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An interaction domain in human SAMD9 is essential for myxoma virus host-range determinant M062 antagonism of host anti-viral function

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ABSTRACT

In humans, deleterious mutations in the sterile α motif domain protein 9 (*SAMD9*) gene are associated with cancer, inflammation, weakening of the immune response, and developmental arrest. However, the biological function of SAMD9 and its sequence-structure relationships remain to be characterized. Previously, we found that an essential host range factor, M062 protein from myxoma virus (MYXV), antagonized the function of human *SAMD9*. In this study, we examine the interaction between M062 and human *SAMD9* to identify regions that are critical to SAMD9 function. We also characterize the *in vitro* kinetics of the interaction. In an infection assay, exogenous expression of SAMD9 N-terminus leads to a potent inhibition of wild-type MYXV infection. We reason that this effect is due to the sequestration of viral M062 by the exogenously expressed N-terminal SAMD9 region. Our studies reveal the first molecular insight into viral M062-dependent mechanisms that suppress human *SAMD9*-associated antiviral function.

1. Introduction

Sterile α motif domain protein 9 (SAMD9) is a cytoplasmic protein with diverse functions including antiviral, antineoplastic, and stress-responsive properties. Human SAMD9, and potentially the pathway that it governs, can be targeted by poxviruses as a general strategy for host evasion. In some poxviruses, proteomic studies have observed protein–protein interactions between SAMD9 and a *C7L* homolog (shown by immunoprecipitation) that has been correlated to the host range function (Liu and McFadden, 2015; Liu et al., 2011; Meng et al., 2015; Sivan et al., 2015). In almost all sequenced genomes of mammalian poxviruses, at least one copy of a member from the *C7L* host range superfamily exists (Liu et al., 2012a; Meng et al., 2008). The host range *C7L* superfamily is conserved among the family of *Poxviridae*, and so far, no sequence homologue has been found in host or other pathogens. This suggests a potentially general functional relevance of the mammalian *SAMD9* pathway to the life cycle of poxviruses.

Several human diseases have been linked to deletion and deleterious mutations in the *SAMD9* gene. Two mutations in *SAMD9*, a missense K1495E (Topaz et al., 2006) and nonsense R344X (Chefetz et al., 2008) mutations, are responsible for a disease called normophosphatemic familial tumoral calcinosis (NFTC) when both alleles are inactivated in the patient. NFTC is an autosomal recessive disorder; patients with NFTC suffer from calcified skin tumors over their extremities and calcium deposits in the mucosa, which are associated with incessant pain and severe infections (Chefetz et al., 2008; Sprecher, 2010; Topaz et al., 2006).

SAMD9 along with two neighboring genes [*Miki* (Ozaki et al., 2012) and *SAMD9L* (Nagamachi et al., 2013)], is found within a region of the chromosome 7 long-arm, which frequently undergoes non-random interstitial deletion in patients with myeloid disorders (Asou et al., 2009). Reduced *SAMD9* mRNA or protein levels (Li et al., 2007; Ma et al., 2014) are often detected in cancerous tissue, which suggests an inhibitory role of *SAMD9* in tumorigenesis.

Most recently, a new form of congenital adrenal hypoplasia, designated MIRAGE (myelodysplasia, infection, restriction of growth, adrenal hypoplasia, genital phenotypes, and enteropathy), was identified and found to be associated with mutations in the *SAMD9* gene (Narumi et al., 2016). Narumi et al. found that patients with MIRAGE are young and most of them did not live beyond 2-year of age. Among these patients, mutations in *SAMD9* are on a single allele at one

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dissimilar residue (including 459, 769, 834, 974, 1195, 1280, and 1286) of evolutionarily conserved amino acids in *SAMD9* (Narumi et al., 2016). In general, these mutations occur postzygotically and are rarely germline-mosaic (Narumi et al., 2016). Nevertheless, these patients present unusually identical syndromes. Potentially similar cases with this disorder have previously been reported in the literature (Le and Kutteh, 1996; McDonald et al., 2010), albeit without direct confirmation of the genetic links to *SAMD9* at the time. The expression of these disease variants in the presence of the wild-type (wt) SAMD9 protein consistently leads to growth restriction in cultured cells (Narumi et al., 2016), suggesting that there is a dominant feature for these mutant proteins compared with the normal function of SAMD9.

Despite these reports on SAMD9's direct functional importance in cell biology and physiology, the molecular mechanism of SAMD9 function that is interrupted by these mutations remains unknown.

Evolutionary studies showed that *SAMD9* and its paralogue, *SAMD9*-like (*SAMD9L*), are derived from a common ancestral gene through a duplication event (Lemos de Matos et al., 2013). Despite a similar 60% amino acid identity between human SAMD9 and SAMD9L proteins, unique signatures of positive selection in both genes strongly advocate their distinct functions (Lemos de Matos et al., 2013). Some species harbor only the *SAMD9L* gene, such as the house mouse, whereas in cow (*Bos taurus*) and pig (*Sus scrofa*) species only *SAMD9* exists (Lemos de Matos et al., 2013). This suggests a certain redundancy in the functions of *SAMD9* and *SAMD9L*. We reason that *SAMD9* and *SAMD9L* could be involved in related cellular processes. Studying the biological function of SAMD9 has been difficult with the lack of a murine model due to the loss of *SAMD9* homolog in mouse genome. Therefore, information related to *SAMD9L* provides some clues on how *SAMD9* performs its cellular functions. SAMD9L is a facilitator of endosomal homotypic fusion and binds to the early endosome protein EEA1, and SAMD9 also shares this feature of binding to EEA1 (Nagamachi et al., 2013). In another study, SAMD9 was shown to interact with RGL2 (Hershkovitz et al., 2011) that is linked to cytokine receptor-associated signaling transduction (Takaya et al., 2007). Thus, it is possible that SAMD9 also plays a role in the endosomal fusion process. Larger vesicles than those found in normal cells can be observed in cells derived from patients with the dominant mutant of *SAMD9* (where MIRAGE is present). These vesicles are RAB7A-positive for late endosomes and may be caused by enhanced endosomal fusion (Narumi et al., 2016), which leads to disrupted receptor recycling back to the plasma membrane for continuous signaling transduction. Although these studies provide information about the potential sequence or residue correlation of *SAMD9*'s intrinsic function, it remains unknown whether the same domain or motif of SAMD9 is responsible for the antiviral function.

