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e-Research: A Journal of Undergraduate Work, Vol 2, No 2 (2011)[HOME](#) [ABOUT](#) [LOG IN](#) [REGISTER](#) [SEARCH](#) [CURRENT](#) [ARCHIVES](#)[Home](#) > [Vol 2, No 2 \(2011\)](#) > [Cyr](#)**Mapping Regions of RNF168 Required for its Degradation by ICP0****Andrea Cyr, Matthew Weitzman*****ABSTRACT**

Viruses establish infection by overtaking host cell processes and developing mechanisms that promote viral replication. Herpes simplex virus undergoes lytic and latent cycles of infection throughout the lifespan of its host. The viral genome is transcriptionally silent during latency, but viral proteins are produced upon reactivation. Herpes simplex virus type 1 encodes the ICP0 protein, an E3 ubiquitin ligase required for reactivation from latency of the infectious virus. The immediate-early protein ICP0 regulates the herpes simplex virus by activating viral gene expression thereby initiating lytic infection. Cellular proteins are degraded by ICP0, promoting the virus to enter the lytic cycle. Two cellular histone ubiquitin ligases, RNF8 and RNF168 that promote recruitment of DNA repair factors to DNA damage sites. ICP0 viral expression counteracts RNF8 and RNF168 as anti-viral factors by their degradation. This study maps regions of RNF168 required for its degradation so that proteins involved in viral reactivation can be determined. RNF168 has been shown to organize the DNA damage response by increasing the local concentration of ubiquitinated proteins in damage sites to prolong the ubiquitination signal. As a chromatin-associated RING finger protein, RNF168 consists of a sequence of two motif interacting with ubiquitin (MIU) domains that allow RNF168 to be recruited to damage sites. Two constructs were created, which separated each MIU to test for degradation by ICP0, thus localizing how ICP0 targets cellular proteins. In understanding how ICP0 targets its cellular substrates, other proteins targeted by ICP0 with similar motifs may be identified.

Keywords: Herpes simplex virus, ICP0 protein, Regulation of Herpes simplex virus, RNF 168

INTRODUCTION

Herpes Simplex Virus Type 1 (HSV-1) is a double stranded DNA virus with a biphasic lifecycle. A vast majority of the U.S. population is seropositive and experience outbreaks of bothersome cold sores on the vermilion border of the lip (Lachmann, 2003). The virus causes primary infection by entering the lytic cycle within the epithelial cells in the skin of its human host. HSV-1 enters the sensory neurons and travels retrogradely via axons to the ganglia to establish latency of the viral genome, thus the relationship between the virus and these neurons are central to viral survival (Lachmann, 2003). In latency, all transcriptional expression of viral proteins is silent. During this state, only latency-associated transcripts (LATs) are expressed, and the function of these remains unclear (Everett, 2000). During ultraviolet exposure or stress, the virus spontaneously travels back through the neuron to re-establish infection in the epithelial cells, to cause recurrent lesions. The virus encodes immediate early (IE) genes, which aid the cells survival during lytic infection (Lachmann, 2003). IE genes are necessary for the activation of a later class of promoters (Everett, 2000). A complex is formed that requires the function of VP16, which uses the transcriptional machinery of the host cell and Oct-1, another cell protein. A third component, HCF, acts with VP16 and Oct-1 to strongly activate the IE gene expression (Everett, 2000). One IE gene in particular, ICP0, acts as a regulating switch between the lifecycles of the virus (Everett, 2000).

ICP0, an E3 ubiquitin ligase, is one of the first genes activated by the viral genome to regulate viral gene expression (Everett, 2000). ICP0 degrades specific proteins that aid viral DNA replication. Although it is non-essential to the virus, its absence negatively affects viral replication initially (Everett, 2000). The zinc-binding RING finger motif of

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ICP0 is necessary for its function. Viral proteins interact with cellular proteins to regulate gene expression. Rather than binding to DNA directly, ICP0 acts by ubiquitin-proteasome pathways of cellular protein and nuclear domain structure degradation (Everett, 2000).

