Identification and Characterization of Human Cytomegalovirus-Encoded Proteins Interacting with Viperin

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Identification and Characterization of Human Cytomegalovirus-Encoded Proteins Interacting with Viperin

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Sincerely,

Hyejin Jeon

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Identification and Characterization of

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(Directed by Professor Jun-Young Seo)

Human cytomegalovirus (HCMV) is a member of beta-herpesvirus family. Infection with HCMV causes acute and chronic diseases in both healthy and immunocompromised hosts. Host employs various defense mechanisms against viral infection. The interferon (IFN) response is the first line of host defense. IFN induces expression of a number of interferon-stimulated genes (ISGs). Viperin is a multifunctional, IFN-inducible protein. During HCMV infection, the function of viperin depends upon when it expresses and where it locates. When viperin is pre-expressed in cells, it localizes to the ER and inhibits viral replication. However, if it is induced directly by HCMV infection, it localizes to the mitochondria through interaction with HCMV viral protein vMIA at the early stage of infection and modulates the cellular metabolism to enhance viral

replication. Viperin finally re-localizes to the viral assembly compartment (AC) at the late stage of infection, but its role in this compartment has not been elucidated yet. Here, I hypothesized that viperin's interaction with HCMV viral proteins at each compartment may play a critical role in determination of viral replication status. In this study, I screened HCMV-encoded proteins interacting with viperin using yeast two hybrid assay. As a result, I identified 10 interacting HCMV protein candidates. An HCMV UL99-encoded tegument protein, pp28, was selected to study its capacity to interact with viperin. The pp28 of HCMV is an essential protein for viral assembly and maturation at the AC. The interaction and co-localization of pp28 and viperin were examined in both infected and transiently expressed cells by performing co-immunoprecipitaion, immunofluorescence, and protein-fragment complementation assays. Viperin was demonstrated to interact as well as co-localize with pp28 to the AC late in HCMV infection. Interestingly, viperin was translocated from the ER to the ERGIC when pp28 was transiently expressed in the absence of viral infection. This study suggests that the pp28 is likely responsible for the translocation of viperin to the AC. Further investigation is required to examine viperin's function on viral assembly process via interaction with pp28 at the AC during HCMV infection.

Key words: Viperin, HCMV, pp28, yeast two hybrid assay, protein-protein interaction, ERGIC, viral assembly compartment

Identification and Characterization of Human Cytomegalovirus-Encoded Proteins Interacting with Viperin

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I. INTRODUCTION

Human cytomegalovirus (HCMV) is a member of the family of herpesviruses and an important human pathogen associated with acute and chronic diseases in both healthy and immunocompromised populations. This virus is widely dispersed in more than 60% of the world population.[1] Although HCMV is usually asymptomatic in immunocompetent population, it causes severe diseases such as allograft rejection, microcephaly and cytomegalic inclusion in immunocompromised population. It has been proposed that HCMV is also associated with chronic diseases including atherosclerotic coronary artery disease, gastric ulcer disease, rheumatologic disease and malignancy.[2]

HCMV is the largest and most complex human herpesvirus. The virion consists of a nucleocapsid, containing a 230-kbp double stranded linear DNA genome, an envelope and a tegument layer in-between.[2] HCMV is also a

prolonged virus. It takes 48-72 hr to complete viral replication. HCMV penetrates host cells by fusion of viral envelope and cellular plasma membrane.[3] It is believed that interactions between several viral glycoproteins and host cellular receptors are involved in the viral penetration process. After the penetration, the deenveloped nucleocapsid and tegument proteins translocate into the nucleus where viral genome replication and transcription occur.[4] HCMV genome expression is sequentially regulated by three categories of viral proteins, immediate early (IE), early (E), and late (L) proteins.[4] The IE gene transcription occurs without de novo protein synthesis. The IE proteins regulate expression of E and L genes. The E proteins play roles in viral DNA replication and virion maturation. Transcription of L gene is stimulated by the E proteins. Leaky and true late gene transcriptions occur at 24-36 and 24-48 hr post infection, respectively. The L proteins contribute to virion maturation and morphogenesis.[2] Viral DNA is replicated and packaged into the newly assembled capsid. The nucleocapsid buds into the cytoplasm where it is associated with many tegument proteins.[5] The tegument-coated particles traffic to the cytoplasmic assembly compartment (AC), a modified compartment of the cellular secretory system where the final envelope is acquired and virons are maturated.[6] Viral structural proteins such as tegument proteins including pp150 and pp28 and envelope glycoproteins accumulate in this compartment.[2,7] Maturated virions egress to finish a replication cycle and release from the cell through exocytosis or cell lysis.[2]

Viral infection induces a variety of host immune responses. The interferon (IFN) response is the first line of defense against viral infection. Production of IFNs stimulates expression of a broad spectrum of antiviral proteins including protein kinase R (PKR), the GTPase Mx1 (myxovirus resistance 1), ribonuclease L (RNaseL), ISG15 (IFN-stimulated protein of 15 kDa), and IFIT (IFN-induced proteins with tetratricopeptide repeats).[8] Although several IFN-inducible proteins have been known as antiviral proteins, the functions of most IFN-inducible proteins remain unexplored.

Viperin (virus inhibitory protein, endoplasmic reticulum [ER]-associated, interferon-inducible) is an IFN-inducible host cellular protein. It is also known as RSAD2 (radical S-adenosyl methionine domain-containing protein 2). Viperin was identified from primary macrophages treated with IFN-gamma.[9] It was identical to cig5 (cytomegalovirus inducible gene 5) induced in fibroblasts infected with HCMV. Viperin is highly conserved in evolution. A homology called vig1 (hemorrhagic septicemia virus [VHSV]-induced gene 1) was induced in fish, and another called BEST5 (bone-expressed sequence tag 5) was expressed in rat. [9,10]

Viperin consists of 361 amino acids with a molecular mass of approximately 42 kDa.[10] It is composed of three domains, N-terminal, central, and C-terminal domains. The N-terminal domain has variability of sequences and length between species. This domain includes amphipathic alpha helix structure which is responsible for association with cytosolic face of ER and the membrane of

lipid droplets.[11,12] The central domain contains a conserved CxxxCxxC motif that is responsible for interaction with iron-sulfur clusters.[13,14] This motif classifies viperin as a radical *S*-adenosyl-L-methionine (SAM) enzyme. It has been also appeared that this motif is required for its function during virus infection.[15,16] The C-terminal domain is highly conserved and might be related to protein-protein interaction,[15] although a specific role of this domain is yet to be known.[10]

Viperin is induced in cells treated with type I, II, and III IFNs, double-stranded (ds) B-form DNA, lipopolysaccharides (LPS), and polyinosinic:polycytidylic acid (poly I:C) or infected with virus. IFN-gamma highly induces viperin in primary macrophages, but IFN-alpha and -beta induce viperin more effectively in the majority of cell types.[9] Viperin induction occurs by both classical IFN-mediated pathway and IFN-independent pathway. B-form dsDNA, LPS, poly I:C, and some viruses such as sendai virus and sindbis virus induce viperin by IFN-stimulated gene induction pathway. However, other viruses such as HCMV and vesicular stomatitis virus induce viperin directly by the IFN-independent pathway.[10]

Viperin is a multifunctional protein. It functions as an anti-viral protein and a regulator of cell signaling pathways or cellular metabolism. Viperin can inhibit a broad spectrum of DNA and RNA viruses, including HCMV, influenza A virus, hepatitis C virus (HCV), alphaviruses, and human immunodeficiency virus.[17] Pre-expression of viperin in human fibroblasts inhibits the replication of

HCMV significantly.[9] In addition, over-expression of viperin alters lipid raft microdomains on plasma membrane and inhibits budding and release of influenza A virus.[18] During HCV infection, viperin localizes to lipid droplets known as HCV replication sites where it suppresses viral replication.[19] Viperin can also regulate Toll-like receptor (TLR) 7 and 9-mediated type I IFN production in plasmacytoid dendritic cells.[20] Interestingly, viperin modulates cellular energy and lipid metabolism when it is induced directly by HCMV, resulting in the enhancement of viral infectivity.[21] These studies suggest that the differential functions of viperin depend upon the time-point and subcellular localization of its expression.

During HCMV infection, viperin localizes to three distinct cellular compartments, ER, mitochondria, and the viral assembly compartment (AC).[16] When viperin is expressed prior to HCMV infection, it localizes to ER where it inhibits viral replication possibly by blocking the secretion of soluble viral proteins including gB essential for virion maturation and assembly.[9] However, if viperin expression is induced directly by HCMV, it interacts with vMIA and translocates from ER to mitochondria. In mitochondria, viperin associates with mitochondrial trifunctional protein (TFP) which catalyzes fatty acid beta-oxidation to generate ATP. This interaction reduces the activity of TFP and thus decreases the levels of cellular ATP, resulting in disruption of actin cytoskeleton and modulation of cellular metabolism to increase lipogenesis and lipid synthesis that are needed for formation of viral envelope and enhancement

of viral replication.[16,21] Late in HCMV infection, viperin is relocalized to the AC where its function still awaits to be elucidated.

