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The N-terminus of vaccinia virus host range protein C7L is essential for function

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Abstract

Vaccinia virus (VACV), a member of the *Poxviridae* family of large double-stranded DNA viruses, is being used as a smallpox vaccine as well as an expression vector for immunization against other infectious diseases and cancer. The host range of wild type VACV is very broad among mammalian cells. C7L is a host range gene identified in VACV and is well conserved in mammalian poxviruses except for parapoxviruses and molluscum contagiosum virus. The molecular mechanisms by which the C7L gene exerts host range function are not well understood. The C7L protein does not have any known conserved domains or show sequence similarity to cellular proteins or viral proteins other than the C7L homologues in mammalian poxviruses. We generated recombinant vaccinia viruses carrying deletion mutants of the C7L gene using NYVAC as a parental strain and found that the N-terminus is essential for host range function of C7L, which is consistent with a previous report that showed homology among C7L homologues are greater near the N-terminus than the C-terminus.

Keywords

vaccinia virus; host range gene; C7L; poxvirus; immunomodulatory gene

Introduction

Vaccinia virus (VACV) is a member of the *Poxviridae* family of large double-stranded DNA viruses [1] and is being used as a smallpox vaccine [2] as well as an expression vector for immunization against other infectious diseases and cancer [3, 4]. The host range of wild type VACV is very broad among mammalian cells. The first host range gene, K1L, was identified by analyzing mutant virus which lost the ability to grow in human cell lines [5, 6]. C7L, the second host range gene identified in VACV, can compensate for K1L function in human cells, but not in rabbit cells [7]. Either the K1L or C7L gene is necessary for replication in human cells [7, 8]. Contrary to the K1L gene, the C7L gene is well conserved in mammalian poxviruses except for parapoxviruses and molluscum contagiosum virus [9, 10]. The C7L homologues *myxoma virus* M62R and *Yaba-like disease virus* 67R genes are functionally equivalent to VACV C7L in human cells [10].

The molecular mechanisms by which the C7L gene exerts host range function are not well understood. Najera et al. reported that C7L has an anti-apoptotic function [11]. When HeLa

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cells were infected with VACV NYVAC strain, a highly attenuated strain which lacks 18 genes including both C7L and K1L [12], there was a translational block of late genes, and infected cells underwent apoptosis [11]. NYVAC-C7L, into which the C7L gene was re-introduced, regained the ability to replicate in HeLa cells and did not induce apoptosis, suggesting the C7L gene is an inhibitor of apoptosis [11]. Meng et al. reported that both K1L and C7L can inhibit antiviral effectors induced by type I interferons [13] and by interferon regulatory factor 1 (IRF1) [14]. However, which interferon-stimulated genes are inhibited by the K1L and the C7L genes are not known. Recently *myxoma virus* M62R protein was found to interact with human sterile alpha motif domain containing protein 9 (SAMD9) [15], which can be induced by interferon- α , β or γ [16]. SAMD9, however, did not interact with C7L or *Yaba-like disease virus* 67R proteins [15].

The C7L protein does not have any known conserved domains or show sequence similarity to cellular proteins or viral proteins other than the C7L homologues in mammalian poxviruses [15]. Figure 1 shows amino acid sequence homology among C7L and its homologues including *myxoma virus* M62R and *Yaba-like disease virus* 67R. In order to determine which part of the C7L gene was required for host range function, we generated recombinant vaccinia viruses carrying deletion mutants of the C7L gene using NYVAC as a parental strain and found that the N-terminus is essential for host range function of C7L.

Materials and Methods

Generation of recombinant viruses

The C7L cDNA was amplified by PCR from genomic DNA of VACV Copenhagen strain, VC-2 (kindly provided by James Tartaglia and Enzo Paoletti of Virogenetics, New York, NY, USA). The cloned DNA fragment was sequenced and confirmed to be identical to the published sequence (GenBank: M35027.1). Additional sequences for Sall and ApaI restriction enzyme recognition sites for subcloning and influenza hemagglutinin (HA) epitope tag, “YPYDVPDYA”, for protein detection by an anti-HA antibody were introduced by PCR. Deletion mutants of the C7L gene were generated by PCR. The first ATG was added when the HA tag was added to the 5' end of the cDNA and when the 5' deletion was introduced to the cDNA. All DNA fragments generated by PCR were sequenced by the Nucleic Acid Facility at the University of Massachusetts Medical School to confirm that no unintended mutations were introduced by PCR. The wild type and all modified C7L genes are summarized in Fig. 2A.