Once primary infection is established, the host cell recognizes the viral genome as DNA damage, thus a network of signaling pathways are coordinated by the cells DNA repair machinery (Lilley et al, 2010). Several factors are sent by the cell to respond to the invasive viral genome as recognized by the cell. A series of phosphorylations produce irradiation-induced foci (IRIF)-like subnuclear structures near the incoming viral genome. IRIF organization involved in marking sites of cellular DNA damage is still being studied (Huen and Chen, 2008). A mediator protein, Mdc1 (Mediator of DNA damage check point protein 1), interacts with gH2AX and ATM (Lou et al, 2006).

Two cellular ubiquitin ligases, RNF8 and RNF168, are important in the formation of IRIF (Huen et al. 2007). RNF8 binds to phosphorylated Mdc1 and ubiquitinates H2A and H2AX histones (Huen et al 2007). The second ubiquitin ligase, RNF168, reinforces this mechanism by further modifying ubiquitinated histones (Doil et al, 2009; Stewart et al, 2009). These mechanisms coordinate the recruitment of DNA repair proteins to sites of IRIF by proteins with ubiquitin interacting motifs and promoting chromatin restructuring (Huen et Chen, 2008). The parallels between IRIF and IRIF-like sub nuclear structures formed near incoming viral genome are a research focus.

In a previous study to determine a link between viral reactivation and damage signaling, ICP0 was found to degrade RNF8 and RNF168 (Lilley et al. 2010). Less DNA repair proteins accumulate at sites of DNA damage once ICP0 induces the degradation of RNF8 and RNF168 (Lilley et al. 2010). Proteasome-mediated degradation of RNF8 and RNF168 is ICP0-dependent (Lilley et al. 2010). In the absence of ICP0 (mutated viral genomes deleted for ICP0), RNF8 and RNF168 are not degraded. RNF8 and RNF168 interact with ubiquitin to target histone H2A and mark regions of DNA damage. Histone H2A directs DNA repair proteins to these sites. In the absence of ubiquitinated H2A, the accumulation of repair proteins is hindered and viral genes are expressed. Degradation of RNF8 and RNF168 is responsible for the loss of H2A ubiquitination and disruption of IRIF in the presence of ICP0 (Lilley et al. 2010).

RNF168 is involved in mediating the recruitment of DNA repair proteins. RNF168 includes a RING finger motif and two motifs interacting with ubiquitin (MIUs) (amino acids 168-191 and 439-571). The RING finger motif (amino acids 15-58) of RNF168 interacts with other cellular factors to add ubiquitin onto target proteins. Mapping the regions required for RNF168 degradation by ICP0 gene expression may indicate a more precise location and a specific motif in RNF168.

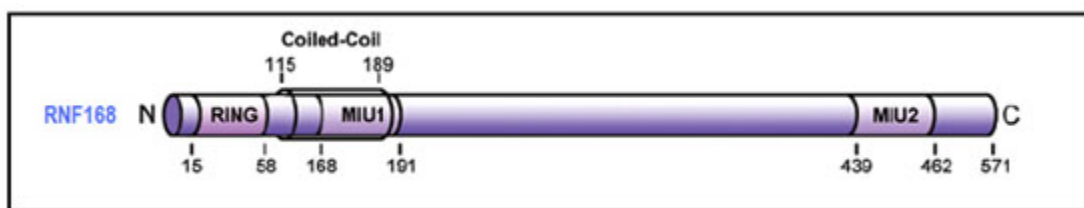


Figure 1: Schematic image of RNF168 containing a RING finger motif, MIU1, and MIU2.

MATERIALS AND METHODS

Cloning

PCR

Used Polymerase Chain Reaction for amplification of two RNF168 fragments. Ordered 2 forward and 2 reverse primer sequences from Eton. Primers prepared from 100 mM stock to 10 mM. Forward primers contain Flag sequence. 2X Mastermix containing dNTPs, polymerase and buffer was added to 100 ng Flag Riddlin DNA, and forward and reverse primers for either F1 or F2. Fragment 1 (F1) contain EcoRV and XhoI restriction sites and

Fragment 2 (F2) contain EcoRI and XhoI restriction sites. Cyclor conditions set to 30 rounds of 95 degrees C for 1 min for initialization, 95 degrees C for 30 seconds to denature, 55 degrees C for 30 seconds to anneal, 72 degrees C for 1 minute for elongation, and 72 degrees C for 7 minutes for final elongation. Samples were run on an 8% agarose gel electrophoresis and stored at 4 degrees C. Followed QIAquick PCR Purification Kit protocol from QIAGEN using a micro centrifuge to purify amplified DNA.

