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The Varicella-Zoster Virus ORF54 Gene Product Encodes the Capsid Portal Protein, pORF54

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Summary

The Varicella-zoster virus (VZV) ORF54 gene was characterized using a guinea pig antiserum prepared to a GST-pORF54 fusion protein. A protein of the predicted size, 87kDa, was detected in VZV-infected MeWo cells but not in mock-infected cells. Sucrose density gradient fractionation of pORF54 expressed in a recombinant baculovirus system resulted in samples containing enriched amounts of pORF54. Electron microscopic analysis suggested that the ORF54 gene encodes a protein that assembles into ring-like portal structures similar to those observed for numerous bacteriophages and other herpesviruses.

Keywords

Varicella-Zoster Virus; ORF54; pORF54; encapsidation; portal

Herpesviral encapsidation entails the packaging of concatemeric viral DNA into a protein capsid via a self-assembled portal structure. Proteins that play a role in herpesviral DNA encapsidation have become promising novel chemotherapeutic targets. Two series of related non-nucleoside compounds that inhibit either Herpes simplex virus (HSV) (van Zeijl et al., 2000; Visalli et al., 2003; Visalli and van Zeijl, 2003) or Varicella-zoster virus (VZV) (Visalli et al., 2003; Visalli and van Zeijl, 2003) DNA encapsidation have been described. A series of N- α -methylbenzyl-N'-aryl thiourea analogs demonstrated specific and potent activity against VZV, but not HSV (Visalli et al., 2003). Structure-activity studies directed the development of a series of α -methylbenzyl thiourea compounds with selective anti-VZV activity yielding IC₅₀ values between 0.1–1.0 μ M when tested by either ELISA or standard plaque reduction assay (Visalli et al., 2003). In the presence of inhibitors, only B-capsids were observed in the nuclei of VZV-infected cells. VZV mutant viruses resistant to thiorurea compounds were found to contain mutations in the putative portal protein, pORF54 (Visalli et al., 2003; Visalli and van Zeijl, 2003). In a separate study, the HSV portal protein homolog, pUL6, was shown to be the likely target of the HSV-specific compounds

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(Newcomb and Brown, 2002; van Zeijl et al., 2000). Previously, pUL6 was shown to localize to a single vertex of the viral capsid and is the likely site or “port of entry” for viral genomic DNA during the encapsidation process (Cardone et al., 2007; Newcomb et al., 2001). Additionally, HSV UL6 deletion mutants are defective in both DNA cleavage and packaging, which results in large numbers of B-capsids in the nuclei of mutant-infected cells. The effect of inhibiting pUL6 or pORF54 function via the thiourea compounds is consistent with the genetic evidence provided by studies with HSV deletion mutants (Patel et al., 1996). Electron microscopy results confirmed the expected phenotype, namely a lack of DNA-filled capsids in the nucleus, for HSV or VZV-infected cells treated with their respective thiourea inhibitor (van Zeijl et al., 2000; Visalli et al., 2003).

It is reasonable to speculate that pORF54 performs a functional role similar to that of pUL6 since pORF54 shows 44% amino acid identity with its HSV-1 homolog (Visalli and van Zeijl, 2003). In addition, the similar results observed via electron microscopy for inhibitor treated, infected cells (van Zeijl et al., 2000; Visalli et al., 2003) are predictive of conserved functions for pUL6 and pORF54.