The 1589 amino acid sequence of SAMD9 has only two small regions that resemble known domains: a 71-residue sterile α-motif (SAM) domain at the N-terminus and a 152-residue P-loop NTPase domain in the middle. We found previously that an essential host range factor, M062 protein from myxoma virus (MYXV), antagonizes the function of human SAMD9. Deletion of *M062R* gene in the viral genome resulted in a defective virus that was unable to mount a productive infection due to an activated SAMD9 response. Knocking down SAMD9 protein synthesis rescued *M062R*-null MYXV infection suggesting a functional antagonism of M062 to SAMD9 (Liu et al., 2011). Considering the fundamental importance of SAMD9 function, we thus utilized MYXV M062 as a probe to examine the regions that are important for the antiviral property of human SAMD9. We also characterize the biophysics and specificity of the interactions between SAMD9 and the viral protein M062. We hypothesize that the N-terminal region of SAMD9, which is targeted by the viral protein, is an important regulatory domain for the intrinsic function of the human protein.

2. Material and methods

2.1. Cell lines and viruses

HeLa (Liu and McFadden, 2015), A549 cells (ATCC CCL-185), HEK-293 (NR-9313 was obtained from the NIH Biodefense and Emerging Infectious Research Resources Repository, NIAID, NIH), and BSC-40 (Liu et al., 2011) were cultured in Dulbecco minimal essential medium (DMEM; LONZA and Invitrogen) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), 2 mM glutamine (Corning), and 100 μg/ml penicillin/streptomycin (Pen/Strep; Invitrogen). Murine DBT cells (Cai et al., 2007) were cultured in 5% FBS containing DMEM supplemented with glutamine and Pen/Strep as described above. Myxoma viruses [vMyxGFP (wt-like MYXV), vMyxM062RV5 (wt-like MYXV with V5-tagged M062R gene) and vMyxM062RKO (M062R-null MYXV)] and vaccinia virus [VACV-$_{E/L}$GFP/$_{L}$tdTr (wt-like VACV) and VACV-C7LK1L-DKO (*C7L* and *K1L* double-knockout virus)] were reported previously (Liu and McFadden, 2015; Liu et al., 2011). BSC-40 cells were used for viral stock preparation (36% sucrose ultracentrifugation) and the titration of viral stocks as described previously (Liu et al., 2012b; Liu et al., 2011).

2.2. Cloning, plasmids, and primers

For protein expression in bacteria, the pET28b vector provided by Dr. Chia Lee [University of Arkansas for Medical Sciences (UAMS)] was used to include SAMD9 fragments by cloning between NdeI and XhoI sites. For mammalian protein expression, a modified pTriEX-4 vector (Cao and Zhang, 2012) containing 3xFLAG at the N-terminal (kindly provided by Dr. Xuming Zhang, UAMS) was used to clone SAMD9 fragments between KpnI and XhoI sites.

To engineer SAMD9-null cells with CRISPR/CAS9 technology, we used pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid #42230) (Kloppmann et al., 2012) and cloned the gRNA1 sequence with following oligo sequences: Forward: 5′-CACCGTAATCCAT ATCGTTACAAGT-3′; Reverse: 5′- AAACACTTGTAACGATA TGGATTAC-3′. We also used pX458-pSpCas9(BB)-T2A-GFP (Addgene plasmid #48138) to clone the gRNA2 sequence targeting SAMD9 with following oligo sequences: Forward: 5′-CACCGTCTCACTATTTGTGGCGAGAT; Reverse: 5′- AAACATCTCGCA CAAATAGTGAGAC. After cotransfection of both constructs, single cells that turned green were individually collected via cell sorting and cultured to expand. PCR screening was conducted using primer set 1 (Forward: 5′-aagctgaagcaaatcggaaa-3′; Reverse: 5′- tatgggcatcacacatg-gac-3′) to examine insertions and deletions (indels) caused by the first gRNA-Cas9. Primer set 2 (Forward: 5′-tttcttctgctgcttttctgg-3′; Reverse: 5′-atgggaaaattgttggcatc-3′) was used to screen for indels caused by the second gRNA-Cas9. Primer set 3 (Forward: 5′-gctgcttttctggactctgc-3′; Reverse: 5′-tatggctaatccgtctgcaa-3′) was used to screen for deletions caused by cleavages at both sites from the first and second gRNA-Cas9 combination. We identified SAMD9-null clones followed by sequencing to confirm the alteration in the genome as previously described (Bauer et al., 2015).

2.3. Antibodies and reagents

Primary antibodies include anti-V5 (R96025, ThermoFisher Scientific), SAMD9 (HPA021319, Sigma-Aldrich), SAMD9L (HPA019465, Sigma-Aldrich), β-actin (A1978, Sigma-Aldrich), FLAG (F3165, Sigma-Aldrich), FLAG-HRP (A8592, Sigma-Aldrich), EEA1 (3288S, Cell Signaling Technologies), and TIA-1 (sc1751, Santa Cruz Biotechnology) that were used for immunoprecipitation (IP), Western blotting, and immunofluorescent (IF) staining according to the manufacturer's standard protocol. Secondary antibodies include goat anti-

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