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Research Article

Molecular characterization of cyprinid herpesvirus 3 (CyHV-3) major capsid protein (MCP) and its impact on immune gene expression *in vitro*

Li K.S.[1], Zhu Y.M.[1], Zhou X.[3], Hu Y.P.[1], Ju C.Z.[1], Wang Y.T.[1], Hu M.J.[1], Zhang C.[1], Zhang J.Q.[1,2*]

1Hubei Key Laboratory of Animal Nutrition and Feed Science, School of Animal Science and Nutritional Engineering, Wuhan Polytechnic University, Wuhan, China
2Fujian Province Key Laboratory of Special Aquatic Formula Feed (Fujian Tianma Science and Technology Group Co., Ltd.) Fuzhou, China
3Technology Center of Wuhan Customs, Wuhan, China
*Correspondence: jiaqizhang1993@163.com

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Abstract

Main chitin protein (MCP) is a crucial component of cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), found on the surface of the viral capsid. It plays a vital role in protecting viral DNA and assembling viral particles, contributing to the virus's high infectivity and lethality. This study aims to characterize the MCP-encoded protein of CyHV-3, investigate its subcellular localization, and assess its impact on the expression of immune factors through in vitro experiments. Our findings revealed that the CyHV-3 MCP protein consists of 579 amino acids. Despite analyzing evolutionary trees of homologous proteins from three carp herpesvirus sources, no significant similarities were observed. Subcellular localization experiments indicated that the green fluorescent signal of the pEGFP-MCP protein predominantly diffused within the cytoplasm. Remarkably, overexpression of MCP significantly suppressed the expression of immune factors in Channel Catfish Ovary (CCO) and Epithelioma papulosum cyprinid (EPC) cell lines, highlighting its role as a viral immune escape factor. These results enhance our understanding of the immune function of CyHV-3 MCP and provide a theoretical foundation for developing new vaccines against CyHV-3 infections. These results provide further insights into the immune function of CyHV-3 MCP and provide a theoretical basis for the development of new vaccines against CyHV-3 virus infection.

Introduction

Carp and koi have been afflicted by a serious viral disease known as Koi Herpesvirus Disease (KHVD) (Chong, 2022). This disease is caused by the koi herpesvirus (KHV), also referred to as carp herpesvirus-3, which leads to interstitial nephritis and gill necrosis in carp (Yanuhar *et al.*, 2020). KHV is a double-stranded icosahedral DNA virus belonging to the family Heteroherpesviridae (Bergmann *et al.*, 2020). With a genome size of 295 kbp, it is the largest known herpesvirus genome. The KHV genome encodes 156 potential protein-coding open reading frames. Each virus particle comprises 40 structural proteins, categorized into capsid (3), envelope (13), periplasm (2), and unclassified (22) structural proteins (Cano *et al.*, 2021a; Mancheva *et al.*, 2023).

The innate immune system serves as a crucial defense mechanism in safeguarding host health and countering infections (Wein and Sorek, 2022). It promptly identifies and eliminates pathogens, initiates inflammatory responses, and provides essential support for adaptive immunity (Carty *et al.*, 2021). Interferons, at the forefront of this system, play a pivotal role by activating antiviral genes and modulating immune cell function to swiftly respond to viral invasions and uphold host well-being (Feng *et al.*, 2021; Kasuga *et al.*, 2021). However, viruses have evolved various strategies to impede interferon production and signaling, evading immune surveillance (Parmigiani *et al.*, 2022). A comprehensive comprehension of these mechanisms is paramount for devising effective antiviral therapies. Notably, herpesvirus infections have shown effective activation of host immune responses. For example, Cyprinid herpesvirus-2 (CyHV-2) infection prompts immune gene expression in goldfish (Das *et al.*, 2021). Conversely, CyHV-3 infection elicits a robust type I interferon (IFN) response in carp (Jiang *et al.*, 2023). Nevertheless, the precise role of CyHV-3-specific proteins in the infection process remains elusive. A deeper understanding of these mechanisms will facilitate a clearer comprehension of disease progression and steer the development of targeted therapeutic interventions.

