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The Molluscum Contagiosum Virus protein MC163 localizes to the mitochondria and dampens mitochondrial mediated apoptotic responses

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ABSTRACT

Apoptosis is a powerful host cell defense to prevent viruses from completing replication. Poxviruses have evolved complex means to dampen cellular apoptotic responses. The poxvirus, Molluscum Contagiosum Virus (MCV), encodes numerous host interacting molecules predicted to antagonize immune responses. However, the function of the majority of these MCV products has not been characterized. Here, we show that the MCV MC163 protein localized to the mitochondria via an N-terminal mitochondrial localization sequence and transmembrane domain. Transient expression of the MC163 protein prevented mitochondrial membrane permeabilization (MMP), an event central to cellular apoptotic responses, induced by either Tumor Necrosis Factor alpha (TNF-α) or carbonyl cyanide 3-chlorophenylhydrazone (CCCP). MC163 expression prevented the release of a mitochondrial intermembrane space reporter protein when cells were challenged with TNF-α. Inhibition of MMP was also observed in cell lines stably expressing MC163. MC163 expression may contribute to the persistence of MCV lesions by dampening cellular apoptotic responses.

1. Introduction

Molluscum Contagiosum Virus (MCV) causes a common skin infection with 122 million cases reported in 2010 (Hay et al., 2014; Randall and Shisler, 2013). A member of the *Poxviridae* family, MCV spreads primarily through skin to skin contact and produces benign skin neoplasms that persist for months in otherwise healthy individuals (Chen et al., 2013). MC lesions are typically 3–5 mm in size. On average, MC lesions are present up to 12 months in otherwise healthy individuals, with 20–100 MC lesions present on the patient (Chen et al., 2013). In immune-compromised individuals, such as those infected with HIV, MC lesions grow much larger and persist indefinitely (Chen et al., 2013; Randall and Shisler, 2013).

Apoptosis is a powerful response to virus infection. Apoptosis can be triggered by two major cellular pathways, the intrinsic and extrinsic pathways (Green and Llambi, 2015; Yang et al., 2015). The extrinsic pathway is triggered by binding of ligands such as FasL, TNF-α, or TRAIL to their respective receptors (Green and Llambi, 2015; Yang et al., 2015). FADD and procaspase-8 are subsequently recruited to the receptor (Micheau and Tschopp, 2003). Upon oligomerization of FADD, procaspase-8 undergoes autocleavage into the active form caspase-8, and subsequently cleaves caspase-3 (Elmore, 2007; Kiraz et al., 2016; Martin et al., 1999; Medema et al., 1997; Muzio et al., 1996). Caspase-3 in turn cleaves additional targets, such as poly(ADP-ribose) polymerase 1 (PARP-1), resulting in apoptosis (Tewari et al., 1995).

The intrinsic apoptosis pathway is induced by numerous intracellular stress responses, including those induced during virus infection, and is mediated through the host cell mitochondria. A key step in intrinsic apoptosis is mitochondrial membrane permeabilization (MMP) (Kiraz et al., 2016). MMP is tightly regulated via the B-cell lymphoma 2 (Bcl-2) family proteins, which include pro-apoptosis proteins (e.g. Bax, Bad, Bid) and anti-apoptosis proteins (e.g. Bcl-2). An intracellular death signal induces the activation and oligomerization of Bax or Bak, which form pores in the mitochondrial outer membrane (MOM) (Kiraz et al., 2016). These pores allow the release of pro-apoptotic factors from the mitochondrial intermembrane space including cytochrome *c* and the second mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein with low pI (SMAC/Diablo)(Kiraz et al., 2016). Cytochrome *c* binds to Apaf1 and

Abbreviations: MCV, Molluscum Contagiosum Virus; MMP, Mitochondria Membrane Permeabilization; TNF, Tumor Necrosis Factor; CHX, Cycloheximide; MPTP, mitochondrial permeability transition pore; CCCP, carbonyl cyanide 3-chlorophenyl hydrazone; SMAC/DIABLO, Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI; STS, Staurosporine

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recruits procaspase-9 to form the apoptosome (Yuan and Akey, 2013). Next, procaspase-9 is converted to active caspase-9, and apoptosis occurs via the activation of effector caspases-3, -6, and -7 (Kiraz et al., 2016). Importantly, the intrinsic and extrinsic pathways are not mutually exclusive in that activation of one pathway typically leads to secondary activation of the other (Green and Llambi, 2015).

