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Vaccinia virus A43R gene encodes an orthopoxvirus-specific late non-virion type-1 membrane protein that is dispensable for replication but enhances intradermal lesion formation

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ABSTRACT

The vaccinia virus A43R open reading frame encodes a 168-amino acid protein with a predicted N-terminal signal sequence and a C-terminal transmembrane domain. Although A43R is conserved in all sequenced members of the orthopoxvirus genus, no non-orthopoxvirus homolog or functional motif was recognized. Biochemical and confocal microscopic studies indicated that A43 is expressed at late times following viral DNA synthesis and is a type-1 membrane protein with two N-linked oligosaccharide chains. A43 was present in Golgi and plasma membranes but only a trace amount was detected in sucrose gradient purified mature virions and none in CsCl gradient purified enveloped virions. Prevention of A43R expression had no effect on plaque size or virus replication in cell culture and little effect on virulence after mouse intranasal infection. Although the A43 mutant produced significantly smaller lesions in skin of mice than the control, the amounts of virus recovered from the lesions were similar.

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Introduction

The *Poxviridae*, a group of large, complex DNA viruses that replicate exclusively in the cytoplasm, are divided into the *Chordopoxvirinae* and *Entomopoxvirinae* subfamilies (Moss, 2007). The chordopoxviruses consist of eight genera of which the orthopoxviruses are best known because of two members: variola virus, the causative agent of smallpox, and vaccinia virus (VACV), used to eradicate smallpox by prophylactic immunization. VACV, the prototype poxvirus, encodes ~200 genes nearly half of which are present in all chordopoxviruses (Upton et al., 2003). The evolutionarily conserved genes tend to be centrally located within the genome and are mostly involved in essential replication functions, while the genus- and species-specific genes are nearer the ends of the genome and typically involved in host interactions. Proteins that are dispensable for poxvirus replication in cell culture can have a wide variety of roles that directly or indirectly affect virulence by enabling virus spread or resisting immune defenses (Fallon and Alcami, 2006; McFadden, 2005). Studies of such genes can contribute to our understanding of cell biology and immunology and are important for development of vaccines and antivirals.

Since not all VACV genes have been characterized, it seems likely that additional proteins involved in host interactions will be discovered. The A43R open reading frame (ORF) is a candidate as it is embedded within the variable region of the genome. For example, the A41L ORF encodes a chemokine binding protein (Bahar et al., 2008) and the protein encoded by A46R inhibits TLR signaling (Stack et al., 2005). In this study we provided the initial characterization of A43, the product of the A43R ORF, which was expressed after viral DNA replication as a glycosylated protein that associated with Golgi and plasma membranes but was not appreciably incorporated into virus particles. The gene was found to be dispensable for VACV replication in cell culture but caused smaller than normal intradermal lesions in mice.

Results

A43R is conserved among orthopoxviruses

The A43R ORF (VACV WR168) is predicted to encode a 194-amino acid protein with N- and C-terminal hydrophobic domains. The SignalP program (Bendtsen et al., 2004) predicted that the N-terminal hydrophobic region is a signal sequence with cleavage occurring between S22 and S23 (Fig. 1). A43R is highly conserved within all orthopoxviruses; the sequence identity is >94% except for ectromelia virus which has a 78% identity (Fig. 1). However, there are no recognized homologs in any other poxvirus genus, nor are there non-poxvirus homologs or functional motifs to help predict the function of A43.

A43 is a glycosylated protein expressed at the late stage of VACV replication

A recombinant virus, in which a V5 epitope tag was added to the C-terminus of A43, was constructed to assist in protein character-

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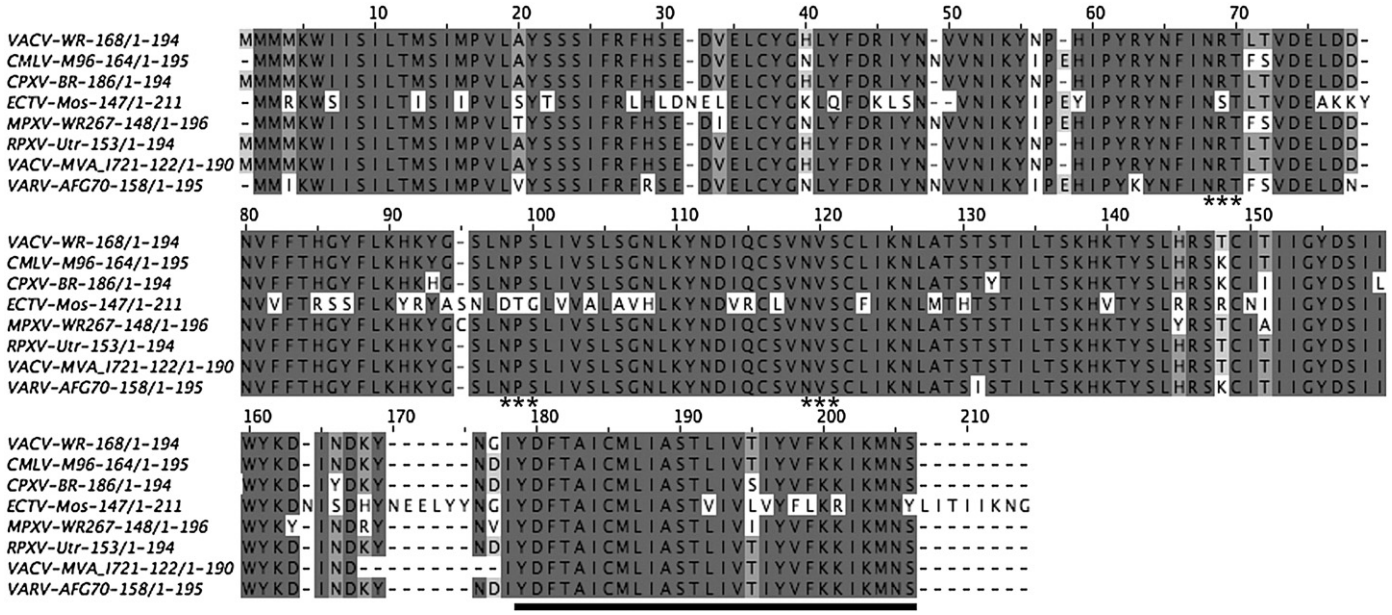

