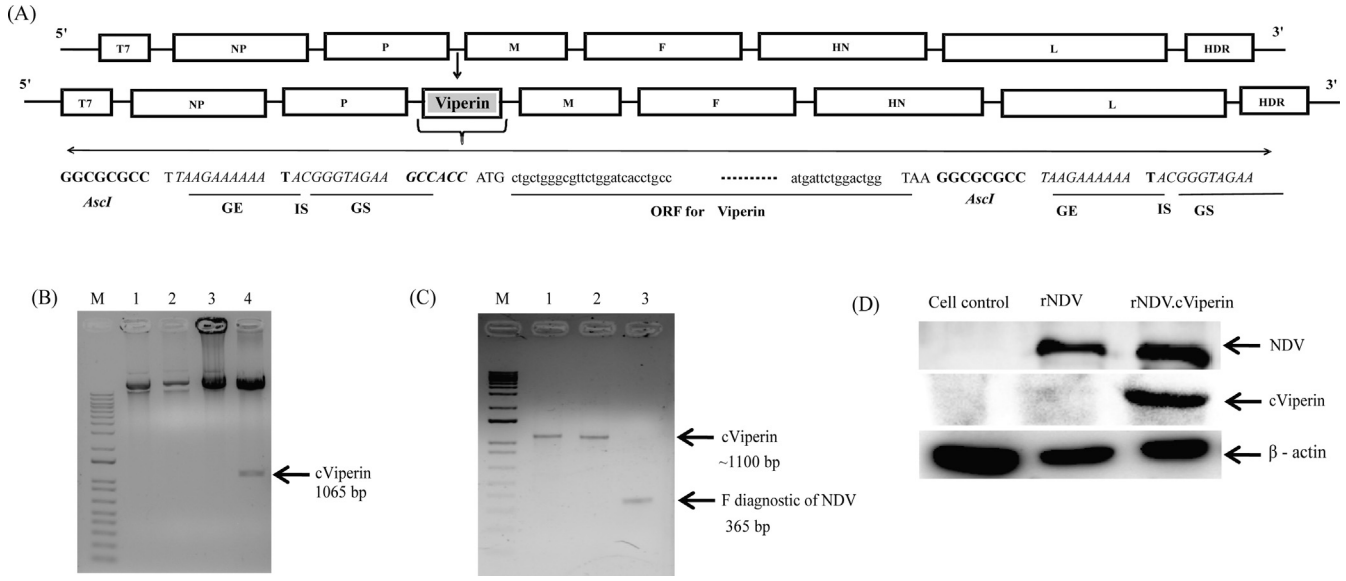

Fig. 7. Construction of recombinant plasmid pNDV.cViperin (A). Upper strand represents pNDV (T7 promoter, NP- nucleoprotein, P- phosphoprotein, M- matrix protein, F-fusion glycoprotein, HN-hemagglutinin-neuraminidase protein, L-large polymerase protein, and HDR- hepatitis delta ribozyme) and lower strand represents pNDV.cViperin. The cViperin cloned at *Asc*I site between the P and M genes of the NDV antigenomic cDNA. The ORF of cViperin has a flanking sequence of NDV gene end (GE), an intergenic T nucleotide, and a gene start (GS). The confirmation of pNDV.cViperin full length clone (B). Lane M: DNA ladder; lane 1: undigested pNDV; lane 2: *Asc*I digested pNDV; lane 3: undigested pNDV.cViperin; lane 4: *Asc*I digested pNDV.cViperin showing the release of a 1065 bp gene. The PCR confirmation of rNDV.cViperin (C). Lane M: DNA ladder; lane 1: amplification by the forward primer of pNDV vector and reverse primer of the cViperin gene; lane 2: amplification by cViperin gene-specific primer; lane 3: amplification by pNDV specific primers. The western blot analysis for the confirmation of NDV and cViperin protein expressions by rNDV.cViperin using monoclonal antibody of HN and polyclonal antibody of cViperin, respectively (D).

