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Ectromelia virus encodes a family of Ankyrin/F-box proteins that regulate NFκB

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ABSTRACT

A notable feature of poxviruses is their ability to inhibit the antiviral response, including the nuclear factor kappa B (NFκB) pathway. NFκB is a transcription factor that is sequestered in the cytoplasm until cell stimulation, and relies on the SCF (Skp1, culllin-1, F-box) ubiquitin ligase to target its inhibitor, IκBα, for degradation. IκBα is recruited to the SCF by the F-box domain-containing protein βTrCP. Here, we show that ectromelia virus, the causative agent of mousepox, encodes four F-box-containing proteins, EVM002, EVM005, EVM154, and EVM165, all of which contain Ankyrin (Ank) domains. The Ank/F-box proteins inhibit NFκB nuclear translocation, and this inhibition is dependent on the F-box domain. We also demonstrate that EVM002, EVM005, EVM154, and EVM165 prevent IκBα degradation, suggesting that they target the SCF. This study identifies a new mechanism by which ectromelia virus inhibits NFκB.

Introduction

The nuclear factor kappa B (NFκB) family of transcription factors regulates the expression of a number of genes, including those involved in proliferation, apoptosis, and the innate and adaptive immune response (Ghosh and Hayden, 2012; Hoffmann et al., 2003; Vallabhapurapu and Karin, 2009). The NFκB pathway can be activated by a number of stimuli; however, the best-studied are the classical pathways that occur following the engagement of the pro-inflammatory cytokines, TNFα and IL-1β, with the tumor necrosis factor receptor (TNFR) and interleukin 1 receptor (IL-1R) or toll-like receptor (TLR), respectively (Ghosh and Hayden, 2012; Hayden and Ghosh, 2008, 2012). Receptor activation results in the recruitment of adapter proteins and ubiquitin ligases that ultimately activate the regulatory kinase complex, the inhibitor of κB (IκB) kinase (IKK) complex, through phosphorylation. Following activation, the IKK complex phosphorylates the inhibitor of κB (IκBα), which normally sequesters the p65/p50 NFκB transcription factor in the cytoplasm. Following phosphorylation, IκBα is recruited to the Skp1, cullin-1, F-box (SCF) ubiquitin ligase by the F-box-containing protein βTrCP. The SCF poly-ubiquitinates IκBα, targeting it for degradation by the 26S proteasome (Hatakeyama et al., 1999; Kroll et al., 1999; Spencer et al., 1999). Left unsequestered by its inhibitor, IκBα, the p65/p50 NFκB transcription factor is free to translocate into the nucleus, where it stimulates gene transcription.

Significantly, many viruses have evolved strategies to modulate the NFκB pathway to ensure their own survival (Brady and Bowie, 2014; Mohamed and McFadden, 2009; Rahman and McFadden, 2011; Smith et al., 2013). The *Poxviridae* are a family of large dsDNA viruses that are notorious for inhibiting the immune response and regulating cellular signaling pathways (Brady and Bowie, 2014; Johnston and McFadden, 2003; Moss and Shisler, 2001; Seet et al., 2003; Smith et al., 2013). Interestingly, a number of inhibitors of the NFκB pathway have been identified in pox-viruses, especially in the prototypic poxvirus vaccinia (Mohamed and McFadden, 2009; Rahman and McFadden, 2011). Activation of the NFκB pathway through the IL-1R is prevented by A52R, A46R, and K7L (Bowie et al., 2000; Schroder et al., 2008). A46R binds the TIR-domain containing adapter proteins MyD88, TRIF, TIRAP, and TRAM, and prevents them from associating with the IL-1R (Bowie et al., 2000; Stack et al., 2005). A52R and K7 bind TRAF6 and IRAK2 to disrupt signaling complexes containing these proteins (Bowie et al., 2000; Graham et al., 2008; Harte et al., 2003; Schroder et al., 2008). The IKK complex is targeted by N1L, B14R, and K1L (Chen et al., 2006, 2008;; DiPerna et al., 2004; Shisler and Jin, 2004). N1L interacts with multiple subunits of the IKK complex (DiPerna et al., 2004), while B14R binds specifically to IKKβ (Chen et al., 2006,