Viperin can interact with many HCMV-encoded proteins including vMIA which determines viperin's function at different subcellular compartments during viral infection. In this study, I screened for HCMV proteins interacting with viperin using yeast two hybrid assay and identified 10 viral proteins. Among them, I selected a viral tegument protein pp28, the strongest interacting partner of viperin from the assay for further investigation. The interaction could suggest a clue to elucidate the function of viperin at the AC because pp28 protein, localized to the AC late in infection, is essential for viral assembly and replication.

pp28 is a true late tegument protein encoded by HCMV UL99 open reading frame (ORF). It is a myristoylated and phosphorylated 190 amino acid (aa) protein with an estimated molecular mass of 28 kDa.[7] Since a mutant virus deleted of this UL99 ORF impairs the production of enveloped and infectious virus particles, pp28 is considered as an essential protein for viral assembly and replication.[22] The myristoylation at aa residue 2 of pp28 is important for the production of infectious virus and the phosphorylation of pp28 is required for its stability.[7] When pp28 is transiently expressed in the absence of viral infection, it localizes to the ERGIC (endoplasmic reticulum-Golgi intermediate compartment) which mediates the trafficking between the ER and Golgi complex.[23] This protein is localized to the AC during HCMV infection. A stretch

of acidic aa (aa residues 44-59) of pp28 is required for its localization to the AC.[24] pp28 proteins are multimerized through self-interaction within the AC during HCMV infection and the multimerization of pp28 is essential for the envelopment and production of infectious virus.[25] It has been also reported that a domain of pp28 (aa residues 37-39) is necessary for interaction with HCMV UL94 protein and their trafficking to the AC. The association of pp28 and HCMV UL94 proteins is required for their trafficking to AC during HCMV infection.[26]

Next, I investigated the capacity of viperin to interact with pp28 in the presence or absence of HCMV infection using co-immunoprecipitation, immunofluorescence, and protein-fragment complementation assays. Viperin was demonstrated to interact with pp28 and translocate to the ERGIC when co-expressed with pp28 transiently in the absence of viral infection. Viperin was also able to interact with pp28 and co-localize to the AC late in HCMV infection. The results in this study indicated that pp28 might be responsible for localization of viperin to the AC during HCMV infection. Further study is required to investigate the role of viperin in virus assembly through the interaction with pp28 in the AC.

II. MATERIALS AND METHODS

1. Yeast two hybrid assay

Saccharomyces cerevisiae strain AH109, Y187, and HCMV ORF libraries were kindly provided by Dr. Jin Hyun Ahn (Sungkunkwan University, Korea).

Human viperin was transferred to pGBKT7 (bait) vector. Each HCMV ORF was transferred to pACT2 (prey) vector.

Each bait and prey construct was introduced into strain AH109 or Y187, respectively. Transformants were selected on minimal synthetic agar medium containing dropout supplements lacking tryptophan or leucine (Clontech, California, USA) and protein expression of bait was confirmed with western blot.

Prior to screening, the bait protein was tested for auto-activation on plates containing 3-amino-1,2,4-triazole (3AT) to check the basal levels of HIS3 activation. No auto-activation was detected from the bait alone. All assays were performed on plates without 3AT.

The interactions between bait and pery were monitored using activation of histidine reporter gene. Bait and each prey were mated on YPD containing all amino acids for overnight at 30℃. Mated yeasts grew on leuicine and tryptophan double deficient plates (Leu^-/Trp^-) for three days at 30℃. The colonies grown on the Leu^-/Trp^- plates were picked, restreaked and selected on

synthetic complete agar medium containing dropout supplements lacking leucine, tryptophan, and histidine ($Leu^-/Trp^-/His^-$) (Clontech, California, USA). The plates were incubated for three to seven days at 30°C and interactions were monitored by formation of colonies. Three independent experiments were performed.

2. Cells, viruses and antibodies

HeLa, COS-7, and HEK-293T cells were used for experiments. Human foreskin fibroblast (HFF) (from Yale Skin Diseases Research Center) and telomerase-immortalized human fibroblast (HFtelo) (from Dr. T. Shenk, Princeton University) cells were also used for experiments.

HCMV strain AD169 (from Dr. W.J. Britt, University of Alabama at Birmingham) was used.

HCMV-encoded proteins were detected with monoclonal antibodies (mAb) as followed: pp28 (41-18), pp65 (28-19), and pp150 (36-14). Polyclonal rabbit Ab to viperin (aa residues 346-361) and mAb to viperin (MaP.VIP, aa residues 262-276) were also used. MAbs to Myc tag (9E10 and 4A6) were purchased from Santa cruz Biotechnology (Texas, USA) and Merk Milipore (Massachusetts, USA). The polyclonal rabbit Abs to HA tag (Y-11) and ERGIC-53 (H-245) were purchased from Santa Cruz Biotechnology. Rabbit Ab to calnexin was purchased from Abcam (Cambridge, UK). MAb to GAL4 (RK5C1) was purchased from Santa Cruz Biotechnology (Texas, USA).

3. Plasmids and transfection

Human viperin was cloned into pCDNA3.1-Myc-His or pCDNA3.1-HA vector. RPS14, UL29, vMIA, and UL148 were cloned into pCDNA.3.1-Myc-His vector. UL38 and IE2 were cloned into pDEST-cs3-MT vector. UL83 (pp65) and UL32 (pp150) were cloned into pCDNA3 vector. UL78 was cloned into pCMV-Myc vector. EGFP and IRF3 were cloned into pCDNA3.1-Myc-His and pCDNA3.1-HA vector, respectively. UL99 (pp28) was cloned intro both pCDNA3.1-Myc-His and pCDNA3.1-HA vectors. The plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, Massachusetts, USA) according to manufacturer's recommendations.

4. Co-immunoprecipitation

HEK-293T cells ($2x10^6$) were seeded on 60 mm dishes and co-transfected with the indicated plasmids. Cells were harvested at 24-30 hr after transfection, washed with PBS three times and lysed with lysis buffer (2% NP40 in TBS) for 1 hr. The cell lysates were precleared with normal mouse or rabbit serum protein G or A beads for 1 hr at 4℃. The precleared lysates were co-immunoprecipitated by incubation with specific antibodies and protein G or A beads for 1 hr at 4℃. Beads were rinsed with washing buffer (0.2% NP40 in TBS) for three times before precipitates were eluted by boiling with 35ul of 2x sample buffer for 5 min.

HFF cells ($5x10^6$) were seeded on 100 mm dishes and infected with

HCMV AD169 for 4 to 5 days. Cells were harvested at the indicated time and lysed. The cell lysates were immunoprecipitated as described above.

5. Immunofluorescence

HFtelo cells were grown on 13-mm-diameter coverslip in 24-well tissue culture plates. The cells were infected with HCMV strain AD169 at multiplicity of infection (MOI) of 0.2 for the indicated time. The coverslips were washed with PBS and fixed with 3% paraformaldehyde in PBS for 45 min at room temperature. The coverslips were washed with PBS and permeabilized with 0.1% Triton X-100 and 0.01% SDS in PBS for 5 min. Then, coverslips were blocked with 0.2% Tween in PBS containing 10% normal goat serum for 20 min at room temperature, followed by primary antibody incubation for 45 min at 37℃. Following washing with 0.2% Tween in PBS, coverslips were incubated with goat anti-mouse Ig secondary Ab diluted in 0.2% Tween in PBS containing 2.5% normal goat serum for 45 min at room temperature. After wash with 0.2% Tween in PBS and rinse with PBS, coverslips were mounted with ProLong Gold Antifade reagent (Molecular Probes, Oregon, USA) and their images were acquired with a Carl zeiss LSM700 confocal microscope.

HeLa and COS-7 cells grown on coverslip were transfected with the indicated plasmids. At 24 to 36 hr posttransfection, cells were fixed and stained with specific Abs as described above and their images were acquired with a Carl zeiss LSM700 confocal microscope (Oberkochen, Gerrnany).

6. Fluorescent Protein-fragment Complementation Assay (PCA)

pCDNA3.1/Zeo(+) vectors containing Venus YFP(Y1) and Venus YFP (Y2) (Dr. S.W. Michnick, University of Montreal, Canada) were used for the experiments. The indicated genes were cloned into these vectors.

HEK 293T cells ($1x10^6$) were seeded on 35 mm glass bottom plates for immunofluorescence and on 6 well plates for fluorometeric analysis and flow cytometry.

HEK 293T cells were transfected with the indicated plasmids. The fluorescence images were examined in living cells at 24-30 hr after transfection using a Carl zeiss LSM700 confocal microscope.

For fluorometeric analysis, the transfected cells were harvested and washed with PBS. Cells were pelleted by centrifugation and resuspended with 1ml of PBS. 100ul of cells were transferred to black 96-well microtiter plates and subjected to fluorometric analysis by using a Molecular Devices Flexstation 3 (Sunnyvale, CA, USA). Excitation and emission wavelengths were 485 and 535nm, respectively.

For flow cytometry (FACS), the cells were washed with FACS buffer buffer (0.5% FBS in PBS) twice. The samples were run on a Beckman coulter FACSVerse flow cytometer (BD Bioscience, San Diego, CA, USA) and analyzed using FlowJo software (Ashland, OR, USA).