In this study, we examined the conservation of CyHV-3 MCP within carp herpesviruses using sequence alignment, phylogenetic trees, and protein tertiary structures. Additionally, co-localization experiments were conducted to determine its specific subcellular localization. Our findings reveal that CyHV-3 MCP exhibits structural distinctions from CyHV-1 MCP and CyHV-2 MCP, showcasing unique evolutionary characteristics. Co-localization experiments demonstrated that CyHV-3 MCP primarily localizes in the cytoplasm, and its overexpression effectively inhibits the expression of interferon and its downstream genes induced by poly(I:C) stimulation. These discoveries offer fresh insights into CyHV-3-host interactions and establish a groundwork for the development of innovative DNA vaccines targeting CyHV-3.

Material and methods

Fish Cell Lines and Viruses

CCO cells were cultured in Minimum Essential Medium (MEM) (HyClone,

USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, New Zealand), 100 μg/mL penicillin, and 100 μg/mL streptomycin, at 25°C in a 5% CO_2 atmosphere. EPC cells were cultured in M199 medium (Gibco, USA) supplemented with 10% FBS, at 28°C in a 5% CO_2 atmosphere. CyHV-3 was propagated in CCO cells until a cytopathic effect (CPE) was observed, after which the virus was stored at -80°C. MCP expression often correlates with the appearance of cytopathic effects in cell cultures. CPEs include cell rounding, detachment, syncytia formation, and eventual cell death.

Plasmids

The open reading frame (ORF) of the carp (CC) MCP gene (GenBank accession number: AVL28280.1) was amplified by PCR using cDNA from virus-infected CCO cells as a template and cloned into the pEGFP-N1 vector. The plasmid pEGFP-MCP and the Renilla luciferase internal control vector (pRL-TK) were constructed as described previously (Lu *et al*., 2020). The luciferase reporter plasmid pmirGLO-MCP was generated by amplifying the MCP gene and cloning it into the pmirGLO vector using the primers listed in Table 1. All constructs were verified by DNA sequencing.

Table 1: The primers for quantitative real-time PCR.

Application	Primer	Sequence (5'-3')
qRT-PCR	EPC-IFN-F	ATGTATCGCTTTGCTGTGTC
	EPC-IFN-R	TTCGGGAAGGTTATTTTAAC
	CCO-IFN-F	TGTACCTCGGCCTTCTCGAT
	CCO-IFN-R	CGAAGCCTGCAACTGGATGA
	EPC-TBP-F	GATGGTGGGAGGTTCAGG
	EPC-TBP-R	GATGGGGGTCATAGGGGT
	EPC-ISG15-F	TAATGCCACAGTCGGTGAA
	EPC-ISG15-R	AGGTCCAGTGTTAGTGATGAGC
	EPC-PKR-F	ACCTGAAGCCTCCAAACATA
	EPC-PKR-R	GCATTCGCTCATCATTGTC
	β-actin-F	GGGCACCTGAACCTCTCATT
	β-actin-R	CTGCTATGTGGCTCTTGACTTTG
Expression plasmid construction	*pEGFP-mcp*-F	ACAAAACCCGAAACCGATCCCGA
	pEGFP-mcp-R	GCGGTTTCGGGTTTTGT

Viral Infection

CCO cells were infected with CyHV-3 at a multiplicity of infection (MOI) of 10, or with an equal volume of PBS as a control. After 2 hours of adsorption at 25°C, the inoculum was removed and M199 medium with 5% FBS was added. Cells were harvested at 12, 24, 36, and 48 hours post-infection, and viral mRNA or host miRNA was detected by qRT-PCR using the primers listed in Table 1.

