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Characterization of a large, proteolytically processed cowpox virus membrane glycoprotein conserved in most chordopoxviruses

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ABSTRACT

Most poxvirus proteins are either highly conserved and essential for basic steps in replication or less conserved and involved in host interactions. Homologs of the CPXV219 protein, encoded by cowpox virus, are present in nearly all chordopoxvirus genera and some species have multiple copies. The CPXV219 homologs have estimated masses of greater than 200 kDa, making them the largest known poxvirus proteins. We showed that CPXV219 was expressed early in infection and cleaved into N- and C-terminal fragments that remained associated. The protein has a signal peptide and transited the secretory pathway where extensive glycosylation and proteolytic cleavage occurred. CPXV219 was located by immunofluorescence microscopy in association with the endoplasmic reticulum, Golgi apparatus and plasma membrane. In non-permeabilized cells, CPXV219 was accessible to external antibody and biotinylation. Mutants that did not express CPXV219 replicated normally in cell culture and retained virulence in a mouse respiratory infection model.

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Introduction

Poxviruses are large DNA viruses that reproduce in the cytoplasm of infected cells and have been widely studied because of their impact on human health, zoonotic spread, historic role as the first live virus vaccine, modern development as recombinant vaccine vectors, and use as model systems to investigate virus replication and host interactions (Damon, 2013; Moss, 2013). Of the 200 or more proteins encoded by poxviruses, nearly 100 are conserved in all members of the chordopoxvirus subfamily and about half of those are also conserved in entomopoxviruses (Upton et al., 2003). The majority of the highly conserved proteins have essential roles in virus replication, whereas most of the less well-conserved proteins of chordopoxviruses are concerned with modulating host interactions (Haller et al., 2014).

The goal of the present study was to characterize an unusual protein that is widely conserved among the chordopoxvirus genera except for members of the parapoxvirus genus, suggesting an evolutionarily conserved but not strictly essential function. Curiously, vaccinia virus is the sole member of the orthopoxvirus genus that lacks an open reading frame (ORF) encoding this protein. With predicted molecular weights of more than 200 kDa, these conserved proteins are larger than any other known poxvirus proteins. Here we characterize the cowpox virus (CPXV) homolog encoded by open reading frame CPXV219 (UniProt Q8QMM9). CPXV is of particular interest because it may have been the original smallpox vaccine, is the cause of an increasing number of zoonoses, contains the largest and most complete genome and has the broadest host range of all known orthopoxviruses (Dabrowski et al., 2013; Gubser et al., 2004). CPXV219 was expressed early during infection, trafficked through the secretory pathway, was N-glycosylated and cleaved into two major fragments and exposed on the plasma membrane. Additional studies indicated that the CPXV219 gene was dispensable for growth in tissue culture and unnecessary for virulence in mice.

Results

CPXV219 is conserved in most chordopoxviruses

The CPXV219 ORF of the Brighton red strain of CPXV is located near the right end of the genome and is predicted to encode a 1919 amino acid protein with a N-terminal signal peptide (SP), a near C-terminal transmembrane (TM) domain, two additional hydrophobic domains, and sites of N-linked glycosylation (Fig. 1A–C). With the exception of VACV, all sequenced orthopoxviruses have similar length orthologs. In VACV strain WR, the absence of DNA adjacent to the expected site of the CPXV219 ortholog suggests the occurrence of a large deletion. The amino acid sequence identities of CPXV219 homologs of the orthopoxviruses ectromelia virus, variola virus,

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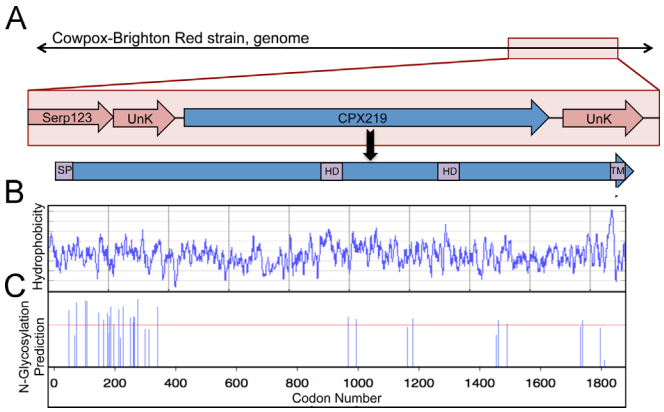

Fig. 1. Predicted features of CPXV219. (A) Diagram of CPXV219 ORF. Top shows the position of CPXV219 (blue) flanked by ORFs of unknown function (pink) near the right end of the CPXV genome. CPXV Serp123 corresponds to VACV C12L. Within the gene is a 20 amino acid N-terminal signal peptide (SP) predicted by the SignalP program (http://www.cbs.dtu.dk/services/SignalP/), a strongly predicted C-terminal transmembrane (TM) domain and hydrophobic domains (HD) that were more weakly predicted TMs by the DAS TM prediction server (http://www.sbc.su.se/~miklos/DAS/). (B) Hydrophobicity plot of CPXV219 predicted by the Protscale program using a window of 9 (http://web.expasy.org/cgi-bin/protscale/protscale.pl Kyle & Doolittle). (C) N-glycosylation sites predicted by the NetNGlyc program (http://www.cbs.dtu.dk/services/NetNGlyc/).