III. RESULTS

1. Screening HCMV-encoded proteins interacting with viperin using yeast two hybrid assay

To screen HCMV viral proteins interacting with viperin, I used yeast two hybrid assay. For this assay, a GAL4 gene was split into two separate fragments, a DNA binding domain (DBD) and an activating domain (AD), and cloned into either bait or prey vector, respectively. Then respective bait and prey candidate genes were fused with DBD and AD. HIS3 reporter gene was used as a selection marker of interaction between bait and a prey **(Fig. 1A)**. Only if a bait protein interacts with a prey protein, the reporter gene, HIS3, is expressed and thus yeasts expressing the bait and prey proteins are selected from the histidine deficient media. To identify viperin's interacting partners amongst HCMV-encoded proteins human viperin was cloned into pGBKT7 bait vector where tryptophan is a selection marker. HCMV library containing 166 open reading frames (ORFs) was cloned into pACT2 prey vector where leucine is a selection marker **(Fig. 1B)**. Recombinant pGBKT7-viperin bait plasmid was transformed into yeast strain AH109 which has MATa allele. Each recombinant pACT2-HCMV ORF prey plasmid was transformed into yeast Y187 which has MATα allele. Viperin bait construct was confirmed by nucleotide sequencing, and the expression of recombinant fusion protein was verified by its antigenic specificities and relative molecular mass.

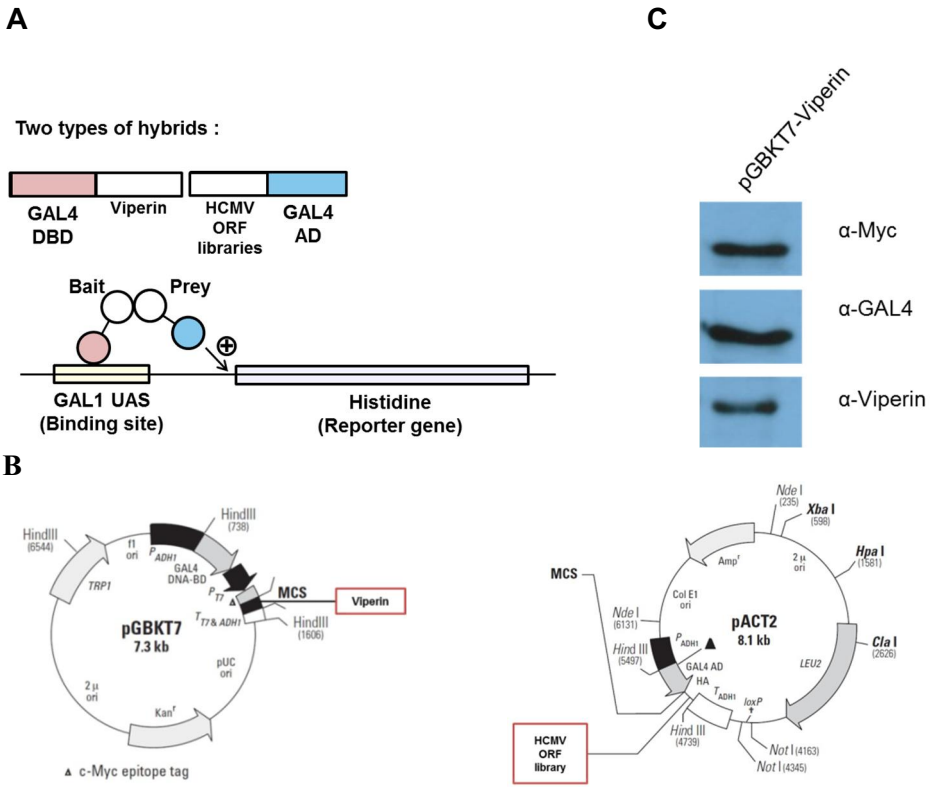


Figure 1. Generation of constructs for yeast two hybrid assay. (A) A schematic diagram of the principle of yeast two hybrid assay used to identify the interacting partners of viperin. Viperin as bait and HCMV ORF library as prey were used in the screen. Histidine reporter gene was used to select interaction of HCMV proteins and viperin. (B) Viperin was introduced into pGBKT7 vector which has GAL4 DNA binding domain and HCMV ORF library was introduced into pACT2 vector which has GAL4 activating domain. Images were adapted and modified from Clontech. (C) Yeast AH109 was transformed with viperin and the expression of protein was verified by western blot with the indicated antibodies.

by western blotting analysis **(Fig. 1C)**.

Using these transformed yeasts, I screened HCMV ORFs encoded viral proteins interacting with viperin. As summarized in Fig. 2A, yeasts transformed of bait were mated with yeasts transformed of each prey, i.e., HCMV ORF. The mated clones were then monitored and selected from triple deficient plates (Leu $^{-}$/ Trp $^{-}$/His^{-}) (Fig. 2B). To verify the selected clones and eliminate false positives, X-Gal filter assay was carried out with a lacZ reporter gene. Although the expression of lacZ reporter gene in AH109 strain is regulated by MEL1 UAS promoter, bait alone as a negative control showed a positive result upon X-Gal treatment, indicating that MEL1 UAS promoter is leaky. Therefore, I had to verify the positives by repeating three times the selection experiments on triple deficient plates. Finally, 10 HCMV-encoded proteins were identified as interacting partners of viperin **(Table 1)**. The interacting proteins were further classified into two groups, strong and weak hits based on the size and number of yeast colonies on triple deficient plates. When the number of colony is over 10 at least twice in three independent assays, the candidate was classified as a strong hit. Finally, 6 strong hits were selected for interacting partners of viperin **(Table 1)**.

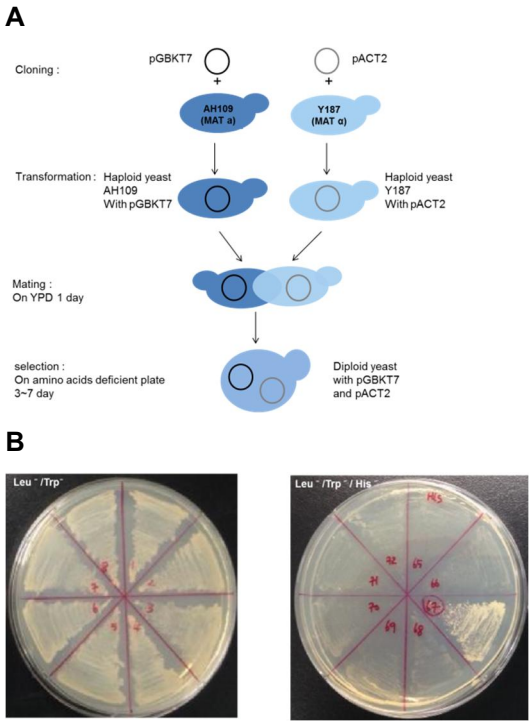


Figure 2. Screening HCMV ORFs encoded proteins interacting with viperin. (A) A schematic diagram of yeast two hybrid assay. The pGBKT7-viperin and pACT2-HCMV ORF library were transformed into yeast strain AH109 and Y187, respectively. Bait and preys were mated on YPD for overnight. The mated colonies were selected on leucine and tryptophan double deficient plates (Leu^-/Trp^-). The protein – protein interaction from the selected clones were mated on leucine, tryptophan, and histidine triple deficient plates ($Leu^-/Trp^-/His^-$). (B) All mated colonies grow on -Leu/-Trp plates (left). Only colonies containing interaction between viperin and HCMV protein grow on triple deficient plates (right). Representative pictures of three independent experiments were shown.

Table 1. Candidates of HCMV proteins interacting with viperin from yeast two hybrid assay

Bait	Hits
Viperin	**UL38**, **UL74**, **UL78**, **UL99**, **UL122**, **UL148**, UL13, UL29, UL40, UL112-113

Strong hits are written in bold.

2. Confirmation of HCMV proteins interacting with viperin

To confirm viperin interaction with HCMV-encoded proteins in mammalian cells, I performed co-immunoprecipitation assay (Co-IP). Viperin was cloned into pCDNA3.1 vector with HA tag. The construct was confirmed by nucleotide sequencing, and protein expression was confirmed by immunoblot analysis. HCMV ORFs including the interacting partners selected from yeast two hybrid assay were cloned into pDEST-cs3-MT vector with 6Myc tag, pCDNA3.1 vector with Myc tag or pCDNA vector. The plasmids containing viperin and the HCMV ORFs were co-transfected into HEK 293T cells. Cells were lysed and the cell lysates were immunoprecipitated with antibodies specific to Myc tag or HCMV proteins. Viperin bound to the viral proteins was accessed with viperin specific antibody by western blot. RPS14 (ribosomal protein subunit 14) was used as a negative control. Viperin was co-immunoprecipitated with vMIA (UL37), pUL38, pp28 (UL99), and pp150 (UL32) but not with pUL29 and pUL122 **(Fig. 3)**, indicating that there is a difference between yeast system and mammalian system. I could not confirm viperin interaction with pUL78 and pUL148 because of the low levels of expression of these proteins. Finally, 4 HCMV proteins, vMIA, pUL38, pp28, and pp150 were identified as interacting proteins of viperin in mammalian cells.

