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Murine gammaherpesvirus M11 gene product inhibits apoptosis and is expressed during virus persistence

Brief Report

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Summary. The murine gammaherpesvirus (MHV-68) M11 gene encodes a protein with BH1 domain homology to Bcl-2. We found that the M11 gene product (MHVBcl-2) protected murine epithelial cells from TNF-α induced apoptosis. M11 was transcribed during early lytic infection in vitro. During early infection of mice, M11 message was detected in spleen and lung along with lytic cycle messages. During persistence, lytic cycle gene expression was undetectable but M11 RNA was still present. This suggests that MHVBcl-2 promotes virus survival by protecting not only productively infected but also persistently infected cells from apoptotic death.

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Murine gammaherpesvirus (MHV-68) infection of mice offers a small animal model relevant to aspects of Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated virus (KSHV) infection (for review see [19]). Following intranasal inoculation, MHV-68 replicates transiently in lungs followed by spread to B cells and macrophages in the spleen [24]. A splenomegaly then ensues in concert with a peak in latently infected splenocytes approximately 14 d post infection (p.i.). Splenomegaly resolves around 21 d p.i. and the spleen then become a reservoir of latently infected B cells and macrophages [22, 25, 32]. The virus also establishes long-term persistence in other tissue sites, primarily alveolar epithelial cells in the lung [22].

Apoptosis or programmed cell death is a controlled process of cell suicide that is essential for embryonic development and homeostasis in adult tissues. However, this process is also important in eliminating cells whose survival might prove harmful to the organism, thereby providing a defence mechanism against virus infection. Many viruses encode proteins that inhibit apoptosis of infected

