Mutation of the Theiler's virus leader protein zinc-finger domain impairs apoptotic activity in murine macrophages

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ABSTRACT

The Theiler's murine encephalomyelitis virus (TMEV) leader (L) protein zinc-finger domain was mutated to study its role in cell death in infection of the murine macrophage cell line M1-D, revealing that an intact zinc-finger domain is required for full apoptotic activity. A functional L zinc-finger domain was also required for activation of p38 MAPK that results in phosphorylation and activation of p53, and in turn, alteration of the conformation of the anti-apoptotic proteins Puma and Mcl-1, leading to the release of pro-apoptotic Bax and apoptosis through the intrinsic pathway. TMEV infection also inhibits host protein synthesis, a stress shown by others to induce apoptosis. Since inhibition of host protein synthesis follows rather than precedes activation of MKK3/6 and p38, it seems less likely that it triggers of apoptosis in infected cells. Finally, we showed that the levels of reactive oxygen species following infection were consistent with apoptotic rather than necrotic cell death. Thus, these experiments support an important role for the TMEV L protein zinc-finger domain in apoptosis in an infected murine macrophage line.

Theiler's murine encephalomyelitis virus (TMEV), a cardiovirus in the family *Picornaviridae*, is a highly cytolytic RNA virus that persists in the central nervous system (CNS) of mice after intracerebral (ic) inoculation. Virus persists primarily in infiltrating macrophages and presumably by continual cell-to-cell spread in the presence of adaptive immunity, resulting in an inflammatory demyelinating disease. Infection of murine macrophages in culture induces apoptosis (Ghadge et al., 1998; Jelachich et al., 1999; Jelachich and Lipton, 1996) in a Bax-dependent manner through the intrinsic or mitochondrial pathway by activation of p53, and in turn, alteration of the conformation of the anti-apoptotic proteins Puma and Mcl-1 and release of pro-apoptotic Bax (Son et al., 2008), restricting infectious viral yields, possibility reflecting the role of apoptosis in attenuating TMEV virulence.

Cardioviruses [encephalomyocarditis virus (EMCV) and TMEV] regulate nuclear trafficking of cellular proteins and RNA through the leader (L) protein, which contains a zinc-finger domain near the N-terminus, an acidic domain with potential Thr and Tyr phosphorylation sites, and in the TMEV, a C-terminal Ser/Thr domain (Fig. 1A) (Dvorak et al., 2001; van Pesch et al., 2001; Zoll et al., 2002). Unlike the L of aphthoviruses, the cardiovirus L protein does not function as a protease. Delhaye et al. (Delhaye et al., 2004) first reported that the TMEV L protein interferes with nucleocytoplamic trafficking. Subsequent studies showed that EMCV L binds Ran-GTPase and hyperphosphorylates nucleoporins (nup), leading to disruption of the nuclear pore complex spanning the nuclear envelope and the Ran gradient (Bardina et al., 2009; Lidsky et al., 2006; Porter et al., 2006; Porter and Palmenberg, 2009; Ricour et al., 2009). Conserved residues within the L zinc

finger and acidic domains are essential for nup phosphorylation, which is mediated by mitogen-activated protein kinases (Porter and Palmenberg, 2010).

The cardiovirus L protein also antagonizes the innate immune response in acute systemic EMCV infection and in the establishment of persistent TMEV infection in the CNS of mice (Hato et al., 2007; Paul and Michiels, 2006; Ricour et al., 2009). The type I interferon (IFN) response is inhibited by the L protein due to inhibition of interferon regulatory factor 3 (IRF-3) dimerization (Hato et al., 2007) and a block in nuclear trafficking which prevents transcription factors, such as IRF-3, from reaching the nucleus (see below). While Hato et al. (Hato et al., 2007) reported that EMCV prevented IRF-3 dimerization, Stavrou et al. (Stavrou et al., 2010) provided evidence that the DA strain of TMEV blocked IFN-β gene transcription upstream of IRF-3 but downstream of mitochondrial antiviral-signaling protein (MAVS; also known as VISA, CARDIF and IPS1). The exact site of low-neurovirulence TMEV interference remains to be identified.