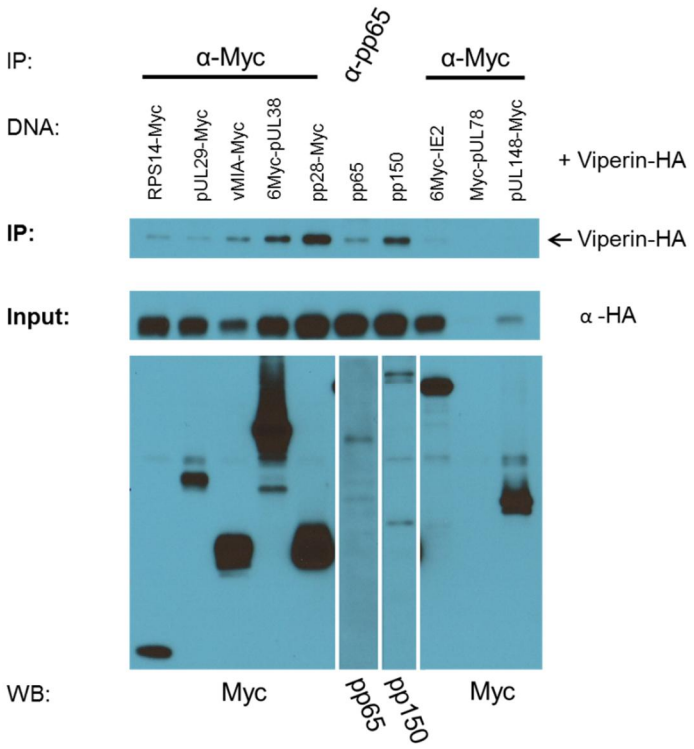


Figure 3. Confirmation of HCMV proteins interacting with viperin. HEK-293T cells were transiently co-transfected with viperin-HA and the indicated plasmids. Cells were lysed and immunoprecipitated with anti-Myc, anti-pp65 or anti-pp150 antibodies. Precipitates were probed by western blot using anti-HA antibody. RPS14 was used as a negative control. Note that HCMV vMIA, pUL38, pp28, and pp150 proteins show stronger interaction when compared to a negative control and other candidates.

3. Viperin interacts and co-localizes with pp28 during HCMV infection

It has been reported that viperin is localized to the assembly compartment (AC) at the late stage of HCMV infection. However, the function of viperin in this compartment has not been elucidated yet. Among 4 HCMV proteins interacting with viperin, pp28 showed the strongest interaction in both yeast two hybrid assay and Co-IP. pp28 has been known as an essential tegument protein for virus assembly and maturation in the AC **(Table 2)**. Hence, I hypothesized that pp28 interacts with viperin and translocates it to the AC during HCMV infection, suggesting possible roles of viperin in the AC.

To address my hypothesis, I initially investigated interaction between viperin and pp28 during HCMV infection using Co-IP assay. Since pp28 is a true late protein, HFtelo cells were infected with HCMV for 4 and 5 days and the expression of pp28 was confirmed by western blot. Cell lysates were immunoprecipitated with rabbit-anti-viperin antibody. Rabbit HA antibody was used as a negative control. pp28 was co-immunoprecipitated with viperin at both 4 and 5 days post infection (dpi) **(Fig. 4A)**, indicating that viperin indeed interacts with pp28 late in infection of HCMV. To examine their localization during infection, the intracellular distribution of pp28 and viperin was monitored by confocal microscopy at 4 and 5 dpi. Viperin was observed with pp28 at the AC late in HCMV infection, although viperin expression is decreased **(Fig. 4B)**, suggesting that pp28 interacts with viperin and transports it

Table 2. Functions and localization of HCMV viral proteins interacting with viperin

HCMV protein	Functions	Localization
pUL37	Antiapoptosis[27], Disruption of actin cytoskeleton[16]	Mitochondria[27]
pUL38	Antiapoptosis[28]	Nucleus, cytoplasm[28]
gO (pUL74)	Component of glycoprotein complex for viral penetration (cell - cell fusion)[29]	TGN, Golgi apparatus[29]
pp28 (pUL99)	Virion assembly and maturation[22]	AC[22]
IE1 (pUL122)	Regulation of viral and host gene expression[2]	Nucleus[30]
pUL148	Maturation of glycoprotein H and O[31]	ER[31]
pUL78	Chemokine receptor[32]	ER, TGN, EE[33]
pp150 (pUL32)	Reorganization of viral assembly compartment[34]	AC[35]

TGN, trans-Golgi network; AC, viral assembly compartment; ER, endoplasmic reticulum; EE, early endosome

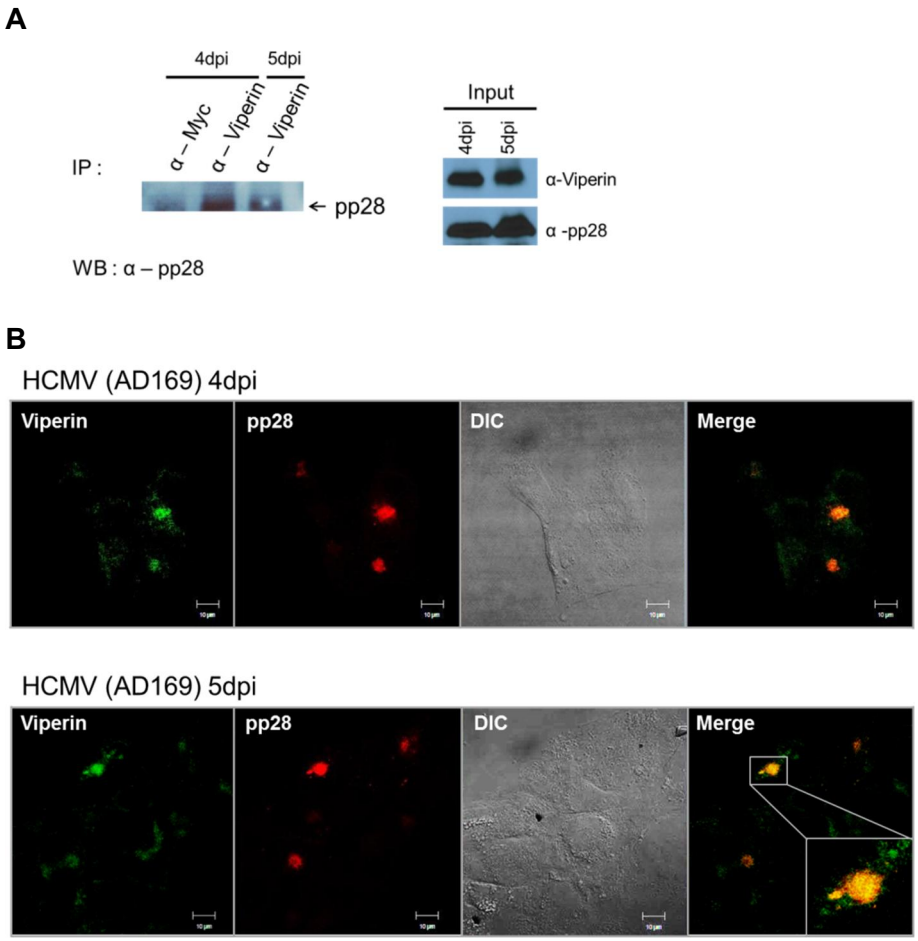


Figure 4. Viperin interacts and co-localizes with pp28 during HCMV infection. (A) HFtelo cells were infected with HCMV (AD169 strain) at a multiplicity of infection (MOI) of 0.2 for 4 and 5 days. Cells were lysed and immunoprecipitated with anti-Myc or anti-viperin antibody. Precipitates were probed by western blot using anti-pp28 antibody. Note that co-immunoprecipitated pp28 was detected at both 4 and 5dpi. (B) HFtelo cells were infected with HCMV at MOI of 0.2 for 4 and 5 days. Cells were fixed and stained with antibodies against pp28 and viperin. A representative image from

three independent experiments was shown. Note that viperin and HCMV pp28 co-localize to the viral assembly compartment at the late stage of infection. Scale bars, 10 um.

into the AC.

4. Viperin associates with pp28 in the absence of other viral proteins

To examine whether viperin interacts with pp28 in the absence of viral infection, Co-IP was performed in transiently expressed cells. It has been reported that pp28 fused with tag at the N-terminus impairs its authentic localization. I first generated a pp28 construct with HA tag at the C-terminus. Since the N-terminus of viperin has ER localization signal sequences, Myc tag was fused to the C-terminus **(Fig. 5A)**. The pp28-HA and viperin-Myc were co-transfected into HEK 293T cells. EGFP-Myc and IRF3-HA were used as negative controls. Cell lysates were immunoprecipitated with anti-HA or anti-Myc antibody and immunoblotted with anti-viperin or anti-HA antibody. Viperin showed specific binding with pp28 **(Fig. 5B, C)**. These results indicated that pp28 and viperin associates in the absence of other viral proteins.

5. The pp28 protein translocates viperin from the ER to the ERGIC

I next tested intracellular distribution of viperin and pp28 in transiently expressed cells. Both viperin and pp28 were transiently expressed in COS-7 cells and HeLa cells. Consistent with previously reported studies, viperin alone localized to the ER and lipid droplet. pp28 protein alone localized to the ERGIC. Interestingly, viperin and pp28 partially overlapped at the ERGIC when both proteins were co-expressed. It appeared that their interaction translocates viperin from the ER to the ERGIC **(Fig. 6A, B)**. In figure 6A,

viperin alone

seemed partially overlapped with an ERGIC marker. To clarify this, the images were analyzed in higher resolution and magnification **(Fig. 6C)**. It showed that viperin alone localizes to the ER but not the ERGIC. The results indicated that only when vipein is co-expressed with pp28, it co-localizes with pp28 to the ERGIC.