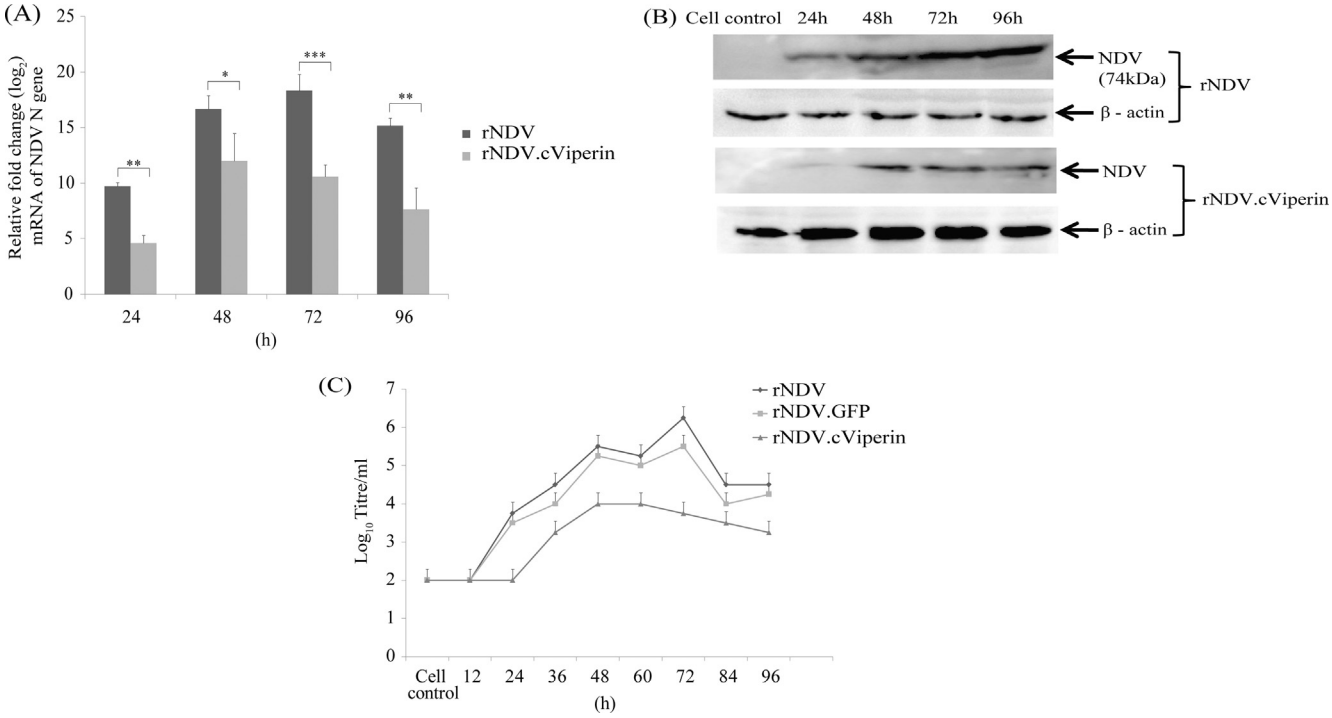

Fig. 8. Reduced replication of rNDV.cViperin as compared to rNDV and rNDV.GFP at different time points post infection in CEF cells. The expression of the N gene was analysed by the qRT-PCR in CEF cells infected with rNDV and rNDV.cViperin. PCR and mRNA levels were normalised to GAPDH mRNA and represented as mean fold expression with respect to control. The qRT-PCR data were transformed in log base 2 (A). The western blot analysis is showing the reduction in the HN protein levels of rNDV.cViperin as compared to rNDV (B). The growth kinetics of rNDV.cViperin as compared to rNDV and rNDV.GFP is showing reduced viral titers (C). The q-PCR was performed three times and statistically analysed using the *t*-test (Microsoft Excel). An asterisk indicates a significant difference (*), (**), (***) of $P < 0.05$; $P < 0.01$; $P < 0.001$ respectively.

and budding process, thus inhibiting viral budding. In our study, bioinformatics analysis showed the possible interaction between viperin and M protein, which was further examined *in vitro*. The isoelectric point of 6.8 suggests no charge in the cViperin at neutral pH, and physiological conditions may play a crucial role in its interaction with M protein of NDV. Nevertheless, other viral proteins might also interact with viperin and could potentiate its antiviral activity.

