Molecular Cloning of a Novel Chaperone-like Protein Induced by Rhabdovirus Infection with Sequence Similarity to the Bacterial Extracellular Solute-binding Protein Family 5*

Previously we demonstrated that a novel stress protein is induced in fish cells by the infection of a fish rhabdovirus (Cho W. J., Cha, S. J., Do, J. W., Choi, J. Y., Lee, J. Y., Jeong, C. S., Cho, K. J., Choi, W. S., Kang, H. S., Kim, H. D., and Park, J. W. (1997) Biochem. Biophys. Res. Commun. 233, 316–319). In this paper, we present the molecular cloning and characterization of a gene encoding this protein named virus-inducible stress protein (VISP). The VISP was purified partially by immunoprecipitation using a monoclonal antibody against the VISP and further purified by the electroelution from a SDS-PAGE gel. The protein was subjected to internal protein sequencing, and the sequence of three peptides was determined. Degenerate oligonucleotides based on the three sequences were used to screen a cDNA library from rhabdovirus-infected CHSE-214 fish cells, and a cDNA of a 2193 bp open reading frame encoding the VISP (VISP) was identified. Whereas the nucleotide sequence of VISP shows no similarity with other genes in the GenBank, the amino acid sequence of the VISP has similarity with the bacterial extracellular solute-binding protein family 5 (SBP_bac_5) that is proposed to have chaperone activity. Thus, we explored whether the VISP also had chaperone-like activity. Purified recombinant VISP expressed in Escherichia coli promoted the functional folding of α-glucosidase after urea denaturation and also prevented thermal aggregation of alcohol dehydrogenase. These results suggest that the VISP has amino acid sequence similarity with SBP_bac_5 and that it has chaperone activity that may play a role in virus infection.

Molecular chaperones are classes of polypeptide-binding proteins that are implicated in protein folding, protein targeting to membranes, protein renaturation, or degradation after stress and in the control of protein-protein interactions (2, 3). They also interact with a wide range of peptides generated by the degradation of polypeptides expressed by the cells, and the chaperone-one-peptide complex activates an immune response by activating antigen-presenting cells (4). The major classes of chaperones comprise heat shock protein (HSP) (60, HSP70, HSP90, and the small heat shock protein (5–7).

Molecular chaperones are induced during the replication of several viruses including cytomegalovirus (8), adenovirus (9, 10), rotavirus (11), and vaccinia virus (12). Although both mechanisms of stress protein induction during virus infection and the subsequent roles these proteins play during infection remain mostly unknown, some have speculated that the function of chaperone proteins expressed during virus infection is to facilitate the folding and/or assembly of viral proteins. Chaperone proteins associate transiently with nascent viral proteins, facilitating the correct folding of polypeptides of many viruses including influenza virus (13, 14), vesicular stomatitis virus (15–17), cytomegalovirus (18), rabies virus (19, 20), rotavirus (21), and human immunodeficiency virus (22, 23). Chaperone proteins facilitate the formation of a ribonucleoprotein complex between the viral reverse transcriptase and an RNA ligand of hepadnavirus, activate reverse transcriptase activity (24, 25), interact with herpes simplex virus type-1 initiator protein, and increase the initiation of viral origin-dependent DNA replication (26). The function of the chaperone proteins is found to be important for the growth of adenovirus (10), vaccinia virus (27), and papillomavirus (28).

We previously reported that a cellular protein named virus-inducible stress protein (VISP) is increased in fish cells by rhabdovirus infection (29). This protein is induced by various kinds of stresses including heat shock, heavy metal, and virus infection and distributed among various kinds of cells, which suggests that this protein has the characteristics of a stress protein, molecular chaperone. However, the molecular mass and the antigenic characteristics of this protein are different from those of other well known chaperone proteins (1). In the present study, the cDNA of this protein was cloned and analyzed to further define its characteristics. Oligonucleotide primers based on the partial peptide sequences of the purified VISP led to the isolation of a 2.7-kb cDNA. Its conceptual translational product of 730 amino acids shares sequence similarities with the bacterial extracellular solute-binding protein family 5 (SBP_bac_5). Members of SBP_bac_5 are involved in the transport of and chemotaxis toward substrates (30, 31). Based on the sequence similarities, they fall into at least eight families that generally correlate with

