Poster B1-1141 : The N1 Vaccinia Virus Virulence Factor Inhibits Host Immune Responses

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Abstract

Background: Poxviruses encode proteins that increase viral virulence by modulating host immune responses, including a few member family of proteins thought to modulate signaling by the innate immune system. N1 was the first member of this family to be identified and N1 enhances vaccinia virus (VACV) vir-plenia by 10,000-fold in animal models of infection. The crystal structure of the N1 protein reveals a bcl-2-like fold. Similarly to cellular bcl-2, N1 inhibits apoptosis. N1 also inhibits signaling by NF-κB and IRF-3 transcription factors. Here we investigated the mechanisms by which N1 inhibits NF-κB signaling. VACV N1 and N1 mutants. Vaccinia virus (VACV) strain WR and N1 mutants were generated. HeLa cells transfected with an N1 and N1 mutants were treated or t 1 nM staurosporine and assessed for apoptosis by Annexin V staining.

Results: N1 is a phosphoprotein that binds IKK-β, a transcription factor key to cell survival, antiviral cytokine production, and cellular and humoral immunity. Furthermore, AP-1 and NF-κB-dependent transcripts such as IL-1alpha, CD83, CCR4 and bcl-xl were suppressed in human macrophages infected with wild type, but not N1 deficient, VACV. In cells infected with wild type VACV, bcl-xl protein was suppressed consistent with an increase in N1 protein. Finally, N1 was also found to be phosphorylated by a host-dependent kinase at an evolutionarily conserved threonine residue. Mutation of this threonine to alanine (T49A) or aspartic acid (T49D) yielded unstable proteins. Mutation of N1 modulated only the antiapoptotic properties of N1. Inhibition of NF-κB signaling by N1 was independent of its phosphorylation.

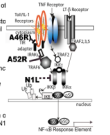
Conclusions: These findings indicate that N1 is consistent with extensive N1-mediated suppression of cellular signaling to anti-viral genes critical to innate and adaptive immune responses. These findings support the hypothesis that inhibition of apoptosis by the N1 phosphoprotein compensates for the anti-survival effects of N1-mediated suppression of NF-κB and AP-1 signaling during VACV infection.

Introduction/Background

Vaccinia virus (VACV) is the poxvirus that was used to eradicate smallpox. Tens of thousands of individuals are innoculated with vaccinia yearly. The canarypox virus (ALVAC) is one of the components of the most effective AIDS vaccine. The vaccinia virus is studied to more fully comprehend mechanisms related to its pathogenicity and to eventually facilitate improved vaccines and antiviral therapeutics.

VACV N1 protein is a potent virulence factor and a strong inhibitor of innate immune signaling

--Toll like receptors (TLRs) are receptors for molecular patterns of microbial pathogens.
--TLRs signal to transcription factc such as NF-kB, inducing antiviral cytokines and interferons.
--All of the characterized A52R family of genes inhibit NF-kB signaling (**bold** right).
--Despite this apparent redundanc in NF-kB inhibition, deletion of A52R, A46R, or N1L significantly attenuates infection.
--N1L is a vaccinia virus gene responsible for a **10,000 fold increase in virulence**. Inhibition c NF-kB and IRF3 signaling, and N1 encodes an anti-apoptotic bcl-2.



Hypothesis

Bcl-2 inhibits signaling to NF-kB and apoptosis. The precise molecular mechanisms for these activities are not fully defined. Since inhibition of NF-kB typically induces apoptosis, we hypothesized that the anti-apoptotic activity of N1 resulted from its inhibition of NF-kB. Further, since N1 inhibits IKK-β, but does not directly associate with IKK-β, we hypothesized that N1 inhibited a kinase distal to IRAK-TRAF6, such as tak1 or MEKK3.

Methods/Results

N1 is a 15 kDa Phosphoprotein

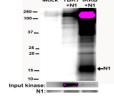


Figure 1. N1L is phosphorylated by a kinase associated with IKK-β. Lysates of HEK cells transfected with control TBK1, IKK-β were added to lysates of HEK cells transfectants, incubated with ATP-32 in a kinase reaction, immunoprecipitated and run on a gel. Lysates containing IKK-β and kinase dead (KD) IKK-β were incubated with phosphorylated N1. These results indicated that N1 was phosphorylated by a kinase other than IKK-β.

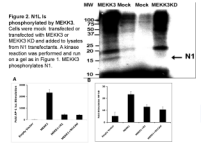


Figure 2. N1L is phosphorylated by MEKK3. Cells were mock transfected or transfected with MEKK3 or MEKK3 KD with equivalent lysates from N1 transfectants. A kinase reaction was performed and run on a gel as in Figure 1. MEKK3 phosphorylates N1.

Figure 3. N1 and its C-terminal Deletion Mutant Inhibit MEKK1 signaling to AP-1 and NF-kB. HEK293 cells were co-transfected with plasmids encoding a TK-Renilla-luciferase, and either empty vector, N1, or N1 C-terminal deletion mutants (N1-1-49) and then were cotransfected with DNA encoding MEKK1 or empty vector. In addition, reporter plasmids encoding AP-1 luciferase were cotransfected in (A) whereas reporter plasmids encoding NF-kB-luciferase were cotransfected in (B).

Results

Figure 5. N1 Solution Structure (Ayogi et al., J. Prot Sci). N1 has an always open thermodynamically unfavorable hydrophobic bcl-2 binding pocket. →Cellular bcl-2/xl inhibit NF-kB signaling and are also phosphorylated.
•Hypothesis: Phospho-N1T49 (shown in red) stabilizes N1's bcl-2 binding pocket, preventing apoptosis.

Figure 6. N1 is phosphorylated at a conserved T49 amino acid. The N terminus of N1 was found to be phosphorylated (not shown); 10 aa deletion mutants of N1 were made. A-deletion mutant of N1 (ΔN1) containing an evolutionarily conserved Threonine 49 (alignment-top) lost detectable phosphothreonine (immunoblot-bottom).

Figure 7. N1, but not N1 mutants, prevent apoptosis as measured by increased Annexin V staining. HeLa cells were co-transfected with the indicated constructs (EV, N1FLAG, T49AFLAG, and T49DFLAG) and a GFP expression plasmid, and analyzed by flow cytometry for Annexin V staining. The bar graph indicate staurosporine treatment.

Conclusions

- **VACV N1 is a potent antiapoptotic virulence factor whose solution structure has the fold of cellular bcl-2/x.**
- **Both N1 and C-terminally deleted N1 inhibit MEKK1 signaling to NF-kB and AP-1.**
- **Mutation at the threonine 49 residue inhibits antiapoptotic functions of N1.**
- **MEKK3, not IKK-β phosphorylates N1, and N1 is phosphorylated at T49.**

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