To identify potential ORF54-encoded polypeptides in VZV-infected cells, a GST fusion protein containing the N-terminal 257 amino acids of pORF54 was purified and used to generate a guinea pig polyclonal antiserum specific for VZV pORF54 (Cocalico Biologicals, Inc., Reamstown, PA). *In vitro* transcription/translation (TNT) products of V5 epitope-tagged pORF54 (pORF54-V5) or β -galactosidase control protein (LacZ-V5) were used to confirm the specificity of the pORF54 antiserum. TNT reactions yielded polypeptides of 91 and 118 kDa, respectively, for pORF54-V5 and LacZ-V5 (Fig. 1A, lanes 7 and 8). The V5-tagged products were immunoprecipitated in RIPA buffer with either a monoclonal antibody specific for the V5 epitope tag, the pORF54 antiserum, or pre-immune guinea pig serum. Immunoprecipitated proteins were detected by immunoblotting with anti-V5. The pORF54 antiserum immunoprecipitated pORF54-V5 but not LacZ-V5. In addition, the pre-immune guinea pig serum did not react with either V5-tagged protein. To confirm expression of pORF54, immunoblot analysis was performed on VZV strain Ellen-infected or mock-infected MeWo cell extracts (Fig 1B). The pORF54 antiserum detected a prominent 87 kDa polypeptide that was unique to the infected cell extract. The observed molecular mass of 87 kDa was consistent with that predicted for the 769 amino acid pORF54 (Visalli et al., 2007). The faint, smaller ~70 kDa band is consistently observed in infected cells but we have not determined its relationship to full-length pORF54.

The Bac to Bac Expression System (Invitrogen, San Diego, CA) was used to create a recombinant baculovirus that expressed the pORF54 gene product, rb-BAC-pORF54. Sf9 insect cells infected with an increasing MOI of rb-BAC-pORF54 yielded increasing amounts of pORF54 detected by the pORF54 antiserum (Fig. 2, lanes 1-4). This 87 kDa band was specific to rb-BAC-pORF54 infected cells and was not detected in uninfected (Fig. 2, lane 5) or two unrelated BAC-infected Sf9 cell extracts (Fig. 2, lanes 6-7). Following the protocol developed by Newcomb et al. (Newcomb et al., 2001), recombinant baculovirus-infected insect cell extracts were processed for the isolation of potential portal structures via sucrose density gradient centrifugation. Sf9 insect cell cultures were infected with rb-BAC-pORF54 or a BAC-GUS negative control virus and incubated with shaking at 28°C for 72 h. All purification steps were performed at 4°C unless otherwise indicated. For each virus, cells were harvested at $1000 \times g$ for 5 minutes (~1.0 ml of packed cells), washed with phosphate buffered saline (PBS, pH 7.4) containing protease inhibitors, pelleted at $1000 \times g$ for 5 min, and resuspended in 0.5 ml of PBS with protease inhibitors. Cells were lysed by three cycles of freezing and thawing at -80°C and 4°C. The lysate was centrifuged for 5 min at $16,000 \times g$ and the supernatant discarded. The pellet was resuspended in 0.5 ml of TNE (pH 8.0) containing 2% Triton X-100, 10 mM dithiothreitol and protease inhibitors and sonicated on

ice with a micro-tip probe. The suspension was centrifuged at $16,000 \times g$ for 5 min and the supernatant discarded. The pellet was resuspended in 0.2 ml of TNE containing 20 mM MgSO_4 , 0.5 mg/ml of DNase I and protease inhibitors followed by a 10 min incubation at room temperature on a rocker-rotator. The suspension was centrifuged at $16,000 \times g$ for 5 min and the supernatant discarded. The pellet was resuspended in 0.5 ml of 1 M arginine (pH 7.4) and incubated for 10 min on a rocker-rotator. The resulting solution was centrifuged at $32,000 \times g$ for 30 min and layered on top of a 10% to 30% sucrose gradient prepared in 20 mM Tris-HCl (pH 7.5) containing 1 M arginine. After centrifugation for 48 h at $105,000 \times g$ in a Beckman SW-41 rotor, the gradient was fractionated into 1.0 ml aliquots from the bottom. The pORF54-enriched fractions were identified by immunoblot using the pORF54 antiserum (Fig. 3). The presence of 87 kDa pORF54 was observed in fractions 10-13 (Fig. 3B) whereas no protein of a similar size was observed in equivalent fraction of the BAC-GUS control-infected samples (Fig. 3A). Fractions that contained full-length pORF54 also contained a 55 kDa protein (fractions 10-12, Fig. 3B). The nature of this 55 kDa band is not currently known but it is likely a proteolytic degradation product of the 87 kDa pORF54 that still reacts with the polyclonal pORF54 antiserum.