We followed a standard procedure to generate recombinant vaccinia viruses [17, 18] with some modification. The wild type and modified C7L cDNAs were subcloned into a vaccinia virus transfer vector pSC11MJ, a modified pSC11ss plasmid vector whose multiple cloning site we replaced with that of another vaccinia virus transfer vector pMJ601 (both plasmid vectors were kindly provided by Bernard Moss of National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA), which has the P7.5 early/late promoter for foreign gene expression (C7L is known to be an early gene [8, 19, 20], but we did not use C7L gene's own promoter to express re-introduced C7L) and the J2R gene (encoding thymidine kinase) fragments for homologous recombination [17]. We, however, were not able to use bromodeoxyuridine for selecting plaques because the protein coding region of the J2R gene was deleted in the NYVAC strain [12]. To perform positive selection we introduced the Neomycin resistance gene into the transfer vector. We amplified the Neomycin resistance gene cDNA from pcDNA3.1(-) plasmid (Invitrogen Corporation, Carlsbad, CA, USA), and introduced it into the transfer vector under the another P7.5 early/late promoter (resultant transfer vectors had two P7.5 early/late promoters) (Fig. 2B). We used CV-1 cells (ATCC® # CCL70), which are permissive to the NYVAC strain [12], for homologous recombination. CV-1 cells were infected with the NYVAC strain at a

multiplicity of infection (moi) of 0.05, incubated for two hours, and transfected with 5 µg of the transfer vector and Superfect® transfection reagent (Qiagen Inc. Valencia, CA, USA). After a two hour incubation, cells were washed and culture medium (minimal essential medium containing L-glutamine, penicillin, streptomycin, and 10% fetal bovine serum (MEM-10)) with Neomycin (G-418 disulfate, Research Products International Corp. Mount Prospect, IL, USA) at 0.125~0.25 µg/ml were added. Cells were harvested after two days of incubation and cell lysates were prepared by freezing and thawing. Recombinant viruses were selected by infecting CV-1 cell monolayers with the cell lysate in the presence of 0.125~0.25 µg/ml of Neomycin for two days, and then agarose containing Neomycin and X-gal (pSC11MJ has the lacZ gene for X-gal staining) were overlaid onto the infected monolayer. After overnight incubation several blue plaques for each transfer vector were picked up. Three rounds of plaque purification in the presence of Neomycin were performed to each plaque. After large scale preparation [21], recombinant viruses were titrated using CV-1 cells and the expression of the HA-tagged C7L proteins was confirmed by Western blotting of infected CV-1 cell lysates. Primary antibody was anti-HA high affinity rat monoclonal antibody (clone 3F10, Roche Applied Science, Indianapolis, IN, USA) and the secondary antibody was goat anti-rat IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bound antibodies were visualized by HyGLO™ Quick Spray Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific, South Plainfield, NJ, USA) and HyBlot CL® Autoradiography Film (Denville Scientific) was exposed to the membrane (Fig. 2C). Western blotting detecting GAPDH protein as a control was previously described [22]. A control recombinant vaccinia virus expressing a fusion protein of DSRred and Neomycin phosphotransferase (Neomycin resistance gene product) was also generated. The DSRred cDNA from the pDSRed-Express-C1 (Clontech Laboratories, Mountain View, CA, USA) and the Neomycin resistance gene cDNA from pcDNA3.1(-) plasmid (Invitrogen Corporation) were combined and subcloned into the pSCMJ11 transfer vector.

Infection of HeLa cells

HeLa cells (ATCC® # CCL-2) were maintained with MEM-10. Monolayers of HeLa cells in 6-well plates were inoculated with vaccinia viruses at an moi of 0.01. After two hours of incubation inocula were removed and fresh MEM-10 medium was added. 24, 48, and 72 hours after infection cells were scraped. Cells and culture supernatants were separated by centrifugation and stored at -80°C until titration.

Virus titration

Infected cell lysates (cell-associated virus) and culture supernatants (virus released into culture medium) were titrated using permissive CV-1 cells. The cell lysates were frozen and thawed three times and sonicated before titration. CV-1 cell monolayers were incubated with ten-fold dilutions of the infected cell lysates or the culture supernatants for 2 days. Agarose containing X-gal was overlaid onto the monolayers in 6-well plates to detect and count small plaques easily. The NYVAC strain and the WR strain (kindly provided by Girish J. Kotwal of University of Cape Town, South Africa and William L. Marshall of University of Massachusetts Medical School) do not have the lacZ gene and, therefore, their plaques were visualized by crystal violet staining [21, 22]. When ten-fold dilutions of the samples did not produce any plaques, undiluted samples were titrated.