To examine the role of the BeAn virus L zinc-finger in infection of cells in culture we mutated the zinc-finger domain from CHCC to RHRC without changing the amino acid sequence of the L* protein which is encoded by an alternative overlapping ORF in both the DA strain (van Pesch et al., 2001) and the BeAn strain (Fan et al., 2009) to inactivate the L protein. The mutated L sequence in a subclone in pGEM13 was then assembled as previously described (Kumar et al., 2004) into a full-length BeAn virus infectious clone to generate a progeny virus stock. Although the one-step growth kinetics in M1-D macrophages was similar for the two viruses and the mutant virus infection produced slightly higher numbers of pfu/cell at 8 to 12 h pi (Fig. 1B), apoptosis was significantly

reduced as determined from counting pyknotic and fragmented nuclei upon light microscopy and DAPI-staining ($p < 0.02$; Fig. 1C). Immunoblot analysis showed that infection with the mutant virus was associated with reduced cleavage of caspases-9 and -3 to their active forms and of PARP as compared to wild-type virus infection (Fig. 1D) consistent with reduced apoptosis. These results indicate a role for the L zinc-finger domain in generating a full apoptotic response in macrophages during TMEV infection, consistent with findings in M1-D cells transfected with L protein constructs (Fan et al., 2009). Our results and those of van Pesch et al. (van Pesch et al., 2001) in DA virus infection of BHK-21 cells contrast with those of Stavrou et al. (Stavrou et al., 2011) who found that DA virus infection in HeLa cells did not require an intact zinc-finger domain but did require an intact L ser/thr domain to induce apoptosis. These results perhaps suggest the importance of the cell type in determining the interaction between viral proteins and cell death pathway.

We previously showed that BeAn virus infection in M1-D macrophages induces apoptosis in a p53-dependent manner, with activation of p38 MAPK resulting in phosphorylation of p53 Ser 15 (Son et al., 2009). Immunoblot analysis of the temporal activation of these molecules in M1-D cells incubated with UV-inactivated or untreated BeAn virus and in mutant virus- compared to wild-type virus-infected cells showed that phosphorylation of p38 requires active virus infection (Fig. 2A) and that the zinc-finger mutant virus infection leads to delayed appearance and reduced levels of phospho-p38 (Fig. 2B) as well as lack of phosphorylation of p53 and accumulation of total p53 compared to wild-type BeAn virus infection (Fig. 2C). Activation of the immediate upstream signaling molecule MKK3/6 prior to activation of p38, not previously reported, was seen only in wild-type virus-infected cells. Thus, apoptosis appears to be initiated by activation of MKK3/6 or

upstream molecules of it and to require a functional L protein zinc-finger for a complete apoptosis.

Since a block in nucleocytoplasmic trafficking is one mechanism by which cardiovirus infections rapidly lead to inhibition of host protein synthesis (reviewed in(Dougherty et al., 2010) and which Chang et al. (Chang et al., 2002) have shown can lead to apoptotic cell death, we examined the temporal relation of initiation of apoptosis in TMEV-infected macrophages with inhibition of host protein synthesis. In wild-type virus- and mutant zinc-finger virus-infected cells, protein synthesis, as measured by ^{35}S[methionine] incorporation into trichloroacetic acid-precipitable counts, began to decline at 4 h pi as compared to mock-infected cells with significant reduction ($p < 0.01$) at $\geq$6 h pi (Fig. 3A). Mutant virus-infected cells showed less marked reduction in protein synthesis than did wild-type virus-infected cells (Fig. 3A). Thus, the finding that inhibition of host protein synthesis appeared to follow rather than preceded activation of p38 or p53 (Fig. 2B,C) suggests that inhibition of host protein synthesis is not the trigger for apoptosis in BeAn virus-infected cells. Finally, levels of reactive oxygen species (ROS) were increased at 6 h pi ($p < 0.05$) for both mutant- and wild-type virus-infected cells, but the increase at 8 to 10 h pi was slight compared to ROS levels in phorbol myristate acetate (PMA)-stimulated cells (Fig. 3B). This result is consistent with apoptotic cell death and not necrosis, in which levels are much higher in BeAn virus-infected BHK-21 (Arslan et al., 2012).