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Wha Ja Cho§§, Won Joon Yoon§§, Chang Hoon Moon‡, Seung Ju Cha‡, Heok Song‡, Hong Rae Cho†, Soo Jin Jang†, Dae Kyun Chung†, Choon Soo Jeong‡, and Jeong Woo Park‡**

From the †Department of Biological Sciences and Immunomodulation Research Center, University of Ulsan, Ulsan 680-749, Korea. ‡Department of Surgery, College of Medicine, Ulsan University Hospital, Ulsan 682-060, Korea, and the ¶Department of Genetic Engineering and RNA Inc., Kyung Hee University, Yongin-gun, Kyungki-do 449-701, Korea

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§ These authors contributed equally to this work.

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The abbreviations used are: HSP, heat shock protein; VISP, virus-inducible stress protein; SBP_bac_5, extracellular bacterial solute-binding protein family 5; ADH, alcohol dehydrogenase; HBV, infectious hematopoietic necrosis virus; mAb, monoclonal antibody; RACE, rapid amplification of cDNA ends.
the nature of the soluble bound. Among them, family 5 proteins are specific for dipeptides and oligopeptides (32). Members of SBP bac 5 are proposed to act in a manner similar to that of molecular chaperones and therefore increase the refolding of unfolded proteins (33, 34). In line with its likely role as a molecular chaperone, we now show that the recombinant VISP increases the refolding of unfolded proteins and protects proteins against thermal denaturation. Therefore, we propose that the VISP has sequence similarity with SBP bac 5 and acts as a molecular chaperone that may play a role in virus infection.

EXPERIMENTAL PROCEDURES

Cells and Viruses—CHSE-214 cells were grown in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum and antibiotics. A Korean isolate of fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV), IHNV-PRT (35), was used.

Immunoprecipitation—The CHSE-214 cells were harvested 24 h after IHNV-PRT infection. The cell lysates were prepared by using lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride) and were preadsorbed with 10 µl of protein G-agarose (50% suspension in lysis buffer) (Invitrogen) for 2 h at 4 °C. Preadsorbed supernatant (200 µl) was then mixed with 10 µl of monoclonal antibody (mAb) AB7 raised against the VISP (29) or normal mouse serum and incubated overnight at 4 °C. The immunoprecipitates were harvested by centrifugation after incubation with 10 µl of protein G-agarose suspension and analyzed by SDS-PAGE.

SDS-PAGE and Western Blotting—The polypeptides were electrophoresed in a 10% separating gel under reducing conditions. The molecular masses were assessed in comparison with molecular mass standards (Bio-Rad). The proteins on acrylamide gels were transferred electrophoretically transferred to a polyvinylidene difluoride membrane (36), and then submitted for internal protein sequencing to the BioResource Center at Cornell University.

VISP cDNA Isolation, Sequencing, and Characterization—Degenerate oligonucleotides were designed based on the amino acid sequence of the three peptides and were synthesized. Then PCR was carried out using as a template cDNA from IHNV-infected CHSE-214 cells (see Table I). Primer sets 5’-7R, 5’-9R, 7F-5R, 7F-9R, 9F-5R, and 9F-7R were utilized for the gene amplification. The gene amplification reaction conditions were as follows: 1 cycle of 94 °C for 5 min; 12 cycles of 92 °C for 30 s, 50 °C for 1 min each and 72 °C for 1 min each; and 1 cycle of 72 °C for 5 min. A unique 186-bp product was isolated from the gene amplification reaction using 9F-3R primer set, cloned into pGEM-T vector (Promega), and sequenced using the chain-terminating, dideoxy method. Conceptual translation of the primary sequence verified that this amplified product corresponded to the VISP. The 186-bp product was labeled with [32P]dCTP by random prime reactions (Stratagene) and was used to screen the cDNA library. Positive clones were plaque-purified, and the clone containing the largest insert was chosen for further characterization. The cDNA insert was excised into pBlueScript (Stratagene), and the sequencing was performed at the Basic Science Research Center (Daejon, Korea) on an automatic DNA sequencer (Applied Biosystems, Inc.) according to the dye terminator procedure (37). The sequence was compared with the protein sequence of the homologous protein and used in the BLAST search Center (Daejon, Korea) on an automatic DNA sequencer.