Fig. 1. Multiple sequence alignment of A43 orthologs. Jalview (Waterhouse et al., 2009) was used to construct a multiple sequence alignment of A43 orthologs from orthopoxvirus species. The ORFs are indicated after the species and strain names. Abbreviations: VACV-WR, VACV strain WR; CMLV-M96, camelpox virus strain M96; CPXV-BR, cowpox virus strain Brighton; ECTV-Mos, ectromelia virus strain Moscow; MPXV-WR267, monkeypox virus Walter Reed 267; RPXV, rabbitpox virus strain Utrecht; VACV-MVA, VACV strain Modified VACV Ankara; VARV-AFG70, variola virus strain Afghanistan 1970. The asterisks indicate the Asn-X-Ser/Thr potential glycosylation sites. The predicted transmembrane domain is underlined. Note that the numbering includes spaces for alignment so does not correspond precisely to the VACV sequence.

ization. The region upstream containing the promoter sequence was unaltered so as not to perturb expression. The growth kinetics and plaque phenotype of vA43V5 were similar to that of the parental virus (not shown). The sequence TAAATG, present at the start of the A43R ORF, is a characteristic of late promoters (Davison and Moss, 1989). To experimentally determine the time of synthesis of A43V5, BS-C-1 cells were infected with the recombinant virus and whole cell lysates were prepared at intervals and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting. A band visualized by binding of the anti-V5 MAb migrated more slowly than the predicted 23-kDa mass of A43V5 (Fig. 2A). The band was detected at 0 time, indicating its presence in the unpurified virus inoculum, but did not increase significantly until 6 h and then continued to increase up to 24 h. This pattern was similar to that of the well-characterized late A3 protein (Fig. 2A). Neither A43 nor A3 was synthesized in the presence of the DNA replication inhibitor AraC (data not shown), which is indicative of VACV proteins expressed at the late stage of infection.

Inspection of the predicted amino acid sequence of A43 revealed three potential N-glycosylation sites that could account for the relatively slow electrophoretic migration of A43. To determine the state of glycosylation of A43, a whole cell lysate from vA43V5 infected cells was divided into portions that were untreated or treated with peptide: N-glycosidase F (PNGase F) or endoglycosidase H (Endo H). PNGase F is capable of removing all types of N-linked oligosaccharides, whereas Endo H removes only high mannose and some hybrid types of oligosaccharides. Both glycosidases caused an increase in the mobility of A43V5 as determined by SDS-PAGE and Western blotting (Fig. 2B), consistent with N-glycosylation of the protein.

A43 has two N-linked glycosylation sites

To determine the number and sites of glycosylation, plasmids (pA43HAN65Q, pA43HAN93Q, pA43HAN114Q) were constructed, each with a mutation in one of the three predicted N-linked glycosylation sites of A43 (Fig. 1). Each plasmid expressed A43 under its natural promoter and with a C-terminal HA epitope tag. An additional plasmid (pA43HA) was constructed with none of the glycosylation sites mutated as a control. To analyze the expression of the mutated forms of A43, BS-C-1 cells were infected with vΔA43GFP (a recombinant virus with a deletion of A43 to be described below) and then transfected with the plasmids. The cells were lysed and the extracts were analyzed by SDS-PAGE and Western blotting using antibody to the HA tag. If each of the N-X-S/T consensus sequences were glycosylated, then we would expect to see an increase in the

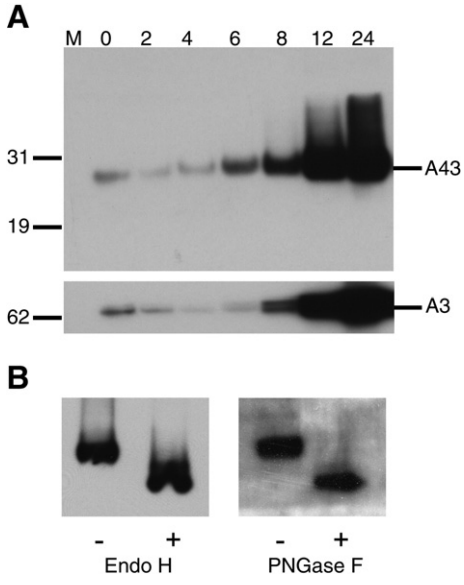

Fig. 2. A43 synthesis and glycosylation. (A) Western blot analysis of A43 expression kinetics. BS-C-1 cells were infected at a multiplicity of 10 PFU per cell with vA43V5. At 0, 2, 4, 6, 8, 12 and 24 h post-infection, whole cell lysates were analyzed by SDS-PAGE and Western blotting with an antibody to the V5 epitope tag. The blot was stripped and reprobed with an antibody to the VACV late protein A3. Position and mass in kDa of marker proteins are shown on the left. (B) vA43V5 infected cell lysates were treated with (+) or without (−) Endo H or PNGase F and analyzed by SDS-PAGE and Western blotting with an antibody to the V5 epitope tag.