Furthermore, our co-IP assay showed the interaction of viperin and M protein. Considering our molecular docking and co-IP, we speculated that inhibition of NDV replication by cViperin is due to its interaction with its M protein. Our results showed localization of cViperin to LDs as reported earlier [20]. Studies with influenza virus have shown that viperin binds to farnesyl diphosphate synthase (FPPS), an enzyme involved in cholesterol biosynthesis and thus, may alter the lipid content [26]. As LDs are derived from ER where synthesis of cholesterol occurs, it is possible that viperin could affect the type and content of lipid in LDs [103]. In the present study, viperin has shown to be induced by NDV infection and inhibits its replication *in vitro*. The localization of viperin to LDs may reflect the mechanism that viperin deploy to inhibits NDV replication. The diverse anti-viral mechanisms of viperin have been studied for various viruses. Viperin is the first reported molecule to affect rabies virus replication by reducing cholesterol and sphingomyelin of the host cell membrane [111]. The anti-viral activity of viperin by inhibiting virus release was shown in the case of influenza and HIV-1 [26,112,113].

Our results of truncated N and C terminus of cViperin showed that only a few amino acids are involved in the interaction and the rest all are a part of the radical SAM domain. The finding suggested that a few amino acids of the C- and N-terminal acting as the pivot points in the interaction between the two proteins. Together we can realise that there are a few regions of amino acids in all the three domains of the cViperin that are responsible for its activity against the matrix protein of the NDV. The regions 60–70 amino acids of the N-terminus, and 100–120 amino acids of the C-terminus are common in all the findings suggesting that the amino acids present in these regions may be important for the interaction between both the aforementioned proteins. However, advancements in the software used in these studies will further enrich the data. The phylogenic study suggested that cViperin showed identity with viperin from other species inferring its conserved nature.

Reverse genetics of NDV has been explored to understand its pathogenicity, vaccine and anticancer activities. In this study, cViperin was cloned, and the rNDV expressing cViperin was recovered to interrogate the effect of cViperin in NDV replication. The recovered rNDV.cViperin showed no enhancement in its pathogenicity as analysed by the MDT and ICPI tests suggesting the virus to be avirulent. The reduced viral titer of rNDV.cViperin was observed as compared to parental NDV in the growth kinetics study which indicates that virus expressing cViperin gets attenuated and its replication rate is diminished. The rNDV.GFP used as a control showed a minimal difference in the replication kinetics of NDV suggesting that the effect is not because of the addition of the foreign protein. Furthermore, the effect was also evident in both RNA and protein level. Understanding the host antiviral activity is essential for designing the therapeutic strategy against NDV infection in poultry. The study will give us an insight into the role of ISGs in curtailing the NDV infection. It will be interesting to explore the other potential ISGs that could target NDV. Perhaps, understanding the chicken immune system will possibly give us a new way to intervene NDV infection therapeutically. Furthermore, these naturally occurring host viral inhibitors could be helpful in the development of novel antiviral therapies against NDV as well as other poultry viruses.

5. Conclusions

Viperin is an interferon-inducible protein that inhibits the replication of a range of viruses by diverse mechanisms. Our data showed the

anti-NDV effect of chicken viperin (cViperin). The modeling of the cViperin protein using I-TASSER and ZDOCK suggested its interaction with the matrix protein of NDV. The interaction was further confirmed by co-immunoprecipitation assay using recombinant matrix and cViperin proteins. Again, the recombinant NDV expressing cViperin showed reduced replication of the NDV. Our results suggest down-regulation of NDV replication by cViperin. The study will be useful to understand the chicken innate immune response towards virus infection.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.04.007.

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