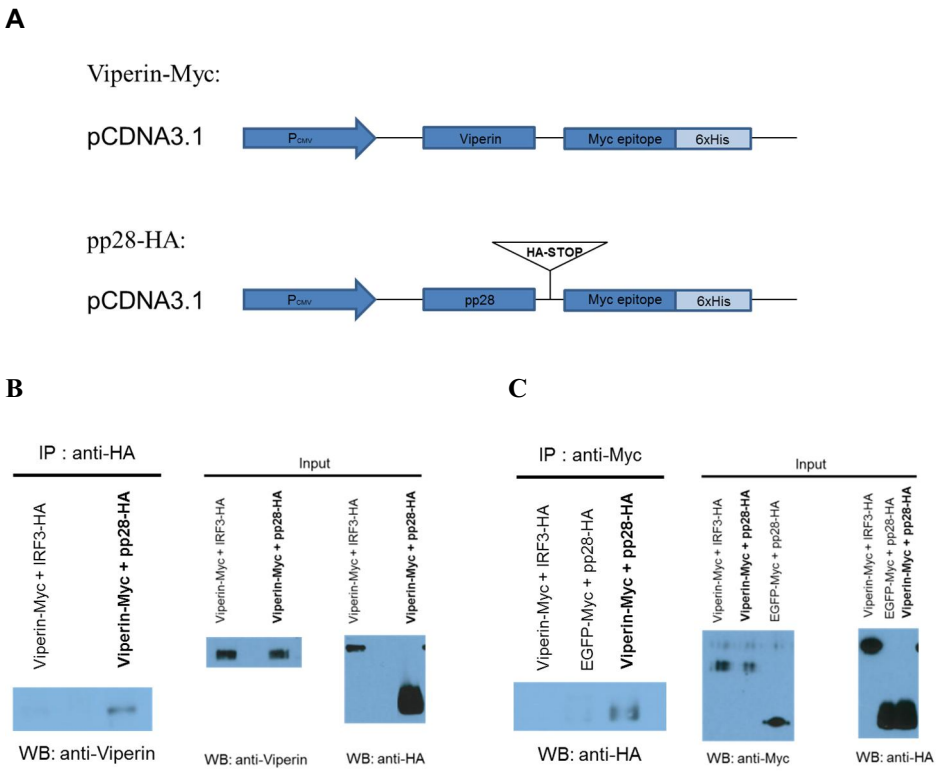
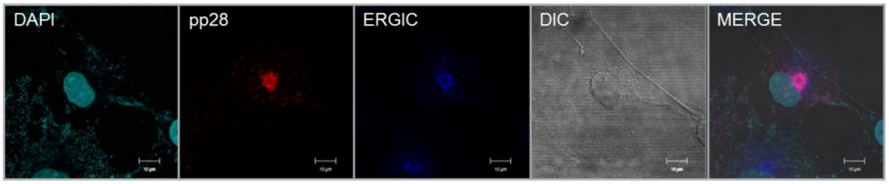


Figure 5. Viperin associates with pp28 in the absence of other viral proteins. (A) Generation of constructs for co-immunoprecipitation. Viperin was introduced into pCDNA3.1 vector with Myc tag at the C-terminus. pp28 was introduced into pCDNA3.1 vector with HA tag at the C-terminus. (B, C) HEK-293T cells were transiently co-transfected with plasmids encoding the indicated proteins. Cells were lysed and immunoprecipitated with anti-HA antibody. Precipitates were probed by western blot using anti-viperin antibody (B). Reciprocal immunoprecipitation was performed with anti-Myc antibody. Precipitates were probed by western blot using anti-HA antibody (C). Interferon regulatory transcription factor 3 (IRF3) and enhanced green

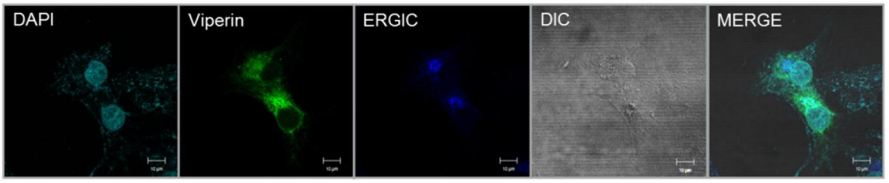
fluorescent protein (EGFP) were used as negative controls. Note that pp28 interacts with viperin in transiently expressed cells in the absence of other viral proteins.

A

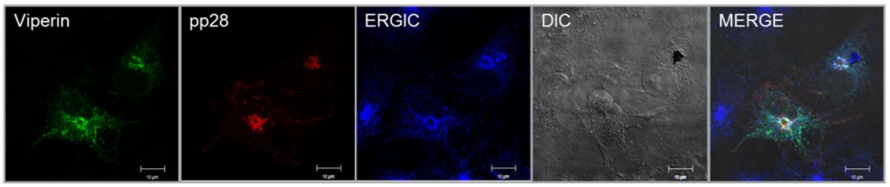
pp28 alone



Viperin alone

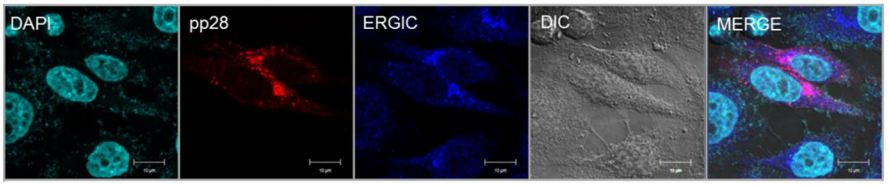


pp28 + Viperin

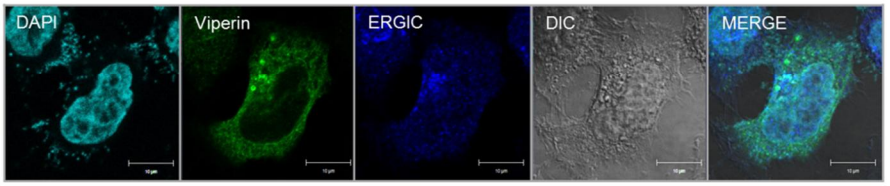


B

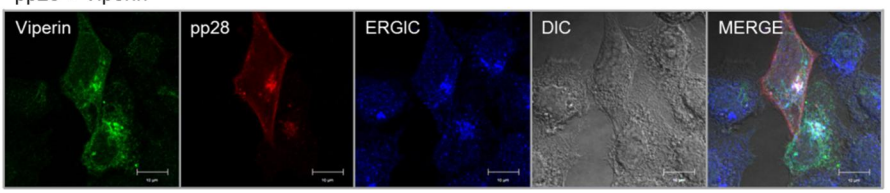
pp28 alone



Viperin alone



pp28 + Viperin



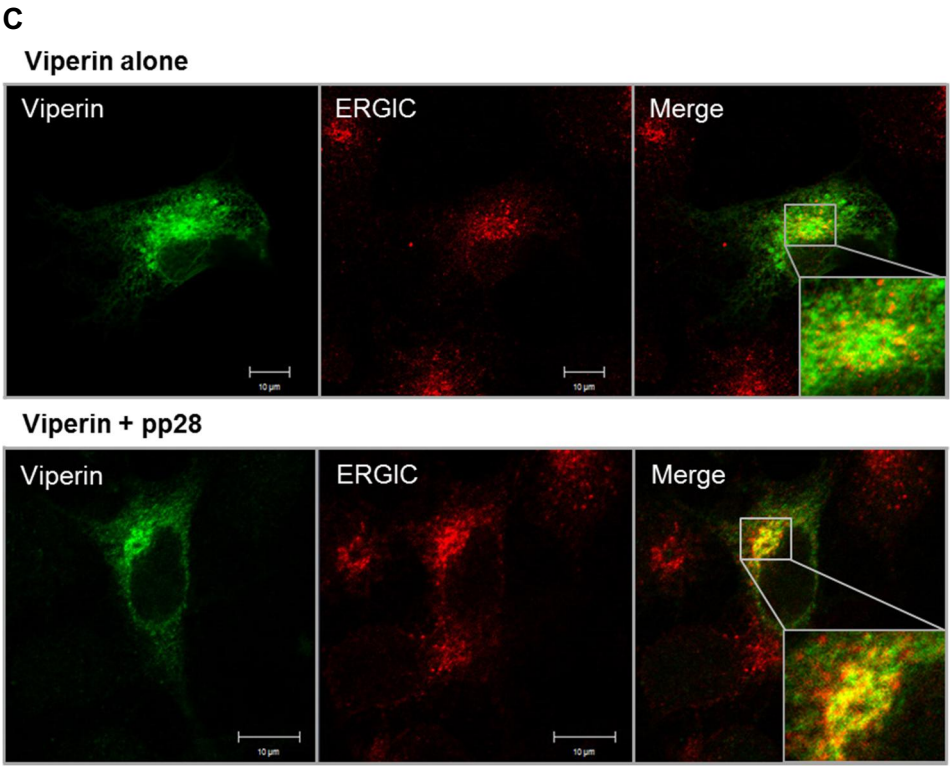


Figure 6. Co-localization of pp28 and viperin in transfected cells. COS-7 cells (A, C) and HeLa cells (B) were transfected with the indicated plasmids. Cells were fixed and stained with anti-ERGIC-53, anti-viperin or anti-pp28 antibody. Viperin and pp28 co-localize to the ERGIC in transiently expressed cells (A, B). Viperin alone localizes to the ER but not the ERGIC. Note that only when viperin is co-expressed with pp28, it has an ERGIC distribution (C). Scale bars, 10 um.