Statistical analyses

For statistical analysis virus titers were converted into log scale (log10), and ANOVA and then t-test (two-sample assuming equal variables) were performed. $P < 0.05$ (two-tail) values were considered statistically significant differences.

Results

We chose the NYVAC strain to introduce C7L deletion mutants because it lacks both the C7L and K1L host range genes but still has the J1R and J3R gene sequences, which can be used for homologous recombination with the transfer vectors we have [12]. The first set of deletion mutants we tested include NYVAC-C7L, NYVAC-N-HA, NYVAC-C-HA, NYVAC- Δ 2-20, NYVAC- Δ 2-40, NYVAC- Δ 111-150 and NYVAC- Δ 131-150 (Fig. 2). We used HeLa cells, which are not permissive to the NYVAC strain, to test the deletion mutants. Consistent with the published study by Najera et al. [11], the NYVAC in which the C7L gene was re-introduced (NYVAC-C7L) was able to grow in HeLa cells, while the control NYVAC expressing the DSRRed/Neomycin phosphotransferase fusion protein (NYVAC-DSRed) was not ($p=0.003$ at 24 hours, and $p=0.0005$ at 48 hours) (Fig. 3). As shown in Figure 3, all of the recombinant vaccinia viruses expressing deletion mutants of C7L (NYVAC- Δ 2-20, NYVAC- Δ 2-40, NYVAC- Δ 111-150 and NYVAC- Δ 131-150) did not grow better than the control NYVAC-DSRed. Both recombinant viruses with the HA epitope-tagged C7L genes (NYVAC-N-HA and NYVAC-C-HA) grew better than the control NYVAC-DSRed (for NYVAC-N-HA $p=0.009$ at 24 hours and $p=0.002$ at 48 hours, and for NYVAC-C-HA $p=0.002$ at 24 hours and $p=0.0005$ at 48 hours) (Fig. 3). The NYVAC-C-HA and the NYVAC-C7L showed no difference in titer at either 24 or 48 hours (Fig. 3). However, the NYVAC-N-HA did not grow as well as the NYVAC-C7L ($p=0.005$ at 24 hours and $p=0.008$ at 48 hours), suggesting the N-terminal HA epitope tag influences or interferes with host range function of the C7L (Fig. 3).

To separate the effect of the addition of the HA epitope tag from the effect of the deletion mutant, we generated C-terminal deletion mutants with the HA epitope tag added to the C-termini rather than the N-termini (NYVAC- Δ 131-150(C-HA) and NYVAC- Δ 141-150) (Fig. 2A). We also generated a smaller deletion in the N-terminus of the C7L gene (NYVAC- Δ 2-10) (Fig. 2A). We tested the new deletion mutants, and the parental NYVAC strain (Fig. 4). To make sure the C7L mutants didn't affect virus release, both cell lysates, which measure cell-associated virus (Fig. 4A and B), and culture supernatants, which measure the virus released into culture medium (Fig. 4C and D), were titrated. Figure 4A and C show growth of the wild type vaccinia virus WR strain and the parental NYVAC strain in HeLa cells measuring cell-associated virus and virus released into medium. There was no statistically significant difference between the NYVAC-C7L and the WR strain, or between the parental NYVAC and the NYVAC-DSRed (the WR and the NYVAC strains do not have the lacZ gene, their plaques were visualized by crystal violet staining, not by X-gal staining). When cell-associated viruses were compared (Fig. 4A and B), the NYVAC- Δ 141-150 deletion mutant grew better than the control NYVAC-DSRed ($p=0.01$, 0.003 and 0.0008 at 24, 48 and 72 hours), but the NYVAC- Δ 131-150 (C-HA) deletion mutant did not grow better than the control. This suggests that last 10 amino acids (141st ~150th) of the C7L protein are not necessary but 10 amino acids prior to the last 10 (131st~140th) are essential for the host range function. In contrast, deletion of the first ten amino acids (excluding the first methionine) completely destroyed the host range function (NYVAC- Δ 2-10) (Fig. 4B and D). Titration of the virus released into culture media (Fig. 4C and D) showed similar results as titrations of cell lysates (Fig. 4A and B).