Table I

Amino acid sequence of internal peptides from the VISP and sequence of degenerate oligonucleotides used in gene amplification

| Peptide | Amino acid sequence | Primer<sup>a</sup> | Degenerate oligonucleotides<sup>b</sup> |
|---------|---------------------|---------------------|---------------------------------|
| VISP-5  | V6TNDVVDNAVnQ       | VISP-5F             | VISP-5F: ACGAYGGAGCNYYGAGYAAY |
| VISP-7  | ITFVvNEAKYERAGK     | VISP-5R             | VISP-5R: RTTTRCNCNCGTTRCTTTG |
| VISP-9  | RGDGAKAVFAPLSP      | VISP-7F             | VISP-7F: GARGCNAARTAYGARGCNNG |
|         |                     | VISP-7R             | VISP-7R: CCCGCGTCTGAYGCTTNC |
|         |                     | VISP-9R             | VISP-9R: GAYCNSGGNATCMTTNYGNC |

<sup>a</sup> Amino acid sequences are listed in the standard single-letter code. Lowercase letters indicate ambiguous amino acids in VISP-5 and VISP-7. Underlined letters indicate amino acids that were used in the design of the degenerate oligonucleotides.

<sup>b</sup> The number refers to the peptide, ‘F’ refers to forward primers, and ‘R’ refers to reverse direction primers.

<sup>c</sup> Nucleotides are denoted with standard single-letter designations and written in 5’ to 3’ format. Degeneracies in the sequence are denoted as ‘Y’ for G or C, ‘R’ for A or G, and ‘N’ for A, G, T, or C.
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were transferred to nylon membranes (Amersham Biosciences, Hybond-N). Hybridization was done with 

Purification of Recombinant VISP—For the expression of the VISP in E. coli, a plasmid (pET-VISP) was constructed by inserting the full-length cDNA corresponding to the VISP into the Ncol-XhoI site of a pET29b in the correct reading frame. E. coli BL21 (DE3) cells were transformed with pET-VISP. VISP was expressed as the His-tagged protein in E. coli and purified on nickel-nitriotriacetic acid His-bind resin, according to the manufacturer's instructions (Novagen).

Refolding of α-Glucosidase—a Glucosidase (Sigma) was denatured at a concentration of 15 μM in 8 M urea, 0.1 M potassium phosphate, 2 mM EDTA, 20 μL dithiothreitol, pH 7.0, at 20°C. Renaturation was initiated by a 50-fold dilution in 40 μL Hepes-KOH, pH 7.5, at 20°C, in the absence of any additional protein or in the presence of various concentrations of VISP, 5 μM DnaK (StressGen), 20 μM bovine serum albumin (Sigma). The enzymatic activity of α-glucosidase was measured as described by Jakob et al. (37).

Multiple Sequence Alignment—Protein data base searches were performed with the National Center for Biotechnology BLAST Network service. The comparison and alignment of the VISP translation product were performed using Clustal W sequence alignment.
RESULTS

Purification and Peptide Sequencing of the VISP—We have shown previously that the expression of a VISP is induced in fish cells by IHNV infection (1, 29). To further characterize this protein, strategies were developed to isolate and purify the protein using IHNV-infected CHSE-214 cells as the starting material. Immunoprecipitation was used to isolate the VISP from the cell lysate of IHNV-infected cells, and this produced a highly purified VISP (Fig. 1A). The protein was confirmed to correspond to the VISP by Western blotting analysis using mAb AB7 against this protein (Fig. 1B). The proteins were further purified by electrophoresis from the SDS-PAGE gel and transferred to polyvinylidene difluoride membrane, and the band was excised and submitted for N-terminal protein sequencing. Alternatively, the protein purified by electrophoresis was subjected to Arg-C digestion and transferred to polyvinylidene difluoride membrane, and several major bands were excised and submitted for internal protein sequencing analysis. N-terminal protein sequencing revealed a blocked N terminus. Analysis of the Arg-C digestion product resulted in the determination of the primary sequences of three peptides, VISp-5, VISp-7, and VISp-9 (Table I).

