Independent Expression and Analysis of the Two Domains of Vaccinia Virus's E3L Gene

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Abstract and Background

Vaccinia virus (VACV) is a large double-stranded DNA virus that has been extensively studied as a safe alternative for a vaccine or gene therapy delivery vector, and can easily be manipulated to construct recombinants. Replication of the VACV virus has been shown to be resistant to pre-treatment of cells with type-I interferon (IFN). The activity of the E3L gene has been shown to cause this phenotype. This gene encodes a 190-amino acid protein has a C-terminal double-stranded RNA-binding domain and a N-terminal half that is a member of the Zα family of Z-DNA-binding domains. Our study, headed by Dr. Bertram Jacobs and Susan Holechek of ASU's Biodesign Institute, will explore the IFN inhibition properties of E3L's two domains when they are expressed independently. The N-terminus will be inserted in the E3L locus of a recombinant VACV virus lacking E3L, while the C-terminus will be inserted into the VACV thymidine kinase locus. Mutants expressing both domains of E3L separately will be constructed and assayed for equivalent expression. Viral replication as well as activation of the IFN system will be evaluated in previously characterized cell lines and under conditions that require both domains to be present in order to inhibit the IFN pathway upon VACV infection. If pathogenesis is retained, it can be concluded that fusion of the two domains is not necessary in order to inhibit the IFN response. This work will provide a better understanding of the function of the E3L protein and how VACV is evading the activation of the IFN pathway. Because VACV is used as a vector against other diseases, further understanding of how VACV evades the immune system could lead to the development of better and safer vaccines.

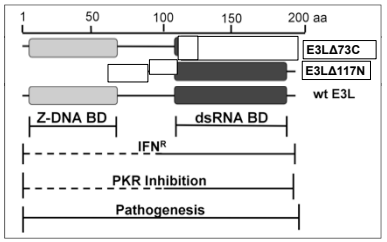

Figure 1. *Vaccinia Virus* E3L gene domains

Introduction

N-terminus of E3L, corresponding to the Z-DNA binding domain, is essential to VACV pathogenicity.

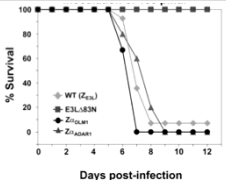

Figure 2. Lethality of viruses on mice after intra-cranial inoculation with 100 p.f.u. From Kim et al 2003.

Mutant Viruses

Table 1. *Vaccinia Virus* mutant constructs

Virus	Locus	Mutant Role
E3LΔ117N-FLAG	E3L	Control, dsRNA binding domain independent expression
E3LΔ73C-FLAG	E3L	Control, and parental viruses for mutants. Z-DNA binding domain independent expression
E3LΔ117N-FLAG TK/ opp	TK: Z-DNA binding domain E3L: dsRNA binding domain	Dual expression of the two domains

Virus Constructs

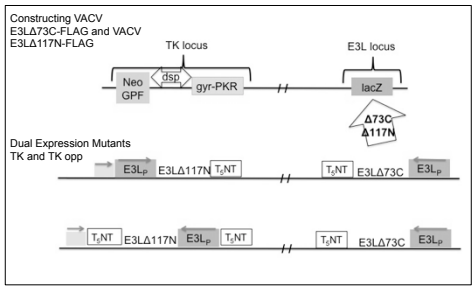

Figure 3. Virus Constructs. All mutant viruses were constructed using the parental mutant shown at the top of figure 3. Neo= neomycin resistance gene; GFP= green fluorescent protein. Gyr-PKR = gyrase-PKR; dsp= double synthetic promoter.

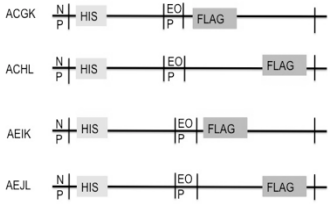

Figure 4. Old virus constructs. VACVΔE3L was used as parental strains to insert the appropriate construct using MPA selection. AC viruses contain amino acids 1-72 and 73-190 and AE viruses contain amino acids 1-83 and 84-190. NP= normal promoter. EOP= early optimized promoter for VACV.

Methods

Plasmid Construction. PCR fragments corresponding to E3L Δ117N and E3LΔ73C were inserted into the *Bam*HI and *Pst*I sites of plasmid JCO1 which has E3L right and left arms flanking the restriction sites, as well as *E. coli* gpt.