Multiple Comparisons and Evolutionary Analyses of Amino Acid Sequences

The MCP 24 gene (GenBank: AVL28280.1) was analyzed and the amino acid sequence of its encoded protein was extracted. To explore the evolutionary

relationships among three carp herpesviruses (CyHV-1 MCP, NC_019491.1; CyHV-2 MCP, NC_019495.1; CyHV-3 MCP, KJ627438.1), multiple sequence comparisons of their homologous proteins were performed using ClustalX1.83 software. Similar sequences were identified in the NCBI database, and conserved structural domains within these proteins were predicted. Data alignment and presentation were meticulously handled using GenDoc software. Additionally, a phylogenetic tree was constructed using MEGA 7.0 software, providing valuable insights into the evolutionary links between these herpesviruses.

Transfection

Poly (I:C) and pEGFP-MCP were incubated with TransIntroTM EL Transfection Reagent (TransGen Biotech, China) in 500 μl Opti-MEM medium (Invitrogen, USA) for 30 minutes at room temperature. Subsequently, the reagents were added to the cultured CCO and EPC cell culture dishes, and the cells were transfected at 25°C. After transfection, the medium was replaced with 1 mL of MEM medium, and the cells were further incubated at 25°C. Finally, the corresponding cell samples were collected for subsequent RNA sample extraction.

Quantitative RT-PCR

The expression of IFN-α, along with key genes in the IFN production pathway (ISG15, PKR, TBP), was quantitatively analyzed using qRT-PCR. Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription and qRT-PCR reagents were purchased from Vazyme Biotech Ltd., and experiments were performed following the provided protocols. To detect immunogenes, 1 μg of RNA was mixed with 1 μL of Oligo (dT), 4 μL of 4 × gDNA wipe mixture, and ribonuclease-free H_2O to a total volume of 16 μL. After incubation for 2 minutes at 42°C, 4 μL of 5×select qRT supermix III was added, followed by incubation for 15 minutes at 50°C and 2 minutes at 85°C. The quantitative PCR reaction was performed in a 20 μL volume consisting of 10 μL of AceQ qPCR SYBR Green Master Mix, 1 μL of cDNA template, 0.4 μL of forward primer, 0.4 μL of reverse primer, and 8.2 μL of ddH_2O. Cycling conditions were as follows: 95°C for 5 minutes, 45 cycles of 10 seconds at 95°C, 10 seconds at 60°C, and 15 seconds at 72°C, followed by a final step at 95°C for 2 minutes to generate a melting curve at a heating rate of 5 °C/s. A gradient PCR instrument (Bioer Technology, Hangzhou, China) was used for reverse transcription experiments, and an Applied Biosystems QuantStudio 1 Plus (Thermo Fisher Scientific, Suzhou, China) was used for PCR experiments. Data were normalized to β-actin levels in each sample using the $2^{-\Delta\Delta Ct}$ method.

Methods of statistical analysis

Data in a completely randomized design were analyzed using a one-way ANOVA procedure with SAS 9.4 software (SAS Institute, Cary, NC, USA), with the significance level set at $p<0.05$. Histograms were plotted using GraphPad Prism 8.0

(GraphPad Software, San Diego, CA, USA).

Results

Sequence alignment and phylogenetic tree analysis of CyHV-3 MCP and other cyprinid herpesvirus homologous proteins

Bioinformatics analysis revealed that the encoded protein of CyHV-3 MCP contains 1268 amino acids (AA) (Fig. 1A). To gain further insights into its properties, we compared its amino acid sequence with homologous proteins from 12 different herpesviruses, including ICHSV-2, Silurid herpesvirus 1, SalHV-1, Lake sturgeon herpesvirus, Acipenserid herpesvirus 1, Ranid herpesvirus 2, Ranid herpesvirus 3, Ranid herpesvirus 4, Anguilid herpesvirus 1, CyHV-1 MCP, CyHV-2 MCP, and CyHV-3 MCP. The results showed that CyHV-3 MCP has the highest homology with CyHV-2 MCP, followed by CyHV-1 MCP, while the differences with other homologous proteins were more pronounced. These findings suggest that CyHV-3 MCP and its homologous proteins may play a crucial role in interacting with the host, potentially leading to similar diseases caused by this type of herpesvirus. Additionally, we constructed an evolutionary tree using these homologous proteins, and the results were consistent with those of the amino acid sequence alignment, further supporting our evolutionary analysis (Fig. 1B).

