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Characterization of the rhesus fibromatosis herpesvirus MARCH family member rfK3

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ABSTRACT

Retroperitoneal fibromatosis-associated herpesvirus (RFHV) is a γ-herpesvirus of macaques that is closely related to Kaposi's sarcoma-associated herpesvirus (KSHV). Herein, we present characterization of the K3 gene from RFHV, a homologue of the KSHV K3 and K5 genes. Like the KSHV proteins, kK3 and kK5, the rfK3 protein decreases cell surface MHC I levels. Similar to kK5, rfK3 also modulates ICAM-1, but not another kK5 target, B7.2. Inhibitors of dynamin or mutations in the rfK3 RING-CH E3 ubiquitin ligase domain block cellular target regulation, implicating a ubiquitin-dependent, endocytosis-mediated mechanism for target down regulation and degradation. Overall, this manuscript presents the first characterization of a non-human primate virus MARCH family E3 ubiquitin ligase contributing important information about the evolution of immune avoidance strategies in primate viruses and paving the way for an animal model examining the importance of kK3 and kK5 *in vivo*.

Introduction

Retroperitoneal fibromatosis-associated herpesvirus (RFHV) is a recently discovered rhadinovirus of rhesus macaques. It was originally identified using degenerate PCR primers directed against conserved regions of the known herpes virus DNA polymerase genes and DNA derived from fibroproliferative tumors of two different macaque species, *Macaca mulata* (Mm) and *Macaca nemestrina* (Mn) (Rose et al., 1997). Thus far, approximately 4.5 kilobases of the genome has been sequenced, demonstrating a high degree of genomic co-linearity and sequence homology with Kaposi's sarcoma-associated herpesvirus (KSHV) (Rose et al., 2003). Included amongst the homologous genes is an open reading frame encoding a protein with homology to the kK3 and kK5 proteins of KSHV, encoded respectively by the K3 and K5 genes. While the overall amino acid homology between the RFHVMm strain and RFHVMn strain homologues is high at 85%, homology with KSHV kK3 and kK5 proteins is only 40% and 38%, respectively (Rose et al., 2003). Although this level of amino acid identity is low, the RFHV proteins contain a number of conserved motifs, including a really interesting new gene (RING-CH) zinc-binding domain at the amino terminal end and two membrane-spanning domains, that identify them as members of the membrane-associated RING-CH-containing (MARCH) family of proteins, a family to which both kK3 and kK5 belong (Bartee et al., 2004).

Previous studies of kK3 and kK5 have shown that they mediate down regulation of a number of immunomodulatory proteins from the surface of KSHV infected cells. Included amongst these proteins are major histocompatibility class I (MHC I), ICAM-1 (CD54), B7.2 (CD86), PE-CAM (CD31), CD1d, the γ–interferon receptor, and most recently BST-2 (Ishido et al., 2000a; Coscoy and Ganem, 2000; Coscoy and Ganem, 2001; Ishido et al., 2000b; Haque et al., 2001; Sanchez et al., 2005; Mansouri et al., 2006; Li et al., 2007; Mansouri et al., 2009). The mechanisms of modulation are complex and depend on the protein being targeted (Means et al., 2002; Means et al., 2007; Mansouri et al., 2006). For MHC I, which is regulated by both kK3 and kK5, down regulation proceeds through a series of steps that begins with an increased rate of endocytosis, likely facilitated through ubiquitylation mediated by the E3 ubiquitin ligase activity of these two viral proteins, an ability that all of the thus far described MARCH family members possess (Means et al., 2002; Duncan et al., 2006; Nathan and Lehner, 2009). Following endocytosis, kK3 and kK5 are able to direct MHC I degradation, and this is dependent on two stretches of acidic amino acids found in the conserved region C terminal of the second membrane spanning domain (Means et al., 2002; Means et al., 2007). For B7.2 and PE-CAM, which are down regulated by kK5 only, the mechanism of modulation is slightly different (Mansouri et al., 2006; Means et al., 2007). While both can be modulated through a pathway that seems identical to the one

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employed for down regulation of MHC I, they can also be shunted directly from the Golgi apparatus into a degradative compartment. Modulation of PE-CAM degradation by this mechanism depends on interactions of the acidic stretches of kK5 with PACS-2 (Mansouri et al., 2006). It isn't clear whether this same interaction plays a role in B7.2 regulation, however, this molecule has been shown not to play a role in MHC I degradation. Overall, there are still a number of grey areas with respect to our understanding of the mechanisms by which these proteins function.

One method for better understanding protein functionality is to study closely related homologues, allowing comparison of the similarities and differences paired with an examination of the homology of primary and secondary amino acid structures. While murine herpesvirus-68 (MHV-68) encodes a MARCH family member protein, its primary sequence and mechanism of action are quite different from both kK3 and kK5 (Stevenson et al., 2000). To date, no non-human primate MARCH family members have been characterized for functionality. The RFHVMm and Mn kK3 homologues possess significantly higher sequence homology than mK3 to kK3 and kK5 and importantly, share high homology in the region downstream of the second transmembrane domain, an area that seems to play a large role in the biology of KSHV kK3 and kK5 (Fig. 1A) (Sanchez et al., 2002; Means et al., 2002). For these reasons, we have undertaken a series of experiments to begin characterizing the functionality of the RFHVMm kK3 homologue.