It was not possible to observe pORF54 protein via Coomassie blue staining of samples from gradient fractions (data not shown). Therefore, concentrated samples of gradient fractionated material were prepared for electron microscopy. Based on previous studies for the HSV and CMV portal proteins (Holzenburg et al., 2009), it was not unreasonable to assume that pORF54 could self-assemble into a larger, multimeric structures. Assuming that the VZV portal behaves similarly to other herpesvirus portals, a dodecameric structure would yield a complex of nearly 1 Mega-Da. Fractions containing pORF54 were concentrated using Amicon Ultra-0.5ml Centrifugal Filters (Millipore, Billerica, MA) with a 100 kDa cutoff (100 kDa mwco). After concentration, immunoblot analysis with the pORF54 antiserum showed that none of the pORF54 passed through the filter (data not shown) and this is consistent with the oligomeric nature of herpesvirus portal proteins. Treatment with SDS resulted in almost the entire sample passing through the 100 kDa mwco filter and likely represented the monomer form of pORF54 (data not shown). Although this does not formally prove the existence of pORF54 oligomers, the results suggest that pORF54 may form oligomeric structures greater than 100 kDa.

A small amount of undiluted concentrated sample was applied to copper grids and imaged using a Philips CM-100 TEM operated at 100 kV. Negative staining using 2% uranyl acetate (Fig. 4A) showed both individual portals with central holes (black arrows) as well as aggregates of portals that appeared as “balls” or groups of portals (white arrows). Portals containing central holes were typical of those observed previously for HSV (Newcomb et al., 2001), HCMV (Dittmer and Bogner, 2005; Holzenburg et al., 2009) and bacteriophages such as Sf6 (Zhao et al., 2010).

Imaging of the concentrated samples proved to be difficult due to what appeared to be large numbers of the portals aggregating (i.e. “balls” or groups of portals). Based on the primary amino acid sequence, the VZV portal is larger than the HSV and CMV portals by ~15% and thus contains additional amino acid sequences at both the N and C termini. It is possible that the properties of some of these sequences contribute to the stickiness or aggregatory nature of the purified VZV portal. Additional studies will be necessary to examine this aspect in more detail.

In an effort to observe an isolated example of the VZV portal, the concentrated sample was diluted and briefly sonicated. Although rare, it was possible to observe independent portal structures. A remarkable example of an axial view of a single VZV portal ring is shown in Fig. 4B. The same portal is further magnified and contrast-enhanced in Fig. 4C. Some detail

of the monomer subunits can be seen as jagged edges or striations along the outside edge and inside channel (Fig. 4C). A schematic representing these features is represented by Fig. 4D. For comparison, an equivalent field of an analogous gradient-purified fraction of the control baculovirus (BAC-GUS) that does not express pORF54 is shown in Fig. 4B and 4C. No portal structures were observed for BAC-GUS.

Newcomb et al. (Newcomb et al., 2001) estimated the width of the crown region of the HSV portal of pUL6 to be ~16.6 nm. The width of the portals observed in Fig. 4A-C, ranged from roughly 18-22 nm. The 87 kDa VZV monomer is approximately 16% larger than the 75 kDa, pUL6 monomer. Assuming the number of subunits required to form the portals is the same, a size of between 18-20 nm is consistent with the predicted size for the VZV portal. Lastly, it is not surprising to see some variation in the size of portal structures. This may be a result of how the portal structure is sitting on the grid surface and/or how many portal monomers make up each particular ring. Newcomb et al. showed previously that the HSV portal can form structures containing different numbers of pUL6 monomers (Newcomb et al., 2001).

This study reports the generation of a VZV pORF54-specific antiserum and subsequent identification of the 87 kDa ORF54 protein encoded by VZV ORF54. Expression of ORF54 in a recombinant baculovirus system allowed for the enrichment of pORF54 via sucrose density gradient centrifugation in the presence of 1M arginine. Enriched fractions analyzed by TEM revealed structures similar to those previously reported for other herpesvirus and bacteriophage portal proteins.