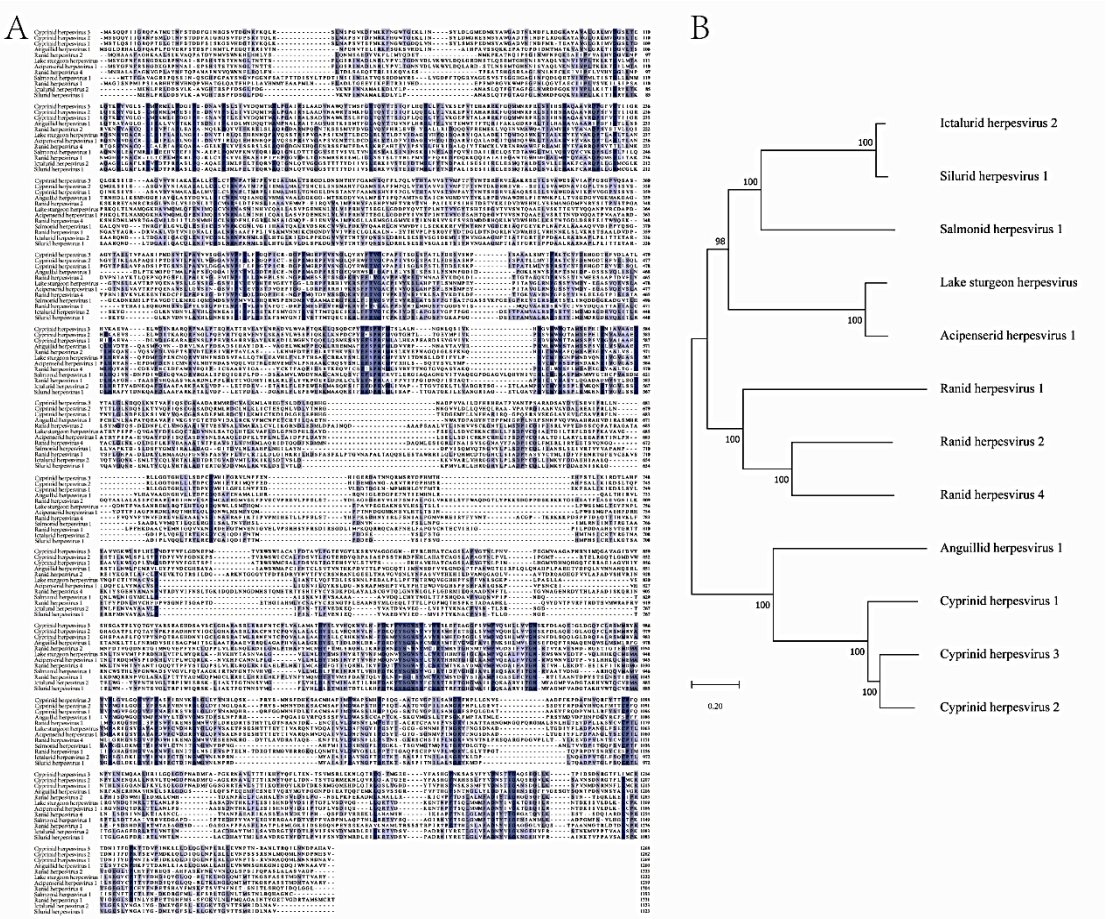

Figure 1: Sequence alignment and phylogenetic tree of CyHV-3 MCP and other Cyprinid Herpesvirus homologous proteins.