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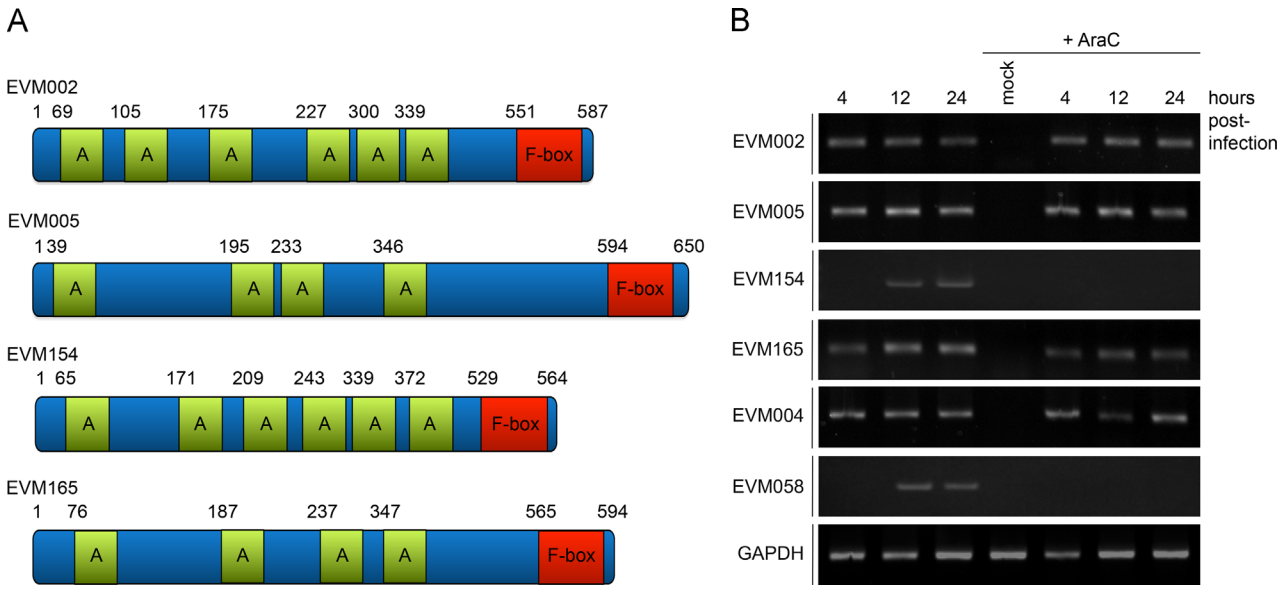

Fig. 1. EVM002, EVM005, and EVM165 are transcribed early during infection and EVM154 is transcribed late. (A) Schematic representation of EVM002, EVM005, EVM154, and EVM165. (B) CV-1 cells are infected with ECTV at a MOI of 5 in the absence or presence of 80 μg/mL cytosine arabinoside (AraC). RNA is harvested at 4, 12, and 24 h post-infection and gene transcription is assessed using gene-specific primers for EVM002, EVM005, EVM154 and EVM165. EVM004 is used as a control for early gene transcription, EVM058 is used as a control for late gene transcription, and GAPDH is used as a loading control.

2008). Phosphorylation and subsequent activation of the IKK complex are prevented by both N1L and B14R (Chen et al., 2008; DiPerna et al., 2004). In contrast to N1L and B14R, K1L does not interact with components of the IKK complex, instead it is thought to inhibit unidentified kinases responsible for phosphorylating the IKK complex (Shisler and Jin, 2004). Recently, A49 has also been discovered to inhibit NFκB by acting as molecular mimic of IκBα (Mansur et al., 2013). Vaccinia also encodes two additional proteins that inhibit NFκB activation; M2L prevents ERK2 phosphorylation and subsequent signaling to the IKK complex (Gedey et al., 2006; Hinthong et al., 2008), while E3L prevents NFκB activation induced by the PKR pathway, following recognition of viral double stranded RNA (Myskiw et al., 2009).