The VISp cDNA Isolation and Cloning—Degenerate oligonucleotides were designed based on the standard triplicate code using the amino acid sequences of the peptides VISp-5,
VISP-7, and VISP-9 (Table I). These oligonucleotides were used for a gene amplification of the IHNV-infected CHSE-214 cell cDNA library. Amplification of the cDNA with primer sets 9F-5R resulted in a unique 186-bp DNA fragment. When this fragment was sequenced and the conceptual translation product was examined, the amino acids from peptides VISP-9 and VISP-5 were revealed, including amino acids that were not utilized in the design of the oligonucleotides (Fig. 2). These results confirmed that this fragment corresponded to a cDNA with sequence homology to the VISP gene. The 186-bp DNA fragment was labeled and used as a probe to screen the IHNV-infected CHSE-214 cell cDNA library. Approximately 200,000 plaques were screened in duplicate, and 20 positive colonies were identified. Ten plaques were subjected to secondary screening, and two of them were purified by tertiary screening. The cDNA inserts were excised into pBlueScript and sequenced completely in both directions. They were found to contain a 1659-bp fragment corresponding to nucleotides 1086–2744 (Fig. 3). The 5′-end of this phage clone lacked an ATG initiation codon and, therefore, represented

FIG. 6. Purification of recombinant VISP. Full-length VISP cDNA generated by gene amplification was cloned into a pET21a vector. E. coli cells were harvested at 5 h post-induction, and His<sub>6</sub>-tagged VISP was purified using nickel-nitrilotriacetic acid His-bind resin under denaturing conditions. A, 10% SDS-PAGE stained with Coomassie Blue; B, Western blotting using monoclonal antibody. Lane 1, purified recombinant protein using nickel-nitrilotriacetic acid His-bind resin; lane 2, purified VISP from CHSE-214 cells by immunoprecipitation. The migration positions for molecular mass markers are indicated on the left. H and L, H and L chains of monoclonal antibody.

FIG. 7. Influence of VISP on the refolding of urea-denatured α-glucosidase. A, kinetics of refolding. α-Glucosidase was denatured in urea and subsequently renatured by dilution of the denaturant at a concentration of 0.3 μM in the absence of additional protein (○) or in the presence of 1 μM VISP (■), 5 μM DnaK (□), or 20 μM bovine serum albumin (●). B, dependence of α-glucosidase refolding on VISP concentration. α-Glucosidase was denatured in urea and subsequently renatured for 20 min by dilution of the denaturant in the presence of VISP at the indicated concentrations.
an incomplete cDNA. To isolate a full-length cDNA clone for the VISP, 5′ RACE was performed on RNA isolated from IHNV-infected CHSE-214 cells. This procedure yielded nucleotides 1–1085 in the composite sequence and included the ATG start codon. The 2193-bp open reading frame (nucleotides 273–2465) encodes a conceptual translation product of 730 amino acids with a calculated molecular mass of 79.84 kDa. The sequences of all three peptides determined by protein sequencing were identified in the conceptual translation product.

**Induction of the VISP Transcription by IHNV Infection**—To determine whether the cloned gene showed an expression pattern matching that previously determined for virus stimulation of the VISP synthesis (1), the amount of mRNA was measured at various times after the virus infection by Northern blotting of RNA (Fig. 4). In mock-infected normal cells, there was no increase in the level of the transcription of the VISP throughout the incubation period of 48 h. However, in IHNV-infected cells, the transcript increased prominently at 24 h post-infection. This pattern of hybridization indicates that the VISP gene expression is induced by virus infection as had been shown previously by analysis of the protein synthesis in IHNV-infected cells.

**VISP Amino Acid Sequence Analysis**—Comparison of the nucleotide sequence of the VISP with those in the GenBank™ data base revealed no similarity with other genes. However, a conserved domain search using reverse position-specific BLAST identified domains of SBP_bac_5 in two regions, Glu5 to Leu257 and Val215 to Asp561. The amino acid sequence of these two regions exhibited similarity with members of SBP_bac_5, including oligopeptide-binding protein (OPPA), dipeptide-binding protein (DPPE), periplasmic dipeptide transport protein (DPPA), oligopeptide-binding protein (APPA), and peptide transport periplasmic protein (SAPA). The region Val215 to Asp561 of VISP exhibits 16–21% identity with members of the SBP_bac_5 and is most similar (21% identity) to the OPPA of *Thermoanaerobacter tengcongensis* (38) (Fig. 5). Although the VISP within these two regions exhibits similarity with members of SBP_bac_5, the VISP outside these regions exhibits little similarity with any other genes in the GenBank™.