Tertiary structure of the CyHV-3 MCP

The tertiary structures of CyHV-1 MCP, CyHV-2 MCP, and CyHV-3 MCP were predicted using AlphaFold2. The results indicate that all three proteins can form various secondary structural elements, including α-helices, β-sheets, and loop structures. Notably, the predominant secondary structure observed in these proteins is the α-helix. Specifically, CyHV-1 MCP has 129 α-helices and 46 β-sheets, CyHV-2 MCP has 79 α-helices and 16 β-sheets, and CyHV-3 MCP has 49 α-helices and 7 β-sheets. These data suggest that the tertiary structures of CyHV-3 MCP and CyHV-2 MCP are more similar to each other compared to CyHV-1 MCP, consistent with the results of our previous sequence comparison (Fig. 2).

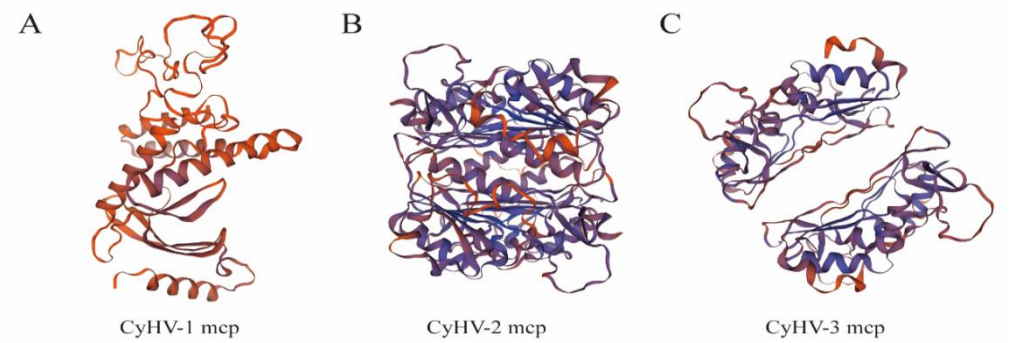

Figure 2: Tertiary structure of the CyHV-1 MCP (A), CyHV-2 MCP (B) and CyHV-3 MCP protein (C).

Subcellular localisation of CyHV-3 MCP protein

When observing cells transfected with the empty plasmid pEGFP-N1, the green fluorescent signal is evenly dispersed throughout the cell. In contrast, the distribution pattern in cells transfected with the recombinant plasmid pEGFP-MCP is significantly different. In these cells, the green fluorescent signal is predominantly located in the cytoplasm, with some fluorescence observed around the nucleus or scattered throughout the cytoplasm (Fig. 3).

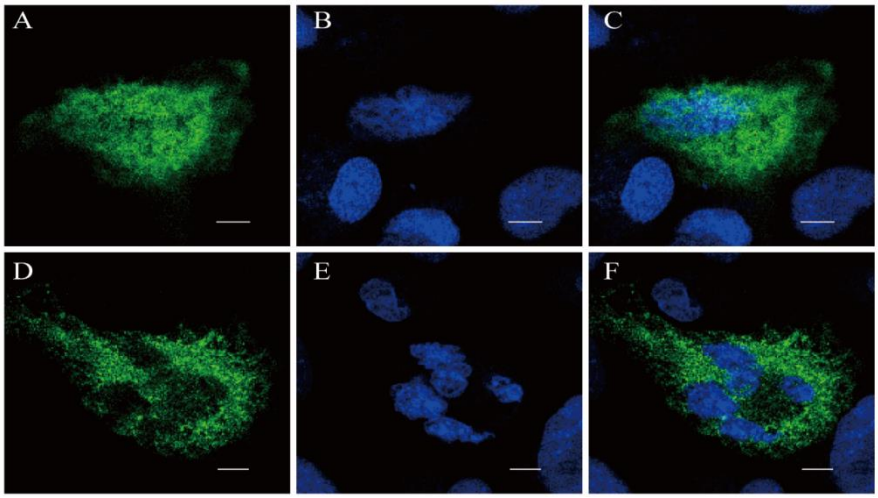

Figure 3: Subcellular Localization of CyHV-3 MCP. 293T cells were transfected with the indicated plasmid. Twenty-four hours post-transfection, cells were analyzed by fluorescence microscopy (original magnification, ×100).