Digests and Ligation

F1 digested with buffer 3, 10X BSA, EcoRV and XhoI restriction enzymes. F2 digested with buffer EcoRI, 10X BSA, EcoRI and XhoI restriction enzymes in 37 degrees C for at least 1 hour. Two vector digests using pcDNA 3.1 prepared for each fragment. 1:20 diluted CAIF Intestinal phosphatase added to vector digests. Digests run on an 8% agarose gel electrophoresis and purified using QIAquick Gel Extraction Kit protocol. F1 ligated with vector digest of EcoRV and F2 ligated with vector digest of EcoRI. Both ligations incorporated 50 ng vectors for 1:5 ratio of vector to insert.

Transformation and DNA Preparation

Competent TAMI bugs stored at -80 degrees C. 50 ul bugs to each ligation and incubated on ice for 30 minutes. Heat shock in 42 degree C water bath for 30 seconds. SOC media added to each ligation and incubated in 37 degree C water bath for 1 hour. Transformations plated on LB-Carb plates and incubated at 37 degrees C overnight. Colonies from F1 and F2 selected and incubated while shaking at 37 degrees C with LB and Amp. Plasmid DNA purified following protocol from QIAprep Miniprep protocol and QIAGEN Plasmid Maxi kit protocol.

Cell lines

HeLa and 293T cells were purchased from the American Tissue Culture Collection. Routine passage of adherent cell lines maintained using PBS buffer as wash and 10% trypsin: EDTA (10 ml trypsin solution in 100 ml versene solution) for cell detachment. Cells resuspended and maintained in Dulbecco modified Eagles medium (DMEM) containing 100 U/ml of penicillin and 100ug/ml of streptomycin, and supplemented with 10% foetal bovine serum (FBS) and antibiotics. Cells were grown at 37 degrees C in a humidified atmosphere containing 5% CO₂. Cells were routinely split as most appropriate in separate 15 cm plates.

Transfections

Cells were subjected to Lipofectamine 2000 (LF 2000) transfection in 6-well plates. 40 ul LF 2000 master mix prepared with 2.5 ml optimem. Mixed with 4ug plasmid in a 3:1 ratio of ICPo to RNF168 of fragment 1-12 (F1-12), fragment 1-15 (F1-15), or full-length RNF168 DNA in 250 ul optimem. Media on cells during transfections contained 10% FBS without penicillin and streptomycin. After 6 hours at 37 degrees C, media was replaced to contain antibiotics.

Whole Cell Lysates

Cells transferred in 1.5 mL eppy tubes and micro centrifuged at 4000g for 5 min. Pellet stored at -80 degrees C. Whole cell lysis buffer prepared (1M PBS, 1000mM Na Vanidate, 1M B-glycerol phosphate 50X, 1M NaF 50X, 1 crushed Complete +EDTA in 1 ml H₂O 50X, 0.1mM PMSF, 10% NP40, 0.1% Triton) and added 40 ul per pellet. Incubated 20 minutes on ice and micro centrifuged 20 minutes at 4 degrees C. Supernatant stored at -80 degrees C.

Lowry Assay

6 standards (final concentrations: 0, 100, 200, 400, 600, 800 ug/ml) prepared in duplicate. Samples added with 125 ul mix of 6.25 mls Biorad protein assay reagent A and 125 ul reagent S. 1 mL Biorad protein assay reagent B added to each sample. Concentrations read on UV spectrometer.