MCV lesions exhibit both cellular hyperplasia and hypertrophy and are generally characterized by reduced signs of inflammation (Callegaro and Sotto, 2009; Shisler, 2015; Simonart et al., 2002; Takahashi et al., 1999). Vermi et al. previously reported two types of MC lesions in healthy individuals, inflammatory MC (I-MC) and noninflammatory MC (NI-MC)(Vermi et al., 2011). I-MC lesions demonstrated clear signs of apoptosis, whereas NI-MC lesions lacked active caspase-3, suggesting MCV immune evasion molecules may dampen apoptotic responses for a period of time during early stages of infection. In support, the MCV MC159 protein is known to inhibit extrinsic apoptosis (Shisler and Moss, 2001). Since other poxviruses encode multiple anti-apoptosis products (Smith et al., 2013), we reasoned that MCV likely encodes additional products that may inhibit cellular apoptotic pathways. Using a bioinformatics approach, we identified MCV gene products that possessed both a potential mitochondrial localization sequence and a transmembrane domain, as a hallmark of potential inhibitors of mitochondrial functions and apoptosis. One such candidate, MC163, was examined for further study. Here, we report that MC163 localizes to the mitochondria and prevents the induction of apoptosis induced by a variety of cellular stressors. To our knowledge, this is the first report to identify a function for the MC163 protein.

2. Results

2.1. The N-terminal region of MC163 is required for localization to the mitochondria

There currently is no means to propagate MCV in cultured cells. Thus, individual MCV proteins must be studied independent of infection. The goal of the current study was to identify novel MCV immune evasion molecules. Bioinformatics analysis revealed the presence of a mitochondrial localization sequence within the MC163 N-terminus (a.a. 1–30). This protein also contains a predicted Cu/Zn binding domain (amino acids 46–108) and a putative transmembrane domain (Fig. 1A). The transmembrane domain was identified previously during the analysis of the MCV genome (Senkevich et al., 1997). The MC163 hydrophobic trans-membrane domain (TMD) is flanked by positively charged residues, similar to that of Tom70, a mitochondrial localizing protein (Rapaport, 2003). The 5' region of the MC163R gene is extremely repetitive and GC rich, making the PCR amplification of this region challenging. To circumvent this problem, the N-terminal region of MC163 (MC163N, corresponding to a.a. 1–132) was synthesized *in vitro* (Genscript), while the C-terminal region (MC163C) was cloned using PCR. Overlapping PCR was used to generate the full-length open reading frame (MC163F, a.a. 1–620) (Fig. 1A). All constructs were engineered to contain a C-terminal HA-tag. MC163R and its truncated mutants were cloned into the pcDNA3.1 vector and correct inserts confirmed by DNA nucleotide sequence analysis. Expression of MC163 wild-type and truncated proteins was verified by immunoblotting with anti-HA antibodies. The predicted size of the epitope-tagged MC163F is 67 kDa, and MC163C was predicted to be 50 kDa. Both proteins were detected with an apparent molecular weight of ~ 67 kDa and 50 kDa proteins, respectively (Fig. 1B). We observed an additional 50 kDa product detected in lysates from MC163F-expressing cells. This band could be the consequence of protease cleavage at a predicted calpain-1 (GTLY-LIPS, a.a. 180–187) or cathepsin K (QVFW-NHSE, a.a. 145–152) protease site located between the N- and C-terminal MC163 constructs. We also detected a doublet band at 50 kDa in MC163F and MC163C lanes, which may