Using a bioinformatics screen, our lab identified four Ank/F-box proteins, EVM002, EVM005, EVM154, and EVM165, in ectromelia virus (Fig. 1A) (van Buuren et al., 2008). The combination of multiple Ank domains in conjunction with a C-terminal F-box was unique to poxviruses (Mercer et al., 2005; Sonnberg et al., 2008), until recently when Ank/F-box proteins were identified in the parasitoid wasp, *Nasonia* (Werren et al., 2010). Ank repeats are present in a number of cellular proteins, and mediate distinctive protein–protein interactions (Li et al., 2006). The F-box domain is necessary for interaction with Skp1 in the SCF ubiquitin ligase, and cellular F-box proteins recruit substrates to the SCF to be ubiquitinated (Kipreos and Pagano, 2000). Interestingly, each protein has variation in the number and location of the Ank repeats that it possesses, suggesting that these unique combinations may allow the four proteins to interact with different substrates.

Previously, our laboratory demonstrated that EVM005 associates with the SCF ubiquitin ligase and inhibits NFκB signaling by preventing degradation of IκBα (van Buuren et al., 2014, 2008). Since degradation of IκBα is catalyzed by the $SCF^{\beta TrCP}$ ubiquitin ligase, we investigated whether EVM002, EVM154, and EVM165 also inhibited NFκB activation. Here, we report that ectopic expression of EVM002, EVM005, EVM154, and EVM165 inhibited TNFα- and IL-1β- induced NFκB activation by preventing degradation of IκBα. However, deletion of the F-box domain abrogated the ability of EVM002, EVM005, EVM154, and EVM165 to inhibit NFκB activation. In addition, our studies revealed that IκBα degradation and NFκB activation were still inhibited by ectromelia virus, even after deletion of the Ank/F-box. Our results suggest that ectromelia virus encodes four additional inhibitors of the NFκB pathway that modulate the $SCF^{\beta TrCP}$ ubiquitin ligase to prevent degradation of IκBα.

Results

Ectromelia virus encodes four Ank/F-box proteins that are expressed at both early and late times during infection

Recently, we demonstrated that EVM005 associates with the SCF ubiquitin ligase and inhibits NFκB signaling by preventing degradation of IκBα (van Buuren et al., 2014, 2008). Since degradation of IκBα is catalyzed by the $SCF^{\beta TrCP}$ ubiquitin ligase, we investigated whether EVM002, EVM154, and EVM165 also inhibit NFκB activation. First, we wanted to verify if and when these genes were transcribed during infection. To do this, we used semi-quantitative reverse transcription PCR to examine mRNA levels during infection. CV-1 cells were infected with ECTV and RNA was harvested at 4, 12, and 24 h post-infection. Gene expression was assessed using primers specific for EVM002, EVM004, EVM154, and EVM165. As controls, we monitored transcript levels of EVM004 and EVM058. EVM004 is a gene that is transcribed early during ECTV infection, while EVM058 is transcribed late during ECTV infection (Wilton et al., 2008). EVM004 transcripts were detected at 4, 12, and 24 h post-infection (Fig. 1B). In the presence of AraC, an inhibitor of late gene expression, EVM004 transcripts were still detected, indicating that EVM004 was transcribed early during infection (Babiuk et al., 1975; De Clercq et al., 1975) (Fig. 1B). In contrast to EVM004, EVM058 transcripts were detected at only 12 and 24 h post-infection, and detection was decreased in the presence of AraC, indicating that EVM058 was transcribed late during infection (Fig. 1B). Transcripts for EVM002, EVM005, and EVM165 were detected at 4, 12, and 24 h post-infection, while EVM154 was not detected until 12 h post-infection (Fig. 1B). Upon AraC treatment, transcripts for EVM002, EVM005, and EVM165 were still detected, while transcripts for EVM154 were no longer detected, indicating that EVM154 was a late gene. Overall, these data indicated that EVM002, EVM005, and EVM165 were transcribed early during infection, while EVM154 was transcribed late during infection.