In contrast to a recent study (Romanova et al., 2009) using an L protein mutant showing that the EMCV L protein has anti-apoptotic activity, our findings indicate an important role for the TMEV (BeAn strain) L protein zinc-finger domain in the induction of

apoptosis during infection of a murine macrophage cell line. While Stavrou et al. (Stavrou et al., 2010) demonstrated that GDVII virus in which L 57Pro was present in the Ser/Thr domain failed to induce apoptosis in HeLa cells nor did DA virus with L 57Ser mutated to Pro, we had reported that GDVII virus with L 57Pro was more efficient in inducing apoptosis in BSC-1 cells (Jelachich, 1996, 683} and found the same apoptotic activity in infected macrophages cell lines (not published), thus the L Ser/Thr motif was not studied further. The intrinsic p38 MAPK/p53-dependent signaling pathway was found to be activated prior to the inhibition of host protein synthesis by the virus through interaction with MKK3/6 or another upstream protein in the p38 pathway. Thus, these experiments support an role for the TMEV L protein zinc-finger domain in apoptosis in an infected murine macrophage line.

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FIGURE LEGENDS

Figure 1. Mutation of the BeAn virus L protein zinc-finger domain decreases apoptotic cell death but not viral yields in M1-D macrophages. (A) Schematic representation of the 71-amino acid L protein showing the zinc-finger domain near the N-terminus, acidic domain in the middle of the protein, and Ser/Thr domain near the C-terminus of the protein. Arrows point to the 5 Ser/Thr residues. The CHCC zinc-finger type domain was mutated to RHRC. (B) Virus titers (pfu/cell) of combined supernatant and cells, assessed by standard plaque assay were only slightly higher ($p > 0.05$) for the mutant than wild-type virus in one-step growth kinetics (moi = 10); note almost complete loss of infectious virus at 20 h pi. (C) Apoptosis was significantly reduced ($p < 0.02$) at 8 and 10 h pi in mutant- vs. wild-type virus-infected cells as based on light microscopy of DAPI-stained nuclei; this assay was not accurate at $\geq$12 h pi because of increasing loss of cells from monolayers. At least 3 randomly chosen fields, each containing ~75 to 100 cells, from a coverslip were photographed at X400 magnification, and the percentage of cells with condensed chromatin and fragmented nuclei was determined by a blinded observer. Each experiment was repeated at least 3 times. Apoptosis measured in mock-infected cells was 2.59 ± 0.36 (n = 3). Error bars in (B and C) indicate SEM (n = 3 experiments). (D) Immunoblot analysis showing delayed and/or reduced cleavage of caspases-9 and -3 and PARP in mutant- compared to wild-type-virus-infected cells at 8 and 10 h pi. β-actin was used as a loading control.

Figure 2. Immunoblot analysis of phospho-p38, p53 ser15 and phospho-MKKK3/6 in infected M1-D. (A) 1- and 10-min UV-inactivation (300 J) of BeAn virus which does not alter viral binding (Jelachich and Lipton, 1996) prevented activation of p38 (10-min activation =

3KJ). (B-D, respectively) Phospho-p38 was detected at 3 h pi, p53 Ser15 at 3-4 h pi, and phospho-MKKK3/6 at 2-3 h pi in wild-type virus-infected cells, while appearance of these phosphoproteins was delayed in mutant-virus infected cells. β-actin provided the loading control.

Figure 3. Host protein synthesis and ROS in infected and mock-infected M1-D cells in 6-well plates (1×10^6 cells/well). (A) Host protein synthesis assessed at indicated intervals by 5-min pulses of 20 μCi of ^{35}S[methionine] incorporated into trichloroacetic acid-precipitable counts began to decrease at 4 h pi in both wild-type virus- and zinc-finger mutant virus-infected cells compared to mock-infected cells ($p > 0.05$) reaching significance ($p < 0.01$) at ≥6 h pi; however, inhibition of host protein synthesis was less in the mutant virus-infected cells. (B) Increased ROS in infected- and mock-infected cells incubated with 10 μM of 5' (and 6')-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate in pre-warmed PBS for 30 min and DCF fluorescence determined by flow cytometry at 6 h pi ($p < 0.05$) for both virus-infected cells. ROS was only slightly increased compared to that of PMA-stimulated cells at 4 to 6 h pi, consistent with lower ROS levels in apoptosis.