PIVR. Recombinant plasmids were used to transfect BHK cells using Lipofectamine and Plus reagent according to manufacturer's protocol (Invitrogen), and using parental virus VACVΔE3LΔTK::GP-GNR. After reaching full CPE (cytopathic effect) cells were harvested and proceeded to three rounds of freeze/thaw.

MPA Selection. PIVR dilutions were used to infect BHK cells pretreated with MPA media containing hypoxanthine, MPA, and xanthine. Following infection, cells were kept on MPA media until plaques were observed. Monolayers were then overlayed with low melting point agarose solution including MPA media and X-gal. During the first 3 rounds of MPA selection blue plaques were picked. Three more rounds without MPA were conducted and clear plaques were picked.

Virus Amplification. Selected plaques were amplified in BHK cells, and viral genomes were extracted and sent for sequencing.

Western Blots. BHK cells were infected at an MOI of 5 and proteins were harvested 24 hours later using the QiaShredder (Qiagen). 20% SDS-PAGE gels were run before transferring overnight at 4 degrees C. Detection was made by Dura and Pico chemiluminescent kits (Thermo Scientific). Anti-FLAG M2 monoclonal antibodies were used (Sigma). E3L detection was made using polyclonal antibodies raised in rabbit (Jacobs lab).

Results and Analysis

Controls: Δ E3L, WT 03, WT FLAG, Δ73C

VACV E3LΔ117N-FLAG (figure 5)
- FLAG western did not detect any Δ117N protein at the expected size of ~9 kDa
- WT FLAG detects 25 and 20 sized proteins
- Wild type (WT 03) does not have FLAG tag
- E3L western did not detect any Δ117N protein at the expected size of ~9 kDa

VACV E3LΔ73C-FLAG (figure 6)
- FLAG Δ117N ~ 14 kDa protein
- Δ73C control does not have FLAG tag
- E3L Δ73C ~14 kDa protein (indicated with*)

Old Virus Constructs (figure 7)
FLAG tag at the N terminus gives better expression

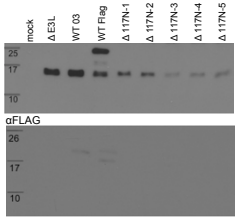

Figure 5. VACV E3LΔ117N-FLAG expression

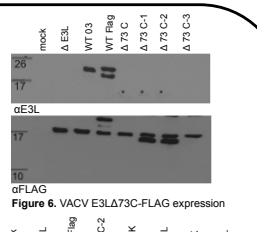

Figure 6. VACV E3LΔ73C-FLAG expression

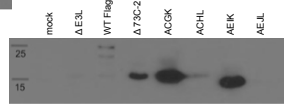

Figure 7. Old Virus constructs expression

Figure 8. Plaque morphology of mutants. VACV E3LΔ117N-FLAG viruses still cause plaques, but morphology is different from WT viruses.

VACV E3LΔ73C-FLAG viruses do not cause plaques to form as expected.

A. mock infection **B.** VACV infection **C.** VACV E3LΔ117N-FLAG **D.** VACV E3LΔ73C-FLAG

Conclusions

- VACV E3LΔ73C-FLAG and VACV E3LΔ117N-FLAG viruses were successfully created.
- VACV E3LΔ73C-FLAG viral proteins of the expected 14 kDa for E3L's N terminus were successfully detected in chemiluminescent westerns.
- VACV E3LΔ117N-FLAG proteins of the expected 9 kDa size for the E3L protein's C terminus were not detected in chemiluminescent westerns.
- Δ117N viruses causes plaques to form in BSC40 cells, but morphology was different than wild type viruses' plaques, indicating that viruses formed but were weaker. Also suggests that the Δ117N E3L protein was expressed but misfolding or degradation prevented full maturation and function of protein.
- Previous studies where E3L proteins lacking the first 83 amino acids were shown to cause normal plaques in BSC40 cells. Therefore, the linker region between both domains of E3L protein is important in protein folding and/or functioning.
- Old virus constructs yielded westerns that showed FLAG tags allowed better protein detection if placed at the N terminus of the desired protein.
- The position of the FLAG tag is important for the correct folding of the C terminus.

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