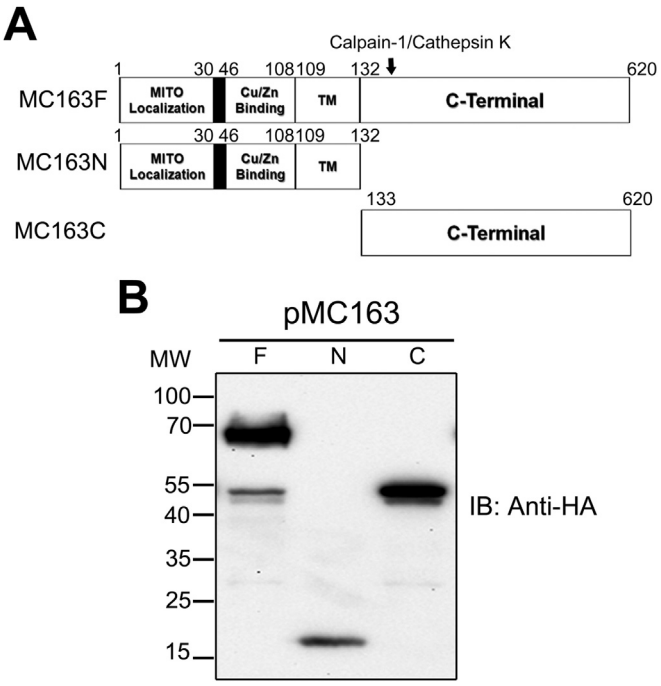

Fig. 1. Cloning and expression of MC163 constructs. (A) Representation of MC163 full length (MC163F), N-terminal (MC163N), and C-terminal (MC163C) constructs. The amino acid residues corresponding to the predicted mitochondrial localization sequence (a.a 1–30), Cu/Zn binding domain (a.a. 46–108), and transmembrane (TM, a.a. 109–132). The predicted Calpain-1/Cathepsin K cleavage site is indicated by an arrow. All constructs were engineered to contain a C-terminal HA tag. (B) 10 μg total protein was resolved by SDS-12% PAGE. Proteins were transferred to a PVDF membrane and blots were incubated with anti-HA antiserum. Bands corresponding to MC163F, N, and C were visualized by chemiluminescence. The relative position of molecular weight markers (MW) are indicated on the left.

represent alternative cleavage products at the calpain-1 and cathepsin K sites. Relative to full-length and the MC163C protein, the MC163N protein was expressed at lower levels, but was detected as a band of approximately 17 kDa (Fig. 1B).

To determine the cellular location of the MC163 construct, we co-transfected HeLa cells with either the wild-type or mutant MC163 constructs and pMTurqoise2-Mito (pMTurq), a plasmid expressing a cyan fluorescent protein engineered to possess a mitochondrial localization sequence (MLS) (Goedhart et al., 2012). As shown in Fig. 2, mitochondrial staining was observed as punctate areas surrounding the nuclei, and this pattern remained the same when MC163 proteins were expressed. The localization of MC163 proteins with respect to the mitochondria was determined by probing with anti-HA conjugated to Alexa Fluor 647. No signal was detected when cells transfected with empty vector (pcDNA3.1) were probed with anti-HA antibodies, as expected. Both MC163F and MC163N, which contain the predicted mitochondrial localization sequence and transmembrane region, showed punctate staining patterns that co-localized with the pMTurq mitochondrial marker (Fig. 2). MC163C, however, which lacks both the MLS and transmembrane region, did not localize to the mitochondria. Instead, MC163C was detected as a diffuse staining pattern throughout the cell (Fig. 2). Interestingly, we also observed a diffuse cytoplasmic staining in MC163F cells as well, which may represent the cleaved C-terminal product.

2.2. MC163 expression inhibits TNF-α-induced mitochondrial membrane permeabilization

Because viral proteins that localize to the mitochondria often inhibit apoptosis, we hypothesized that MC163 expression might antagonize apoptosis. Treating HeLa cells with TNF-α/cycloheximide