Figure 5 depicts HeLa cell monolayers 72 hours after infection. The NYVAC-C7L, C-HA, and Δ 141-150 mutants produced large plaques, while the NYVAC-N-HA produced smaller plaques. Consistent with virus titrations shown in Figure 4, the NYVAC- Δ 2-10, Δ 2-20, Δ 131-150 (C-HA) and DSRRed did not produce visible plaques by crystal violet staining.

Yeast two-hybrid (Y2H) screening for protein-protein interactions between vaccinia virus proteins and human proteins detected an interaction between the C7L protein and the YY1-

associated factor 2 (YAF2) protein [23, 24]. YAF2 is a zinc finger protein that interacts with the transcription factor Yin Yang 1 (YY1) [25], and regulates cell survival during zebrafish embryogenesis [26]. YAF2 is also known to inhibit caspase 8-mediated apoptosis [26], which suggests that YAF2 is involved in the anti-apoptotic function of C7L. YY1 is a multifunctional transcription factor known to have a fundamental role in normal biologic processes such as embryogenesis, differentiation, replication, and cellular proliferation [27]. YY1 also negatively regulates vaccinia virus promoters by binding to their initiator elements [28]. Since the YAF2 protein was found to have anti-apoptotic function in zebrafish [26], we tested to see if the NYVAC strain expressing the YAF2 protein (NYVAC-YAF2) would grow better than NYVAC-DSRed. There was, however, no difference in virus growth between NYVAC-YAF2 and NYVAC-DSRed (data not shown).

Discussion

We showed that the last ten amino acids of the C7L protein are not necessary for its host range function in HeLa cells. The N-terminus is more important than the C-Terminus. When the N-terminal ten amino acids were deleted (excluding the first methionine) (Δ 2-10), the C7L protein loses its host range function. Even the addition of the nine amino acid epitope tag at the N-terminus of C7L reduces the host range function. These data are consistent with the previous report by Meng et al. that homology among the C7L homologues are greater near the N-terminus than the C-terminus [10]. Figure 1 shows multiple sequence alignment of C7L and its homologues. *Myxoma virus* M62R protein and *Yaba-like disease virus* 67R protein are found to be able to compensate for C7L in mammalian cells [10]. The last ten amino acids of the C7L protein, which are not necessary for the host range function, have no homology with M62R or 67R, while nine of the first ten amino acids are identical or similar among these three proteins. Meng et al. used epitope tags (V5-TAP tag, not the HA epitope tag) that were added to the C-termini of these C7L homologues and found that the homologues were functional, confirming that the C-terminal amino acids are dispensable for the host range function. Our data also showed that 10 amino acids from 131st to 140th are necessary for the host range function, which is consistent with a recent finding by Meng et al. that replacing tyrosine and isoleucine residues (135th and 136th) with alanines abrogated host range function of C7L in HeLa cells [14].

Although the YAF2 protein has anti-apoptotic function, the NYVAC strain expressing YAF2 (NYVAC-YAF2) did not regain host range function. In the Y2H screening the bait (in this case the C7L protein) was fused to the C-terminus of the GAL4BD and the prey (YAF2 protein) was fused to the C-terminus of the GAL4AD [23, 24]. Our attempt to detect YAF2-C7L interaction in HeLa cells by mammalian two hybrid or co-immunoprecipitation assays were not successful (data not shown), although it is possible that the experimental conditions were not optimal to detect the interaction. It should be pointed out that the C7L protein used in the Y2H screening is not full-length. It lacks the first nine amino acids and the last 20 amino acids [23] and, based on our findings, is unlikely to retain host range function. We also performed pull-down experiments using NYVAC-C-HA and anti-HA antibody to detect cellular protein(s) interacting with the C7L protein in infected cells, as we did to detect K1L-ACAP2 interaction [29]. However, we were not able to detect any interacting protein (data not shown). Meng et al. suggested that type I interferon-inducible gene(s), especially gene(s) regulated by IRF1, is a likely target of C7L's host range function [13, 14]. However, the interaction may not be strong enough to be detected by a pull-down experiment or the target of direct interaction may not be a protein. It is also likely that different C7L family members may bind to different target proteins which are involved in the similar cellular function. Alternative approaches may be necessary to determine C7L's cellular target(s).

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[illegible]

Fig. 1. Amino acid sequence alignment of C7L and its homologues
Multiple alignment was performed by CLUSTALW (available at <http://www.genome.jp/tools/clustalw/>). Protein accession numbers are shown in parentheses next to species names. The top nine are highly homologous members of the C7L family. The middle four are less homologous but were experimentally shown to function equivalently to vaccinia C7L in human cells [10, 14]. *Myxoma virus* M63R and M64R and cowpoxvirus CPXV020 proteins also show some homology to C7L, but they did not compensate C7L function [10, 14]. “***” shows the tyrosine and isoleucine residues found important for C7L function [14].