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Western Blotting

10-well and 12-well 4-12% Bis-Tris Acetate gels with MOPS running buffer to run 18 ul samples of 30 ug samples and 60 ug samples respectively and full range rainbow recombinant protein marker. 1:5 loading dye prepared with LDS sample buffer (4X) and IM DTT (20X). Gels ran at 150 V for 1.5 hours. Transfers ran at 30 V for 2 hours in 20X NUPAGE transfer buffer, methanol, H₂O, NUPAGE antioxidant. Membranes soaked in 5% milk with 100 ul Na₃N after transfer in 4 degree C cold room. Membranes washed with 1X PBST. Membranes soaked with mouse ICPO, mouse GAPDH, or mouse Flag primary antibodies then horse-radish peroxidase-conjugated mouse secondary antibodies in 5% milk. Equal volumes of Western Lightning Plus-ECL oxidizing reagent and enhanced luminal reagent distributed over membranes for film development.

RESULTS

Cloning

Cloning the two separate constructs of RNF168 was carried out as described in the methods section. Correct constructs of the clones were identified by restriction digest analysis and computer-based DNA sequencing. The verified clones of F1 and F2 were purified and used in transfection experiments in the HeLa cells. As seen in figure 2, the F1 construct (~850bp) contained the RING finger motif and MIU1, and the F2 construct (~1Kbp) contained MIU2.

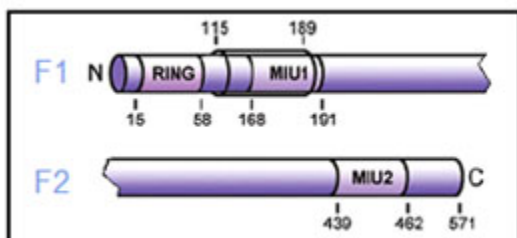


Figure 2: Schematic images of F1 and F2 constructs of RNF168 cloned and transfected +/- ICPO.

F2 is degraded by ICPO expression

ICPO expression degrades several cellular substrates, but this study focused on RNF168 degradation specifically. Degradations of the fragments from the separated RNF168 were compared to each other and to the full-length RNF168. The lysates of ICPO co-transfections in HeLa cells were analyzed by a western blot for F2. F2 fragment degradation is evident with ICPO expression as seen in Figure 3. The F2 fragment is not degraded with pcDNA3.1 vector alone in the absence of ICPO. F2 is sized at approximately 50kDa.

F1 may be degraded by ICPO expression less efficiently

The western blot in Figure 3 indicates moderate degradation of F1 by ICPO expression. Two separate lysates of F1 (F1-12 and F1-15) were analyzed on the western blot to express F1 in duplicate due to uncertainty of the quality of the sequences of the clones. F1 degradation appears to be less efficient in comparison to F2 degradation. Both F1-12 and F1-15 fragments suggest no degradation in the presence of pcDNA3.1 vector alone. F1 is sized at approximately 38kDa.