**VISP Increases the Amount of Correctly Folded α-Glucosidase**—Our previous results suggest that the VISP has characteristics of a stress protein (1). In addition, the amino acid sequence of this protein shows similarity with OPPA and DPPE, members of SBP_bac_5 proposed to have chaperone activity (33, 34). Thus, we investigated whether the VISP acts as a molecular chaperone in the folding of proteins. Overexpressed recombinant VISP was purified from *E. coli* BL21 as a His-tagged protein. This recombinant protein was recognized by mAb AB7 raised against the VISP (29), but its band was a little shifted up because of the His tag (Fig. 6). α-Glucosidase, whose refolding is facilitated by several chaperones, such as GroEL, HSP90, and small HSPs (37, 39, 40), was chosen as substrate for this reaction. It was unfolded in the presence of 8 M urea and allowed to refold upon the dilution of the denaturant in the absence of added protein or in the presence of the VISP (protein folding in the presence of DnaK and bovine serum albumin was comparatively studied). Under our experimental conditions, the refolding yield of 0.3 M α-glucosidase was increased from 4% in the absence of added proteins to 48% in the presence of 1 μM VISP and to 16% in the presence of 5 μM DnaK. The addition of 20 μM bovine serum albumin did not affect the refolding of α-glucosidase (Fig. 7A). The dependence of α-glucosidase reactivation on the concentrations of added VISP is shown in Fig. 7B. The maximal recovery of enzyme activity attains 52% in the presence of 3 μM VISP, and half-maximal reactivation occurs at 0.5 μM.

**VISP Protects the Thermally Induced Aggregation of ADH**—The chaperone-like activity of the VISP was also determined by its ability to prevent the thermally induced aggregation of ADH at 37 °C. ADH loses its native conformation and undergoes aggregation during incubation at 37 °C. Fig. 8 shows the kinetic traces of the apparent absorbance at 360 nm of this protein in the absence and presence of VISP. In the absence of VISP, the 5 μM ADH undergoes aggregation. However, in the presence of VISP, aggregation was suppressed. Partial protection, ~56%, was found in the presence of 1 μM VISP, and 81% protection occurred in the presence of 2 μM VISP. These results suggest that, like molecular chaperones, the VISP interacts with unfolded proteins and increases their productive folding.

**DISCUSSION**

The purpose of this study was to investigate the nature of the VISP that was identified previously as a cellular protein induced by rhabdovirus infection (1, 29). The current study presents the cloning and initial characterization of the VISP. Using the information from the amino acid sequence, we have cloned a cDNA that corresponds to the VISP gene. The evidence supporting this conclusion includes an analysis of the primary sequence of the isolated protein and conceptual translation product, gene expression profiles, and an expression of the recombinant protein. The 730-amino acid open reading frame of the cloned cDNA has the sequences of all three peptides that were determined in the analysis of the isolated protein. These sequences include one peptide not utilized for the library screening. The expression pattern of the VISP gene as detected by cDNA hybridization to mRNA is consistent with the expression profile of the VISP established using Western blotting assays (1). The recombinant protein expressed in *E. coli* has the same size as the VISP in cells and is recognized by the mAb raised against the VISP.