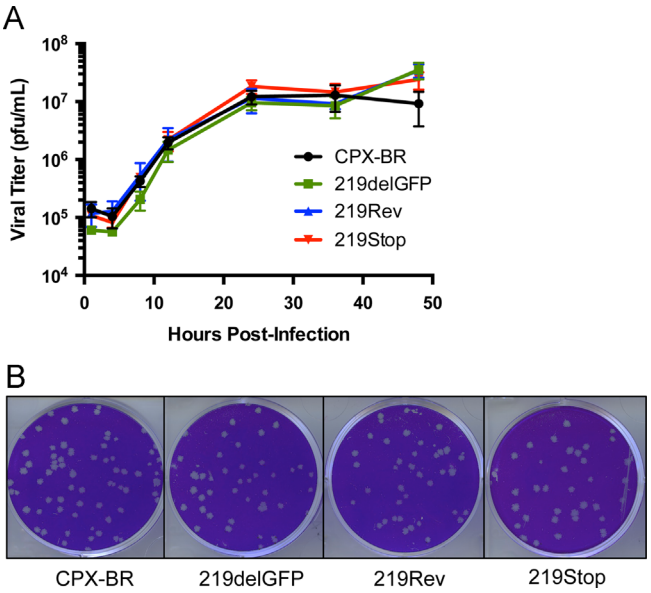

Fig. 2. CPXV219 is not essential for virus replication. (A) One step growth curve. HeLa cells were infected with 3 PFU/cell of the parent CPXV strain Brighton (CPX-BR) and CPXV 219delGFP, 219Rev and 219Stop and the cells plus media were collected at the indicated times. The cells were lysed by repeated freeze-thawing and the virus titers were determined by plaque assay on BS-C-1 cells. (B) Plaque formation. BS-C-1 cells were infected with the indicted viruses, overlaid with semi-solid medium and stained with crystal violet after 72 h.

monkeypox virus and horsepox virus compared to CPXV are 93%, 93%, 85% and 86%. Except for parapoxviruses, at least one strain of all other chordopoxvirus genera encodes a CPXV219 ortholog with sequence identity of 30–45% relative to CPXV219. The highest sequence conservation of CPXV219 orthologs is in the C-terminal region and some sheeppox strains have a N-terminal deletion. Avipoxvirus strains contain 5–6 duplicated full-length orthologs in the center of the genome and crocodilepox virus has 3 copies near the left end of the genome. No homologs have been recognized in entomopoxviruses, non-poxvirus species or prokaryotic or eukaryotic organisms.

CPXV219 is non-essential for replication

The absence of a CPXV219 homolog in VACV suggested that the protein is not essential for replication and a recent study determined that CPXV219 could be deleted from CPXV, though the growth properties of the mutant were not described (Xu et al., 2014). To more fully analyze the requirement of the 219 protein for CPXV replication, homologous recombination was used to make a deletion mutant (CPXV 219delGFP) by replacing the entire approximately 6000 bp ORF in CPXV with one encoding the green fluorescent protein regulated by a VACV promoter in the same orientation as CPXV219. Recombinant virus plaques were identified by fluorescence microscopy and clonally purified by repeated plaque isolation. In addition, the GFP of CPXV 219delGFP was replaced with the original CPXV219 gene in order to make the control virus CPXV 219Rev and with one containing stop codons near the N-terminus to make the mutant CPXV 219Stop. PCR and DNA sequencing confirmed the genomic modifications (data not shown). In addition, we confirmed that the stop codons arrested translation by constructing and testing a recombinant CPXV that had the same stop codons in the CPXV219 gene with a C-terminal epitope tag (shown in Fig. 3D). The CPXV 219delGFP and CPXV 219Stop viruses replicated under one step growth conditions and formed plaques as well as the parent and CPXV 219Rev control viruses in BS-C-1 cells (Fig. 2A, B) or HeLa, RK13, BHK21 and MRC5 cell lines (data not shown), demonstrating that the 219 protein was not required for CPXV replication in cell culture.

CPXV219 is expressed early in infection as full-length and N- and C-terminal fragments

Initial Western blotting experiments employing a recombinant CPXV with a FLAG tag at the C-terminus of CPXV219 revealed a minor band with an apparent mass greater than the 260 kDa marker, a major band of about 160 kDa and additional more rapidly migrating products (data not shown). To further investigate synthesis and apparent processing of CPXV219, we constructed a recombinant CPXV containing specific epitope tags at both the N- and C-termini. Because of the large size of the ORF, a stepwise green-to-white and white-to-green fluorescence screen was used to first replace the C-terminus with GFP and then replace the GFP with the C-terminus of CPXV219 containing an HA tag just before the termination codon to form CPXV 219-HA. A similar two-step procedure was used to replace the N-terminus of 219-HA with GFP and then with the N-terminus of CPXV219 containing a V5 tag downstream of the predicted SP to form CPXV V5-219-HA. In this manner, we obtained a recombinant virus with a specific tag at each end of CPXV219 (Fig. 3A). The addition of the epitope tags did not affect plaque formation or growth kinetics (not shown).

To analyze protein synthesis, HeLa cells were infected with CPXV V5-219-HA, collected at sequential times and lysed directly in buffer containing SDS and β-mercaptoethanol. The proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose filters and probed with antibodies to the epitope tags followed by infrared dyes, which allowed the two tags to be visualized in the same gel. The major CPXV219 bands were prominent at 4 h, increased at 6 h and unchanged at 8 h suggesting an early promoter (Fig. 3B), which was consistent with the putative promoter sequence preceding the ORF (not shown). Expression of CPXV219 in the presence of cytosine arabinoside (AraC), an inhibitor of DNA replication that prevents intermediate and late gene expression, confirmed regulation by an early promoter (Fig. 3B). Antibodies to both N- and C-terminal tags recognized the slowest migrating band with an estimated mass of >260-kDa (which appeared yellow due to the overlap of red and green), indicating that it was the full-length