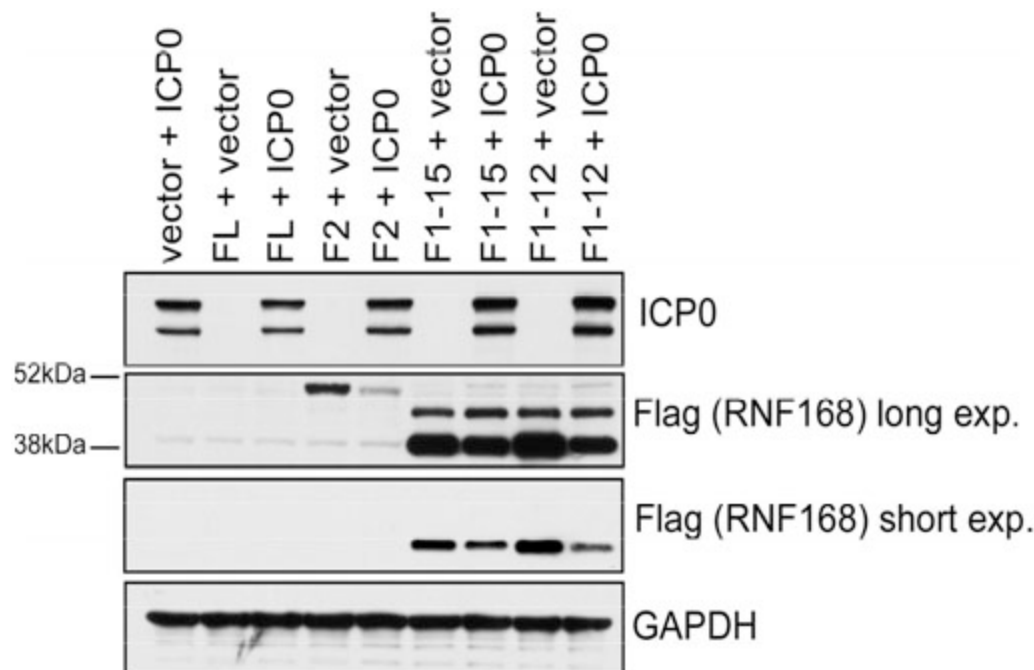


Figure 3: ICP0 expression results in Fragment 2 (F2) degradation at ~50kDa. ICP0 expression results in possible moderate degradation of Fragment 1 (F1) at ~38kDa with blots exposed for 1 min and 3 seconds. HeLa cells were co-transfected with ICP0 and harvested for 24 hrs post transfection. Lysates were analyzed by western blotting and assessed for levels of F1 or F2. Full length RNF168 controls show no expression, likely due to experimental error. GAPDH was used as a loading control. Flag-tag was added onto all RNF168 fragment clones for detection.

DISCUSSION

Based on previous studies, RNF168 is a target for ICP0-mediated degradation (Lilley et al. 2010). This study constructed fragments of RNF168 by truncating the substrate into two fragments to determine their degradation by ICP0. F1 was truncated to contain both the RING finger domain and MIU1. F2s prominent feature was the MIU2. By mapping the region of degradation, the target location on RNF168 can be better determined. Due to the fact that F2 is effectively degraded, it has led us to believe that the target location of RNF168 is found within F2. MIU2 may potentially be the target location for ICP0-mediated degradation, yet further truncations need to be analyzed before reaching more specific conclusions.

Figure 3 indicates F1 degradation with ICP0 expression, but less efficiently than F2, which is clearly degraded. Since both fragments are degraded to some degree, other interacting factors involved in substrate degradation could be present. ICP0 may not directly target RNF168, but other factors are involved by interacting on multiple locations on the RNF168 protein. F1 fragment lysates were tested in duplicate because their quality, as determined by computer-based DNA sequence analysis, was questionable. Both F1 fragments, however, appear to have been successfully transfected and show very similar results.

No bands were found on the full length RNF168 fragment on the western blot as seen in Figure 3. Based on previous studies, full length RNF168 is degraded in the presence of ICP0 expression. The lack of bands for this control is attributed to experimental error. Possible explanations include that the full-length RNF168 construct may not have contained the flag sequence necessary for antibody detection, or an erroneous transfection occurred.

A separate study investigated a mutated RNF168 protein in the RIDDLE syndrome (Stewart et al, 2009). This severe immunodeficiency syndrome, from altered development of the immune system, results in dimorphic features and learning difficulties. Two different RNF168 truncations of the protein affect repair protein accumulation at DNA

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damage sites. Both truncations lack MIU2 indicating its possible necessity for full RNF168 function. Those specific studies suggest that both Ubiquitin binding and ligase activity are necessary for full RNF168 function. Partial RNF168 activity in human syndromes such as in the RIDDLE patient may allow only certain cellular processes to proceed. Such clinical cases could benefit from improved understanding of the protein degradation mechanism of RNF168.

After mapping regions of RNF168, further investigations should research a mutant virus, which cannot degrade RNF168, and how the substrate's absence would affect viral replication and its lifecycle. Based on how ICPO targets cellular substrates that are likely important in transcriptionally silencing the viral genome, other proteins targeted by ICPO with similar motifs could be identified. Once the location that ICPO interacts with the cellular substrate RNF168 is determined, future studies would include mapping the region responsible for degradation on the viral protein ICPO.

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