The *Herpesviridae* encode a family of protein homologs whose function is to act as the “port of entry” for insertion of the viral DNA into preformed capsids during encapsidation. The process of DNA encapsidation is best understood in HSV and HCMV where the genes encoding the encapsidation proteins were first characterized. The identification of encapsidation-specific antiviral inhibitors for HSV, HCMV, and VZV suggests that encapsidation is a valid antiviral target for herpesviruses (Biron, 2006; Bogner, 2002; Buerger et al., 2001; Di Grandi et al., 2004; Reefsclaeger et al., 2001; Underwood et al., 1998; van Zeijl et al., 2000; Visalli et al., 2003). Previous reports have identified the capsid portal proteins VZV pORF54 (Visalli et al., 2003) and HSV pUL6 (van Zeijl et al., 2000) as viable antiviral targets. Reagents, including pORF54 expressing baculoviruses and the thiourea small molecule inhibitors, will be crucial for detailed studies of the VZV portal protein. It will be of interest to examine portal formation in the presence of inhibitors to potentially identify the precise mechanism of action of the thiourea series. Further investigation of the herpesvirus portals may yield new targets for antiviral drug development.

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Fig. 1.

(A) Specificity of the pORF54 polyclonal guinea pig antiserum. V5 epitope-tagged transcription/translation (TNT) products of Lac Z (118 kDa; lane 8) or ORF54 (91 kDa; lane 7) were synthesized *in vitro* and immunoprecipitated with anti-V5 monoclonal antibody (lanes 1 and 4), pORF54 antiserum (lanes 2 and 5), or pre-immune guinea pig serum (lanes 3 and 6). Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with anti-V5 monoclonal antibody. The V5 specific antibody (lanes 1 and 4) precipitated both Lac Z-V5 and pORF54. However, only the pORF54 antiserum precipitated pORF54-V5 (lane 5). Pre-immune guinea pig serum did not precipitate the Lac Z-V5 (lane 3) or pORF54-V5 proteins (lane 6). For reference purposes, a sample of each of the *in vitro* synthesized pORF54-V5 (91 kDa) (lane 7) and Lac Z-V5 (118 kDa) (lane 8) products was loaded directly on a gel and detected with anti-V5 antibody. (B) Identification of pORF54 in VZV-infected MeWo cells. MeWo cells were infected with VZV strain Ellen (Inf) or mock infected (Mock) for 72 hours. Total cell extracts were prepared and analyzed via immunoblotting with the pORF54 antiserum. A prominent band of the predicted molecular mass of 87 kDa was detected in infected (Inf) but not in mock-infected (Mock) cell extracts.

Fig. 2.

Detection of pORF54 in recombinant baculovirus infected Sf9 cells. Sf9 insect cell cultures were infected with increasing MOI of rb-BAC-pORF54 VZV (lanes 1-4). Control cultures were prepared with uninfected cells (lane 5) and two baculoviruses that do not express pORF54, rb-BAC-pORF30 (lane 6) and BAC-GUS (lane 7). The presence of pORF54 was detected by immunoblotting cell extracts with the pORF54 antiserum. A dose-dependent increase in pORF54 (87 kDa) expression was observed in rb-BAC-pORF54-infected cells (lanes 1-4) but not in any of the control samples (lanes 5-7).

Fig. 3.

Sucrose gradient fractionation of processed Sf9 insect cell extracts. Sf9 insect cell cultures were infected with either rb-BAC-pORF54 or BAC-GUS. Fourteen different gradient fractions were analyzed for the presence of the 87 kDa pORF54 protein. pORF54 was strongly detected in fractions 10-13 of the rb-BAC-Page 12 pORF54 sample (B) but not in the BAC-GUS control samples (A).

Fig. 4.

(A) Sucrose gradient purified portals were concentrated and negatively stained with 2% uranyl acetate. Black arrows represent examples of individual portals. White arrows show aggregates or clusters of portals. The central channel is clearly visible. (B) Axial view of an individual portal stained with 2% uranyl acetate. A similar field of material from an analogous fraction of BAC-GUS control is provided for comparison. (C) Enlarged and contrast enhanced field of axial view of portal from panel B. (D) Schematic of axial view of portal observed in panels B and C.