CyHV-3 MCP inhibits interferon production

In this study, the ability of the MCP to inhibit IFN production was examined using qRT-PCR. As shown in Figures 4A and 4B, CyHV-3 MCP significantly inhibited poly(I:C) induced IFN production in CCO and EPC cells (Fig. 4). CyHV-3 MCP significantly inhibited PKR, TBP and ISG15, key genes of the IFN synthesis pathway (Fig. 5).

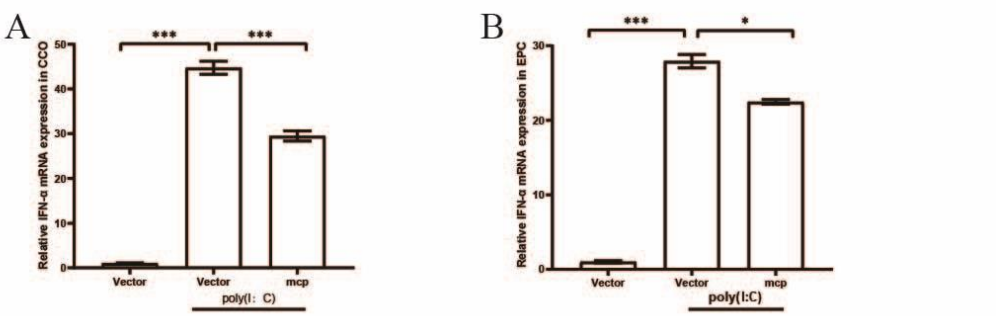

Figure 4: illustrates the inhibition of IFN production by CyHV-3 MCP (A-B). CCO (A) or EPC cells (B) were transfected with CyHV-3 MCP or empty vector, along with poly (I:C). Cell harvest was conducted 24 hours post-transfection. β-actin served as an internal control (*, p<0.05; **, p<0.01; ***, p<0.001).

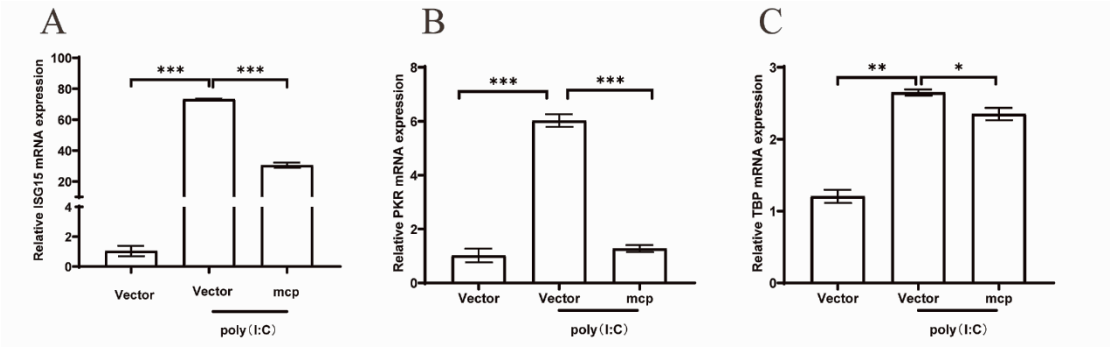

Figure 5: illustrates the inhibition of ISG15, PKR, and TBP production by ORF24. (A-C) EPC cells were transfected with ORF24, empty vector, and poly (I:C). Cells were collected after 24 hours. β-actin served as an internal control (*, p<0.05; **, p<0.01; ***, p<0.001).