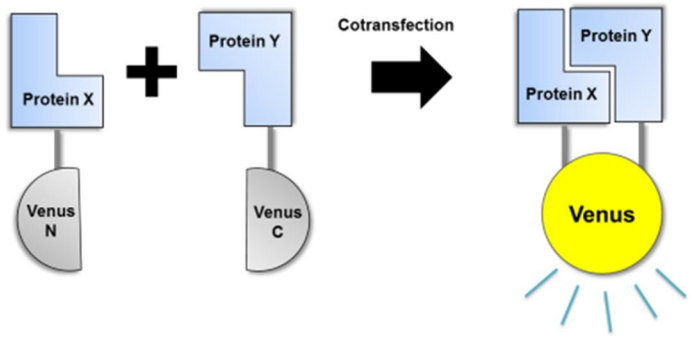
6. Confirmation of intracellular interaction between viperin and pp28

To verify the results from the Co-IP assay, I performed fluorescent protein-fragment complementation assay (PCA). This assay allows visualization and detection of protein-protein interaction in living cells **(Fig. 7A)**. Venus YFP (239 aa) was used as a reporter protein and split into two fragments (Y1 and Y2). Y1 includes the N-terminal sequences (aa residues 1-158) and Y2 contains the C-terminal sequences (aa residues 159-239). Each protein was fused with Y1 or Y2. RPS14 and pUL38 were used as negative controls and GCN4 leucine zipper as a positive control for this assay **(Fig. 7B)**. Cells were grown on glass bottom plate and the fluorescence was monitored by confocal microscopy **(Fig. 7C)**. YFP fluorescence was also examined by fluorometry and flow cytometry (FACS) **(Fig. 7D, E)**. In contrast to negative controls, strong YFP fluorescence was observed upon co-expression of viperin and pp28. Together with the result form Co-IP assay, this result indicated the specificity of viperin interaction with pp28 in live cells.

To confirm the location for their interaction in transiently expressed cells, the transfected cells were monitored after staining with specific antibodies for calnexin, as an ER marker, ERGIC-53 as an ERGIC marker, viperin and pp28. When viperin alone is expressed, it co-localizes with calnexin but not ERGIC-53, indicating that viperin has an ER distribution. However, when viperin is co-expressed with pp28, it co-localizes with pp28 and ERGIC-53,

indicating that pp28 interacts with viperin and translocates viperin into the ERGIC. This result confirmed that viperin interacts with pp28 and co-localizes to the ERGIC without any other viral function or proteins **(Fig. 7F)**.

A



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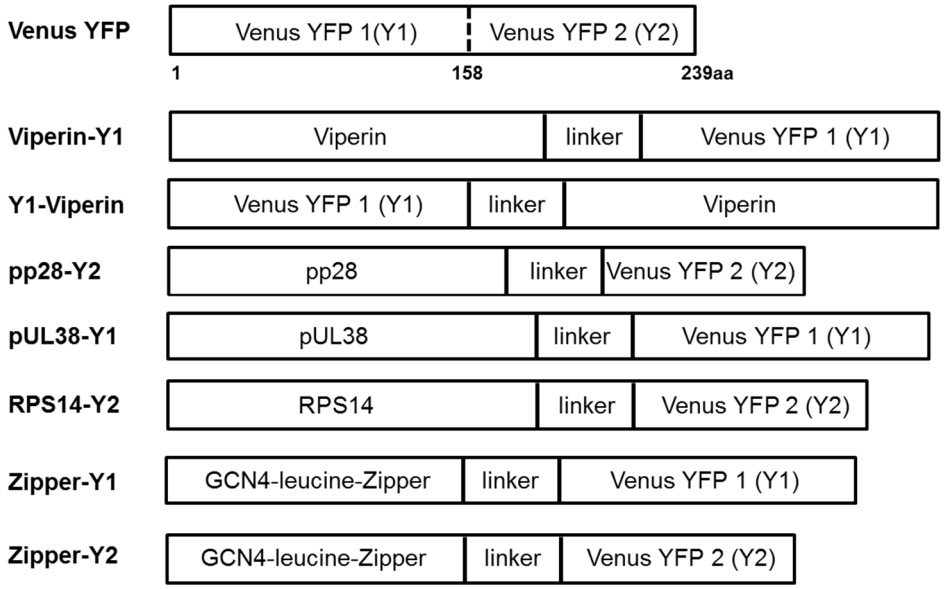
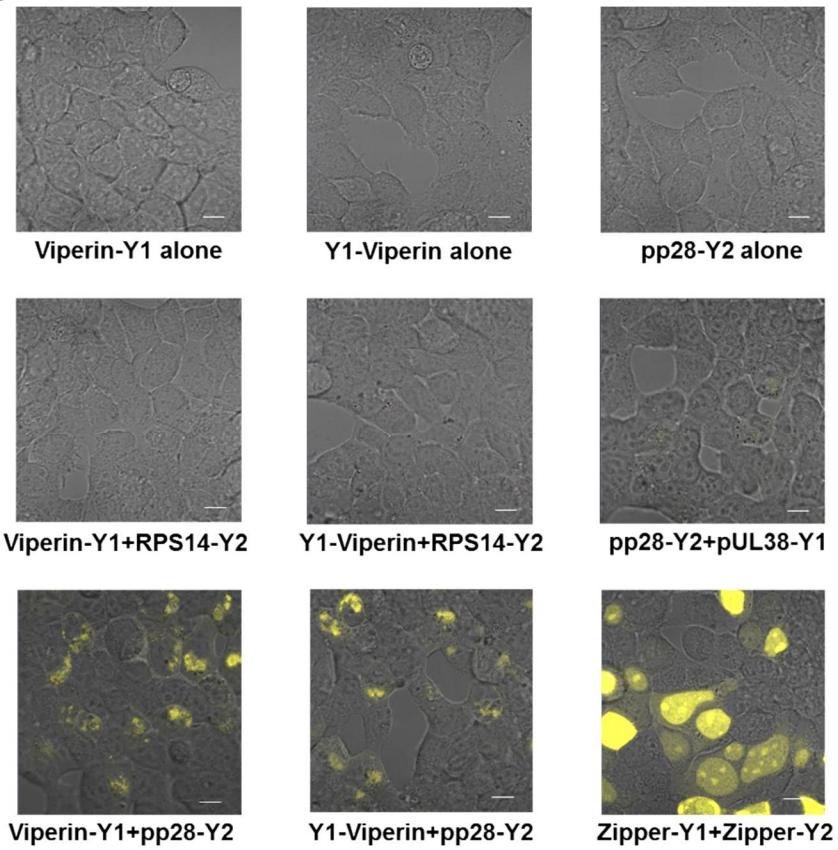


C



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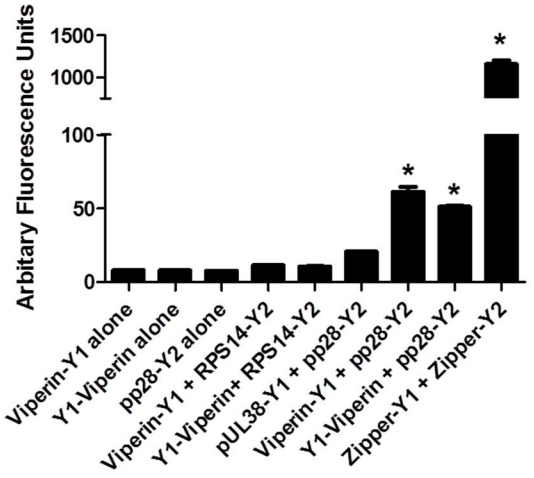
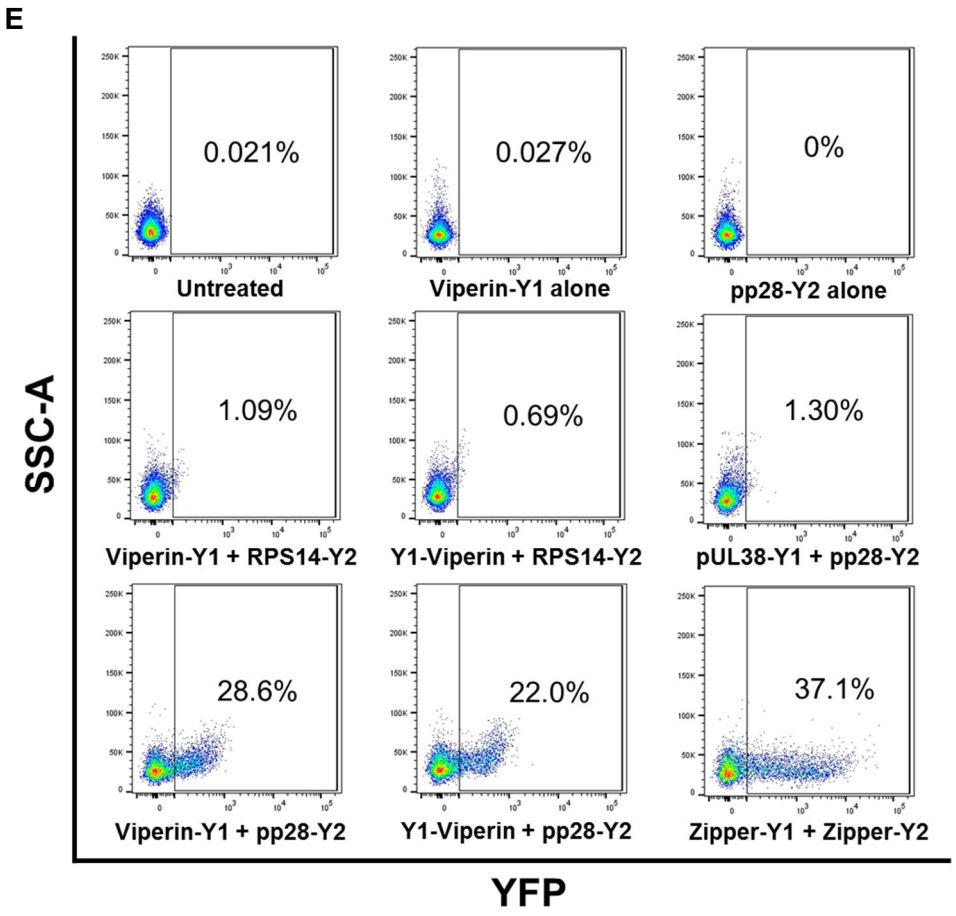