Results and Discussion

Cloning of RFHVMm K3, rfK3

Due to a lack of sample availability for cloning the kK3 homologue, we elected to begin our study of rfK3 by direct gene synthesis and arbitrarily, decided to begin with examination of the Macaca mulata strain of virus, isolate YN91 (accession AF005479). As shown in Fig. 1A, the Mm rfK3 shows considerable identity and greater homology with both kK3 and kK5. At the time, only the amino acid sequence was available, so overlapping primers were designed based on a reverse translation of the available sequence. Codons for each amino acid were based upon a codon bias table for humans (shown in Table 1, determined from the Codon Usage Tabulated from Genbank website (www.kazusa.or.jp/codon/)), but the resulting sequence was not optimized for any codon pair bias. Comparing the resulting synthetic sequence to the now released nucleic acid sequences revealed approximately 75% identity. Each of the synthesized oligomers was approximately 70 bases in length and overlapped by 18 base pairs with the proceeding and following oligomers (Fig. 1B). The oligomers were subjected to multiple rounds of PCR in order to “stitch” them together, as detailed in the Materials and methods section. The resulting product was then used as a template for further amplification. After cloning into pEGNP-N1 vector, the PCR product was sequence confirmed in its entirety. During the course of this screening, in addition to a correct, full-length clone, an additional clone with three single base pair insertions was identified. The location of these base changes is such that the reading frame is shifted following the second cysteine of the RING-CH domain until just prior to the first membrane-spanning domain effectively substituting this important domain while retaining the rest of the rfK3 sequence (Fig. 1C). We elected to utilize this clone, rfK3 FS, to explore the necessity of the RING-CH domain for protein functionality. To further address the role of the RING-CH domain, we deliberately created a clone of rfK3 containing alanine residues substituted for the critical central cysteine and histidine residues of the RING-CH domain, called rfK3 mZn (Fig. 1C). This was easily created, as all of the base changes were located within the second oligomer used to create the original clone. Thus, the mutant was created in an analogous manner to the original, with simple splice-overlap mutagenesis, but with this one oligomer substituted by a mutated version.

Following creation, expression of the constructs was examined by transient transfection into HEK 293T cells. As controls, both the kK3 and kK5 wild-type and mZn constructs, which are homologous to the rfK3 mZn construct, were also transfected. Approximately 48 h post-transfection (hpt) cells were harvested, and subjected to western blot (WB) with an anti-GFP antibody. As expected, we were able to readily visualize the wild-type and mutant versions of kK3, while levels of kK5 and kK5 mZn were at the limits of WB detection and required a longer exposure for detection (Fig. 2A, lanes 5,6 and 7,8, respectively, plus inset panel). This low level of kK5 expression is commonly seen in our experiments (data not shown). Both K3- and K5-RING-CH domain-GST fusion proteins have been shown to auto-ubiquitylate and full-length K5 is highly ubiquitylated ((Coscoy et al., 2001) data not shown). It is possible that this ubiquitin modification results in K5 degradation and overall low levels of protein. All three of the codon-optimized rfK3 constructs were expressed at a very high level, most likely due to the codon optimization, at the expected molecular weight. Intriguingly, rfK3 and kK5 constructs were expressed to nearly identical levels in the African green monkey kidney cell line COS-7 (Fig. 2B). This could be due to a lack of a co-factor in non-human primate cells that allows for high-level auto-ubiquitylation by K5, such as differences in available E2-ubiquitin conjugating enzymes. Equally, it could be because of the lack of a restricting factor found in human cells. Either possibility has numerous implications for animal models of KSHV and will be followed up on by additional experimentation.

rfK3 is localized to the endoplasmic reticulum

Our lab and others have previously demonstrated that kK3 and kK5 localize primarily to the endoplasmic reticulum (ER) with a small amount trafficking to the plasma membrane (Means et al., 2002; Sanchez et al., 2002). Given the high homology of rfK3 to these two proteins, we sought to determine if it localized to the same region of the cell. A7 cells, seeded into 4-well chamber slides, were transiently transfected with a plasmid encoding the rfK3-EGFP fusion protein. At approximately 36 hpt, cells were fixed, permeabilized, and stained with antibodies against protein disulphide-isomerase (PDI), an ER-resident protein, followed by fluorescent secondary antibody. Visualization by confocal microscopy revealed a high degree of co-localization between rfK3 and PDI (Fig. 3). Even though the rfK3 protein was highly expressed, no localization to the plasma membrane was observed.

rfK3 can down regulate human and non-human primate MHC I

The kK3 and kK5 proteins are both able to down regulate surface expression of MHC I. To determine whether this was the case with rfK3, we transiently introduced the wild-type and mutant rfK3 constructs, along with the kK3 and kK5 wild-type and mZn constructs as controls, into one human cell line, HeLa, and two non-human primate cell lines, telomerase-immortalized rhesus macaque fibroblasts (teloRF) and COS-7 (African green monkey). Approximately 48 hpt the cells were harvested, stained for cell surface levels of MHC I, and examined by flow cytometry. As expected, expression of either the kK3 and kK5 wild-type proteins in HeLa cells decreased surface MHC I levels, while the kK3mZn and kK5mZn proteins did not (Fig. 4A). Likewise, rfK3 was able to decrease MHC I even though it is of human origin, while the rfK3 mZn and rfK3 FS constructs lost this ability. Interestingly, changes in MHC I surface levels in the teloRF and the COS-7 cells both mirrored those seen in the human cell line for all of the constructs (Fig. 4B and C). For each cell line the wild-type rfK3 was able to mediate a decrease in cell surface MHC I while the rfK3 mZn and rfK3 FS mutants lacked this ability. This was mirrored by the kK3 and kK5 wild-type and mutant constructs. As shown in the Fig. 4B dot plots, the rfK3 constructs were expressed to a higher level than the