Discussion

Koi herpesvirus disease (KHVD) is caused by Cyprinid herpesvirus 3 (CyHV-3), which mainly damages the gills, liver, and brain tissue of koi. In the early stages of infection, fish may exhibit symptoms such as gill hemorrhagic necrosis, sunken eyes, and grayish-white patches on the skin (Zrnčić *et al.*, 2020). Outbreaks of this viral disease can result in mortality rates of up to 80 to 100 percent, causing severe economic losses to the aquaculture industry (Pokorova *et al.*, 2005). CyHV-3 MCP plays an important role in CyHV-3 virus infection. Studies have shown that CyHV-3 MCP may be involved in the process of viral adhesion and invasion, as well as the activation of immune responses in host cells (Tolo *et al.*, 2021; Akbar Hasan Al-Jaf *et al.*, 2024). These findings contribute to

an in-depth understanding of the mechanisms of CyHV-3 infection and offer the possibility of developing new therapeutic strategies (Cano *et al.*, 2021b).

In this study, we delved into the structure and cellular localization of CyHV-3 MCP and their effects on interferon expression. Sequence comparison identified similarities and relationships between multiple proteins, revealing conserved regions of the protein across species that represent functional specificity (Yang *et al.*, 2020). Constructing phylogenetic trees based on the results of multiple sequence comparisons helps us understand how species have evolved and diverged over time and can be used to infer features of common ancestry (Kapli *et al.*, 2020). In this study, sequence alignment showed that the CyHV-3 MCP is more closely related to the CyHV-1 MCP and CyHV-2 MCP, suggesting that CyHV-3 MCP and its homologous proteins may interact with the host, leading to similar diseases caused by this type of herpesvirus. Through evolutionary tree analysis of the homologous proteins, we found that the CyHV-2 MCP is more closely related to the CyHV-3 MCP and more distantly related to other homologous proteins, corroborating the results of the sequence comparison and emphasizing the uniqueness of the CyHV-3 MCP. The folding pattern of the protein chain in three-dimensional space represents the tertiary structure of the protein, which determines the active site and the mode of interaction with other molecules (Guo *et al.*, 2021). Our tertiary structure analysis further supports the conserved spatial structure of CyHV-3 MCP and highlights their unique role in evolution.

Interferons (IFNs) play a crucial role in viral infections. They rapidly initiate an antiviral innate immune response upon viral infection. When a virus invades a cell, Pattern Recognition Receptors (PRRs) recognize the nucleic acid and protein components of the virus and activate IFN-I signaling, which in turn induces the expression of Interferon-Stimulated Genes (ISGs). IFNs work by binding to IFN receptors on the cell surface and initiating the downstream JAK-STAT signaling pathway, leading to the transcription of ISGs. These ISGs can target different stages of viral replication, thereby resisting viral infection (Wang *et al.*, 2020; Koike *et al.*, 2021; Zhang *et al.*, 2022). In this experiment, overexpression of CyHV-3 MCP significantly reduced the mRNA levels of IFN, classifying it as an immune evasion factor utilized by CyHV-3. This study not only reaffirms the uniqueness of CyHV-3 MCP in the evolutionary history of the virus but also reveals its immune evasion capabilities.

The innate immune system plays an important protective role in host immunity by rapidly recognizing and destroying pathogens, initiating and modulating inflammatory responses, and providing the necessary support and regulation for adaptive immunity to ensure that the organism can effectively defend itself against a wide range of infections and maintain internal stability (Kaur and Secord, 2021). Therefore, we focused on the regulatory mechanisms of CyHV-3 MCP in innate immunity. Previous studies have shown that other proteins of CyHV-3 reduce signaling that initiates the antiviral response by blocking the activation of RIG-

I-like receptors (RLRs) and MDA5 receptors. Specifically, these proteins reduce activation of the Mitochondrial Antiviral Signaling Protein (MAVS) on the mitochondrial outer membrane. Activation of MAVS normally initiates the transcription of antiviral genes (e.g., type I interferon) by recruiting and activating TANK-binding kinase 1 (TBK1), which in turn phosphorylates the transcription factors IRF3 and IRF7, ultimately leading to interferon synthesis (Wicherska-Pawłowska *et al.*, 2021; Malkowska and Niedzwiedzka-Rystwej, 2022; Huang *et al.*, 2022; Wu *et al.*, 2024). This is one of the key defense mechanisms of cells against RNA virus infection.