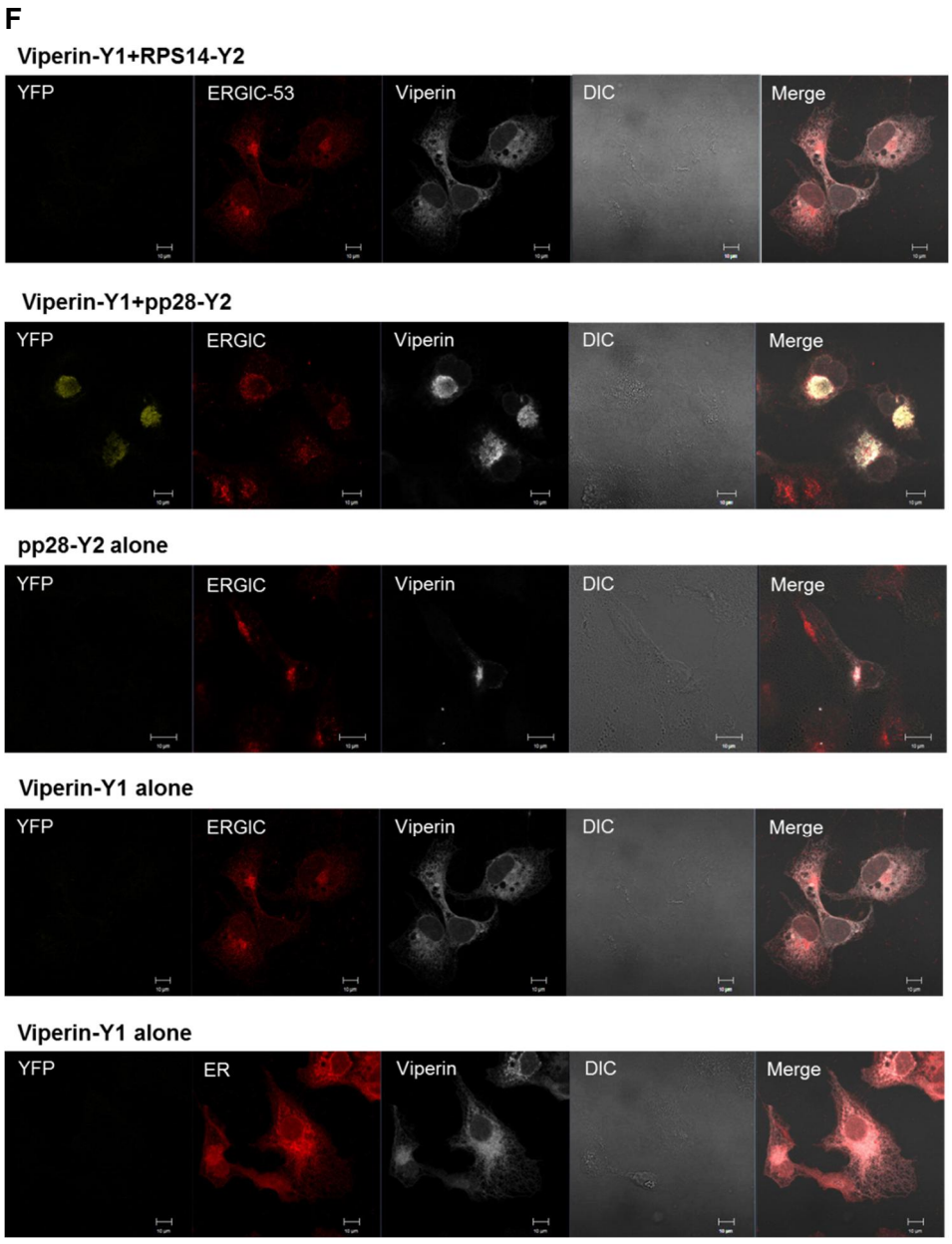


Fig. 7. Intracellular interaction between pp28 and viperin. (A) A schematic diagram of fluorescent protein-fragment complementation assay (PCA). Venus yellow fluorescent protein (YFP) is divided into two fragments, N-terminal and

C-terminal. Each fragment is fused to the interacting proteins. Cells are co-transfected with PCA plasmids. Fluorescence observed only when co-expressed proteins interact with each other. (B-E) Intracellular interaction of viperin and pp28 was confirmed by PCA. Constructs encoding the fusion proteins with Venus YFP fragment 1 or 2 were used as indicated (B). RPS14 and pUL38 were used as negative controls and the GCN4 leucine zipper as a positive control. HEK-293T cells were transiently transfected with the indicated plasmids. YFP fluorescence was examined by confocal microscopy (C), fluorometric anlaysis (D), and flow cytometry (FACS) (E). Co-expression of viperin and pp28 show stronger fluorescence when compared to negative controls. Error bars indicate standard errors (SEM). *, $P < 0.001$. Scale bars, 10 um. (F) COS-7 cells were transfected with the indicated PCA constructs. Cells were fixed and stained with anti-ERGIC-53, anti-calnexin, anti-viperin or anti-pp28 antibody. Note that viperin localizes to the ERGIC when it is co-expressed with pp28, while viperin alone localizes to the ER. Scale bars, 10 um.

IV. DISCUSSION

Viperin is a multifunctional, interferon-inducible protein of host. It has antiviral function against infection of diverse viruses. However, in HCMV infection, viperin shows multiple functions depending on its expression time point and localization during HCMV infection.[10] Overexpressed viperin prior to HCMV infection localizes to ER where it inhibits soluble proteins trafficking and thus decrease viral replication.[9] However, when viperin is expressed directly by HCMV infection, it localizes to mitochondria via interaction with vMIA and enhances viral replication.[16,21] In this study, 10 HCMV proteins were identified as interacting partners of viperin in yeast two hybrid assay. The pp28 protein, an essential true late tegument protein, was selected as an interacting partner of viperin. I found their interaction in both transiently expressed cells and HCMV infected cells using Co-IP. When the cells were infected with HCMV, their co-localization was observed at the AC at the late stage of infection as reported previously. Importantly, I also observed their co-localization at the ERGIC which is abnormal localization for viperin alone in transiently expressed cells.

According to the reproducibility, size, and the number of colonies from the yeast two hybrid assay, the interacting partners of viperin were classified into two groups, strong and weak hits. pp28 was selected as the strongest interacting partner of viperin and examined their interaction with

various assays in this study. pUL37 was one of the reliable interacting partner. HCMV UL37 has three splicing variants. UL37 exon 1-encoded protein (pUL37x1) is known as vMIA which has anti-apoptotic activity. In previous studies, it has been shown that vMIA interacts with viperin during HCMV infection.[16] Among the strong hits, pUL38 is another anti-apoptotic protein. It has been reported that pUL38 interacts with TSC1/2 to maintain mTOR activity.[28,36] I attempted to examine viperin interaction with pUL38. I confirmed their interaction in the Co-IP assay. However, pUL38 did not show interaction with viperin in the PCA. The discrepancy of these assays might be from differences of assays' sensitivities. The interaction of viperin and pUL38 needs to be further studied. The pUL148 protein is an integral component of membrane and might be related to maturation of glycoprotein H and O. It has been reported that this protein has an ER distribution,[31] suggesting that viperin interaction with pUL148 might be related to the antiviral function at the ER. Unfortunately, it was not able to detect their interaction by Co-IP because of the poor level of pUL148 expression. Yeast two hybrid assay has false negatives as well as false positives. That is, there is a possibility that some proteins are omitted from interacting candidates. One of such proteins is pp150. It showed interaction with viperin in the Co-IP assay but not yeast two hybrid assay. This interaction between viperin and pp150 also needs to be further studied **(Table 2)**.

Viperin shows diverse functions and localization patterns during

HCMV infection.[16] Its roles in ER and mitochondria have been reported, although the mechanism is not completely elucidated yet.[9,16,21] However, the role of viperin in the viral assembly compartment (AC) has been unknown. pp28 as an interacting partner of viperin is localized to the AC late in HCMV infection, suggesting possible roles of their interaction in the AC. The interaction and co-localization of both proteins were confirmed in mammalian cells using Co-IP and immunofluorescence microscopy. It has been reported that pp28 localizes to ERGIC and viperin localizes to ER in transiently expressed cells. Here I showed that viperin is co-localizes with pp28 to the ERGIC when both proteins are co-expressed. Their interaction and co-localization were verified using PCA. Moreover, viperin was completely overlapped with pp28 in the ERGIC. Since PCA is an irreversible reaction, the viperin interacted with pp28 could be trapped in the ERGIC without recycling to the ER. Taken together, the data provided evidence that viperin re-localizes from the ER to the ERGIC through the interaction with pp28.