Molecular blast analysis of the VISP revealed no nucleotide sequence similarity with other known genes in the GenBank™ as well as chaperone proteins, suggesting that the VISP is a novel protein. However, its amino acid sequence showed similarity with those of the members of SBP_bac_5. SBP_bac_5 binds solutes, such as dipeptides and oligopeptides, and it translocates the solutes by an interaction with the external...
sites of the integral membrane proteins (7, 11). Until now, among the sequenced eukaryotic proteins, nothing is homologous to the SBP_bac_5. Thus, the VISP is the first eukaryotic protein that has sequence similarity with the SBP_bac_5. Besides its role in transport, SBP_bac_5 has chaperone-like activity (33, 34). The fact that the VISP has characteristics of a stress protein (1) and has an amino acid sequence similarity with SBP_bac_5 prompted us to check whether this VISP also possesses chaperone properties. We now present biochemical evidence suggesting that the VISP has a chaperone-like function in protein folding and protection against thermal aggregation. The VISP effectively increases the yield of active α-glucosidase renaturation at a lower concentration than molecular chaperone DnaK does. Half-maximal reactivation of α-glucosidase occurs in the presence of 0.5 μM VISP, a concentration lower than the concentration of DnaK (1 μM) required for half-maximal reactivation of α-glucosidase in similar conditions (33). The VISP also protects ADH from thermal aggregation, as demonstrated by its 81% suppression of the thermal aggregation of ADH at a molar ratio of 1:0.4 for ADH:VISP. We have thus identified the VISP, which is shown by rhabdovirus infection to have chaperone-like functions.

The precise function of the VISP in rhabdovirus infection remains unclear. However, the chaperone-like activities afforded by this protein suggest that the VISP, like the other chaperone proteins, might facilitate the correct folding and/or assembly of viral proteins. It has been shown that chaperone proteins are induced by virus infection (8–12) and that they are necessary for efficient folding, for the assembly of viral peptides (13–26), and for viral growth (10, 27, 28). Even though we do not have evidence that the VISP is essential for the growth of rhabdovirus, we have proved that the VISP is induced by rhabdovirus infection and bound to the vishovirus virion (29). This supports the possible chaperone function of the VISP in the folding and/or assembly of rhabdovirus. It is, however, not clear how important the chaperone activity of the VISP is in the folding and assembly of rhabdovirus in vitro. In an eukaryotic cell there are many kinds of proteins that possess chaperone activity, including protein-disulfide isomerases (41), tubulin (42), α-hemoglobin stabilizing protein (43), αβ-crystallin (44), as well as HSP60, HSP70, HSP90, and small heat shock proteins. Thus, the VISP may not be the sole cellular protein but one of the chaperone proteins involved in the folding and assembly of viral proteins. We are currently undertaking the question of whether rhabdovirus replication is affected by the absence of the VISP.

Even though the VISP is induced by rhabdovirus infection and possesses chaperone activity, the main role of the VISP in virus infection may not be in assisting the folding of viral proteins. In the case of vaccinia virus, whereas HSP70 is dramatically induced by vaccinia virus infection, vaccinia virus replication proceeds normally in the absence of this protein (45). Another possible function of the VISP is that it may play some role in the host defense against viral infection by binding degraded peptides of viral proteins. This is based on the fact that the VISP has amino acid sequence similarity with SBP_bac_5, including the dipetide-binding protein and the oligopeptide peptide-binding protein. Members of SBP_bac_5 interact with and deliver their ligands to other membrane proteins (46, 47). A similar function was found in eukaryotic chaperone proteins. Chaperone proteins bind degraded peptides and facilitate the loading of the peptides onto MHC class I (48–50). In addition, the chaperone-peptide complex binds to the CD91 receptor on the antigen-presenting cell and stimulates the antigen-presenting cell, CD8+ T cells, and CD4+ T cells (reviewed in Ref. 4). Previously, we reported that fish produce antibodies against VISP after virus infection and that these antibodies possess neutralizing activity against rhabdovirus infection (29). Thus, it is possible that VISP binds viral peptides degraded by the ubiquitin-proteasome system and that this VISP-viral peptide complex may stimulate host immunity against a virus either directly or indirectly. The function of this VISP does not seem to be specific to virus infection because, even though it is very low, this protein is present in the absence of specific inducers, and this protein is also induced by stresses other than virus infection, including heat shock and heavy metals (1).

In summary, we have cloned cDNA coding a novel chaperone-like protein whose expression is induced by rhabdovirus infection and whose amino acid sequence shares similarity with that of SBP_bac_5. The information, which suggests a possible role of VISP in facilitating the folding of viral protein or in the host defense against viral infection, may be significant in understanding the multiplication of a virus or the host defense mechanism against a viral infection.

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