In this study, we explored the effects of CyHV-3 MCP on the expression of IFN and its downstream immune genes. Protein Kinase R (PKR) recognizes and binds to double-stranded RNA produced by CyHV-3 MCP infection, activating the TATA-binding Protein (TBP), which inhibits the transcription of interferon and Interferon-Stimulated Gene 15 (ISG15). Since CyHV-3 MCP, like other viral proteins, can evade surveillance and clearance by the immune system by suppressing the innate immune response, it is not clear whether CyHV-3 MCP may affect IFN expression by interacting with these key "IFN-regulated genes". In the present study, CyHV-3 MCP also effectively inhibited the poly(I:C)-stimulated increase of IFN and showed some immunosuppressive effects. However, its possible interaction with the "IFN-regulated genes" and its molecular mechanism are not clear and need to be further explored (Fig. 6).

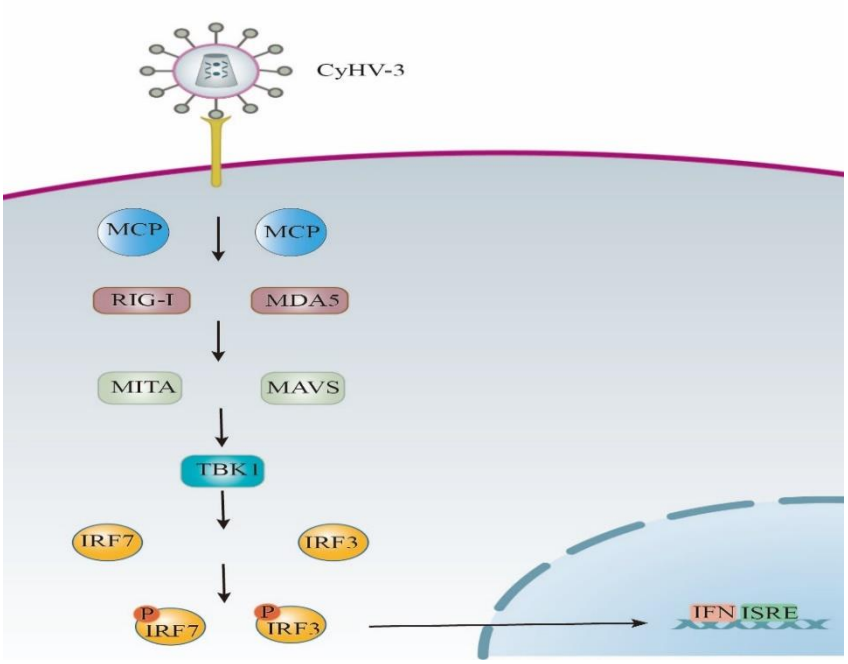

Figure 6: A model depicting the regulatory role of CyHV-3 MCP in the IFN signaling pathway.

Conclusion

In summary, we have provided a comprehensive analysis of the characteristics of the CyHV-3 MCP and verified its ability to regulate IFN expression. This indicates that CyHV-3

MCP plays a crucial role as a functional protein in virus-host interactions and immune evasion. These findings offer valuable insights into the role of this protein in herpesvirus infections and lay the groundwork for the development of future anti-CyHV-3 drugs and diagnostic methods. Furthermore, these results are significant for the prevention and treatment of herpesvirus-associated diseases and provide useful references for future related studies.

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Conflicts of interest

The authors have declared that they have no conflict of interest.

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