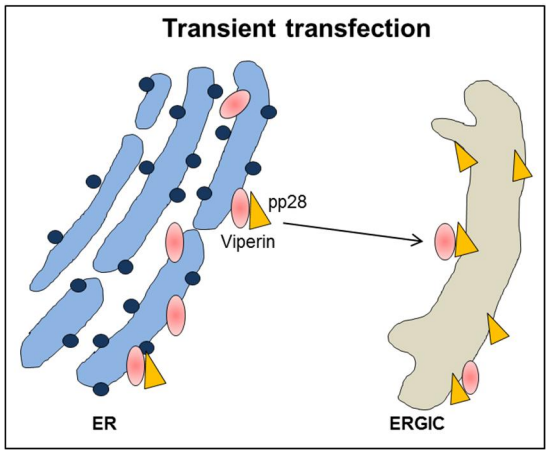
It has been reported that pp28 mutant having an impaired ERGIC localization is not able to traffic to the AC during HCMV infection, suggesting that the ERGIC localization of pp28 is a prerequisite for its trafficking to the AC late in infection.[24] Here, I could suggest a possibility that viperin interaction with pp28 and re-localization into the ERGIC are responsible for re-localization of viperin to the AC. Viperin functions on viral assembly or viral incorporation through interaction with pp28 in the AC late in HCMV infection **(Fig. 8)**. It has

been reported that various host proteins affect the virion assembly through interaction with viral proteins. For example, a host 14-33-3 protein interacts with parainfluenza virus 5M protein and inhibits the virion particle formation.[37] A trans-Golgi network-associated protein, hPOSH is required for HIV-1 Gag localization to the plasma membrane and production of viurs-like particle.[38] Like these proteins, viperin interaction with pp28 might play a role in virus assembly and production of infectious virus. It has been shown that both viperin and pp28 are required for the formation of virion envelope.[21,22] Deficiency of either protein produces non-enveloped viral particles during HCMV infection by different mechanism. It suggested that their interaction is necessary for viral envelopment. Viperin is also able to be incorporated into the virions through interaction with pp28 during viral assembly process. It has been shown that numerous host proteins are incorporated into virions and affect viral infection. Incorporation of the cellular ICAM-1 in HIV-1 virions increases the infectivity of the virus. Embedded ICAM-1 modifies HIV-1 entry routes by enhancing the release of viral components into the cytosol.[39] In contrast, the interferon-induced transmembrane (IFITM) proteins are incorporated into HIV-1 virions to limit entry into new target cells.[40] Thus, if viperin is incorporated into the virions, it could affect viral infectivity and viral spreading.

Taken together, the data indicated that viperin interacts with pp28 and translocates from the ER to the ERGIC in the absence of viral infection, suggesting that this interaction is responsible for viperin's localization and

function in the AC during HCMV infection.

A



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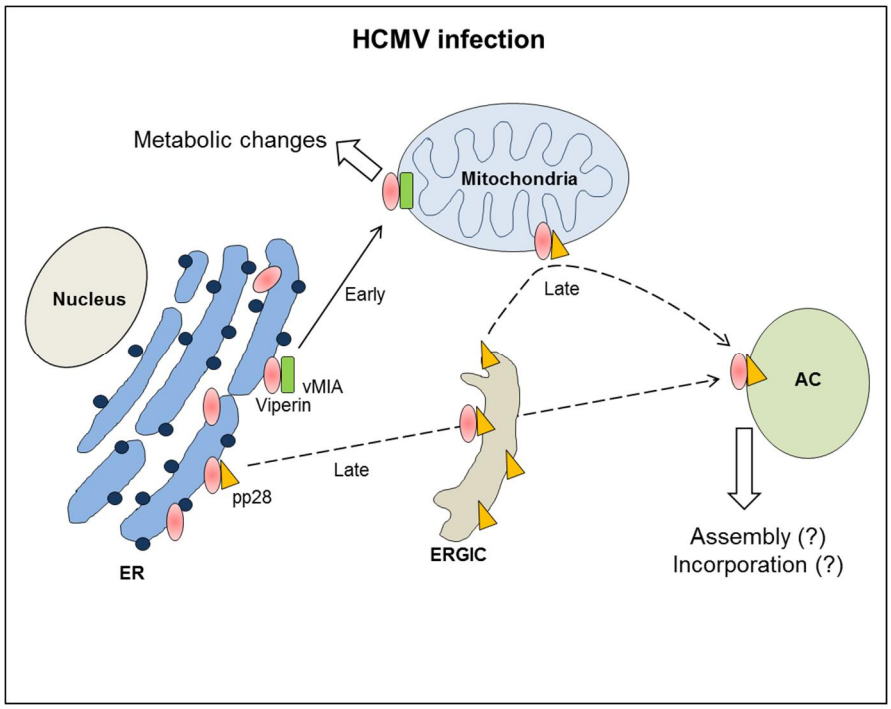

Fig. 8. A model of viperin interaction with pp28 and its possible roles in the AC during HCMV infection. (A) Viperin alone localizes to ER, but it

translocates to ERGIC via interaction with pp28 in transiently expressed cells. (B) At the early stage of HCMV infection, viperin interacts and co-localizes with vMIA to mitochondria and enhances viral replication. At the late stage of HCMV infection, viperin interacts and translocates with pp28 to the AC. There are two possible pathways. A pathway is that pp28 is targeted into mitochondria via ERGIC and interacts with viperin and re-localizes to the AC. The other pathway is that pp28 interacts with viperin in the ER and then finally re-localizes to the AC via ERGIC. Viperin might play roles in viral assembly or incorporation through interaction with pp28 in the AC.

V. CONCLUSION

I identified 10 HCMV proteins interacting with viperin using yeast two hybrid assay. HCMV pp28 protein, an essential true late tegument protein, was verified to be an interacting partner of viperin in this study. I observed their interaction in both HCMV infected cells and transiently expressed cells using co-immunoprecipitation and confirmed intracellular interaction in live cells with protein-fragment complementation assay. I also observed their co-localization to the viral assembly compartment (AC) at the late stage of infection. Furthermore, I found that pp28 interacts with viperin and translocates it from ER to ERGIC in the absence of viral infection. My data suggest that viperin interaction with pp28 might be responsible for localization of viperin to the AC and thus play a role in virus assembly at the AC during HCMV infection.

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ABSTRACT (IN KOREAN)

Viperin과 상호작용하는 Human Cytomegalovirus 단백질의 동정 및 특징 분석

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Human cytomegalovirus (HCMV)는 beta-herpesvirus의 일종으로 다양한 질병을 일으킨다. 특히 어린이나 노약자, 장기이식 환자, HIV 감염자와 같은 면역능이 약화된 사람에게 감염될 경우 심각하고 다양한 급성 및 만성질환의 원인이 된다. 바이러스 감염에 대하여 숙주는 다양한 방어 기작을 작동시킨다. 인터페론 반응은 숙주의 일차적인 방어 기작이다. 인터페론은 수 많은 인터페론 유발성 유전자의 발현을 유도한다. Viperin은 인터페론 유발성 다기능 단백질이다. Viperin은 HCMV 감염 시 발현 시기와 세포 내 위치에 따라 그 기능이 달라진다. HCMV 감염에 선행적으로 viperin을 발현시킬 경우, 이는 소포체에 위치하여 바이러스의 복제를 억제한다. 하지만 HCMV에 의해 직접적으로 발현이 유도된 viperin은 감염 초기에 HCMV 단백질인 vMIA와의 상호작용을 통하여 미토콘드리아로 위치하고 세포의 에너지 및 지방 대사를 조절하여 결론적으로는 바이러스의 복제를 촉진시킨다. Viperin은 감염 후기에 바이러스 assembly 와 maturation에 관

여하는 바이러스 assembly compartment로 이동한다. 하지만 assembly compartment내에서 viperin의 기능은 아직 밝혀진 바가 없다. 여기서, 나는 viperin과 HCMV간의 상호작용이 viperin의 기능을 결정짓는 중요한 요소라는 가설을 세웠다. 이를 증명하기 위하여 효모단백질접종법을 이용하여 viperin과 상호작용하는 10개의 HCMV 단백질들을 동정하였다. 이들 중에서 HCMV pp28 단백질을 viperin과 상호작용하는 단백질로 선정하고 연구를 진행하였다. pp28은 HCMV의 감염 후기에 발현되고 assembly compartment에 위치하며 바이러스의 maturation과 envelopement에 작용하는 바이러스 복제에 필수적인 단백질로 알려져 있다. 나는 viperin과 pp28간의 상호작용을 HCMV 감염 조건과 형질주입 조건에서 항체면역침강법과 protein-fragment complementation assay (PCA)를 통하여 검증하였다. 또한 viperin과 pp28은 HCMV 감염 조건에서는 assembly compartment 에 함께 위치하며, 형질주입 조건에서는 소포관상군에서 같이 위치한다는 것을 면역형광법으로 확인하였다. 중요하게도 viperin이 pp28과 같이 발현되었을 경우 소포체로부터 소포관상군으로 이동하는 점은 바이러스 감염 시 pp28과의 상호작용에 의해 viperin이 assembly compartment로 이동하게 될 가능성을 제시한다. 또한, 이러한 상호작용을 통해 viperin이 바이러스 assembly 과정에 기능을 하게 될 가능성을 시사한다. 이번 연구 결과는 HCMV의 감염 후기에 assembly compartment에서의 viprein의 새로운 기능을 밝히는 실마리를 제공한다.

핵심되는 말: Viperin, HCMV, pp28, 효모단백질접종법, 단백질간 상호작용, 소포관상군, Assembly compartment