Fig. 2. Recombinant vaccinia viruses used in this study

(A) Schema of the modified C7L genes. A codon for a first methionine (Met) was added when the HA tag was added at the N-terminus of the C7L (N-HA, $\Delta 111-150$ and $\Delta 131-150$) and when N-terminal deletion was introduced ($\Delta 2-10$, $\Delta 2-20$ and $\Delta 2-40$). (B) Schema of the inserted DNA fragment into the NYVAC strain. The LacZ cDNA is in the opposite orientation compared to the C7L and Neo^r cDNAs. (C) Western blotting detecting the HA-tagged wild type and modified C7L proteins. CV-1 cells were infected with indicated viruses at an moi of 0.1 for two days. Cell lysates were separated by SDS-PAGE (4~15%) and transferred onto PVDF membranes. Primary antibodies were anti-HA high affinity rat monoclonal antibody (Roche Applied Science) and mouse anti-GAPDH (6C5) antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Fig. 3. Virus growth in HeLa cells (1)

HeLa cells were infected with the NYVAC viruses carrying the wild type (C7L) or the HA epitope-tagged (N-HA and C-HA) C7L genes, C7L deletion mutants ($\Delta 2-20$, $\Delta 2-40$, $\Delta 111-150$ and $\Delta 131-150$) or the DSRRed/Neomycin phosphotransferase fusion protein (DSRed). Titers of cell-associated viruses 24 and 48 hours after infection of HeLa cells are shown. 5000 plaque forming unite (pfu) of the virus were inoculated onto each well in 6-well plates. Cell lysates were titrated using permissive CV-1 cells. Results are shown as log10 pfu per well. Averages and standard deviations of three independent experiments are shown. $\Delta 2-40$ (light green) line is overlapping with $\Delta 131-150$ line (dark green).

Fig. 4. Virus growth in HeLa cells (2)

HeLa cells were infected with the NYVAC viruses carrying the wild type (C7L) or the HA epitope-tagged (N-HA and C-HA) C7L genes, C7L deletion mutants ($\Delta 2-10$, $\Delta 2-20$, $\Delta 131-150$ (C-HA) and $\Delta 141-150$), or the DSRed, depicted in Fig. 2 as well as the wild type WR strain and the parental strain NYVAC. Cell-associated virus and virus released into culture medium were titrated using permissive CV-1 cells. **(A)** Titers of cell-associated viruses in HeLa cell lysates infected with the WR and the NYVAC strains. Plaques were visualized by crystal violet staining of CV-1 cell monolayers. **(B)** Titers of cell-associated viruses in HeLa cell lysates infected with the NYVAC viruses carrying the wild type (C7L) or the HA epitope-tagged (N-HA and C-HA) C7L genes, C7L deletion mutants ($\Delta 2-10$, $\Delta 2-20$, $\Delta 131-150$ (C-HA) and $\Delta 141-150$), or the DSRed. Plaques were visualized by X-gal staining of CV-1 cell monolayers. **(C)** Titers of viruses released in culture medium from HeLa cells infected with the WR and the NYVAC strains. Plaques were visualized by crystal violet staining of CV-1 cell monolayers. **(D)** Titers of viruses released in culture medium from HeLa cells infected with the NYVAC viruses carrying the wild type (C7L) or the HA epitope-tagged (N-HA and C-HA) C7L genes, C7L deletion mutants ($\Delta 2-10$, $\Delta 2-20$, $\Delta 131-150$ (C-HA) and $\Delta 141-150$), or the DSRed. Plaques were visualized by X-gal staining of CV-1 cell monolayers.

Fig. 5. Plaque formation in HeLa cells

HeLa cells were infected with the NYVAC viruses carrying the wild type (C7L) or the HA epitope-tagged (N-HA and C-HA) C7L genes, C7L deletion mutants ($\Delta 2-10$, $\Delta 2-20$, $\Delta 131-150$ (C-HA) and $\Delta 141-150$), or the DSRed. Crystal violet staining of HeLa cell monolayers in 6-well plates 72 hours after infection at an moi of 0.01 are shown. $\Delta 2-10$, $\Delta 2-20$, $\Delta 131-150$ (C-HA) and DSRed wells do not have any visible plaques. N-HA well has plaques but they are less clear than plaques in C-HA and C7L wells due to smaller size.