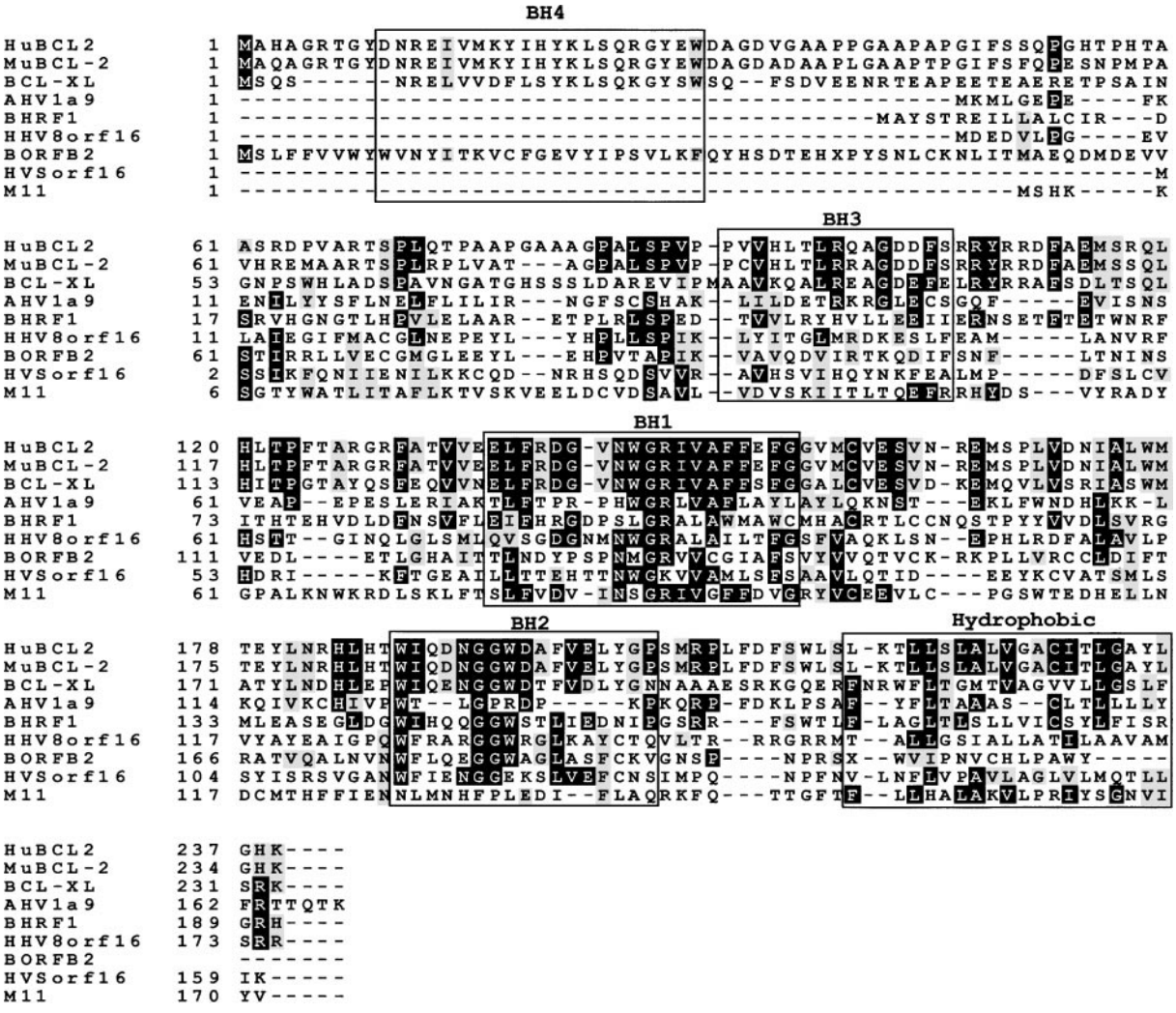

Fig. 1. Protein sequence alignment of cellular Bcl-2 along with gammaherpesvirus Bcl-2 homologs. Sequences were aligned using the CLUSTAL W program [27]. The resulting multiple alignment was presented using the program BOXSHADE (Isrec, Switzerland). Areas of identity are shown in black and conserved regions in grey. Key: HuBCL2, human Bcl-2α; MuBCL2, murine Bcl-2α; BCL-XL, murine Bcl-X_L; AHV1a9, AHV-1 A9; BHRF1, EBV BHRF1; HHV8orf16, HHV-8 ORF16; BORFB2, bovine herpesvirus 4 BORF2; HVSorf16, HVS ORF16; M11, MHV-68 M11

cells (reviewed in [15, 35]). This may extend the survival of lytically infected cells to maximize virion production and the establishment of virus persistence. Anti-apoptotic strategies employed by viruses vary but gammaherpesviruses all encode homologs of the cellular Bcl-2 protein.

The Bcl-2 family members share strong conservation within four domains (BH1–4). In particular, the BH1 and BH2 domains are important for the function of Bcl-2 [3, 10]. All gammaherpesvirus homologs contain identifiable BH1 and BH2 domains but lack BH3 and BH4 domains (see Fig. 1). Despite this, all

Table 1. Primers used for PCR analysis

Gene	Primer	Genome coordinate	Sequence	Product size (bp)
ORF50	sense	68409	AAAAGTTCTGCATCCCAGACCC	293
	antisense	68701	AGGGCTAATGGGTGAAAATGGC	
ORF72	sense	102708	ATGCAGGATGTAACAGATGAG	239
	antisense	102947	GAACTATTCCTTAGCACACGG	
ORF73	sense	103927	TGTCTGAGACCCTTGTCC	450
	antisense	104373	ACACATTTTAAGTCAGCTGTT	
M11	sense	103560	TTAGAAGGCACTATGACAGC	219
	antisense	103779	TGTGTCATGCAATCGTTCAA	
gp150	sense	69770	CACCTCAGAACCAACTTC	337
	antisense	69992	TCTGATGTGTCAGCAG	
Mouse β-actin	sense		TGTGATGGTGGGAATGGGTCA	514
	antisense		TTTGATGTCACGCACGATTTC	

demonstrate anti-apoptotic activity in vitro [3, 4, 6, 8, 14, 18, 26]. MHV-68 also encodes a gene (termed M11 [30]) whose predicted product has homology to Bcl-2. However, this differs in that the homology only extends to a BH1 domain (Fig. 1). Thus, M11 may not be a *bona fide* homolog and may have distinct biologic roles [30]. The aim of this work was to determine whether the MHV-68 M11 gene product had anti-apoptotic function and study its pattern of expression.

M11 gene product protects cells from TNF-α induced apoptosis: To test whether the M11 gene product (MHVBcl-2) could modulate apoptosis, M11 was inserted into the retroviral expression vector pBabe/puro [13]. This vector system was chosen because the level of expression from its Moloney murine leukaemia virus promoter is lower and thus more physiological than that achieved using other promoters such as CMV IE. This vector was then transfected into murine epithelial C127 cells (ATCC CRL-1616) via electroporation (Easyject, Equibio). Several independent puromycin resistant clones were isolated and tested for M11 transcription by RT-PCR as described previously [5] using primers specific for M11 as shown in Table 1. Three lines (3.1, 3.4 and 3.11) positive for M11 RNA were selected, along with a negative control line (W.1) containing empty vector.

MHVBcl-2 activity was tested by measuring the relative sensitivity of M11 expressing cells to apoptosis induced by TNF-α. Confluent monolayers were treated with 500 pg/ml murine TNF-α (PreproTech EC, London) in the presence of 15 μg/ml cycloheximide for 8 h, and cell death monitored microscopically. Cell death was marked in control clone W.1 under these conditions (Fig. 2a). Cells became rounded, detached and exhibited a blebbing of the plasma membrane indicative of apoptotic cell death. To confirm the induction of apoptosis, terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end labelling (TUNEL) analysis was performed on cytospin preparations of detached cells as described

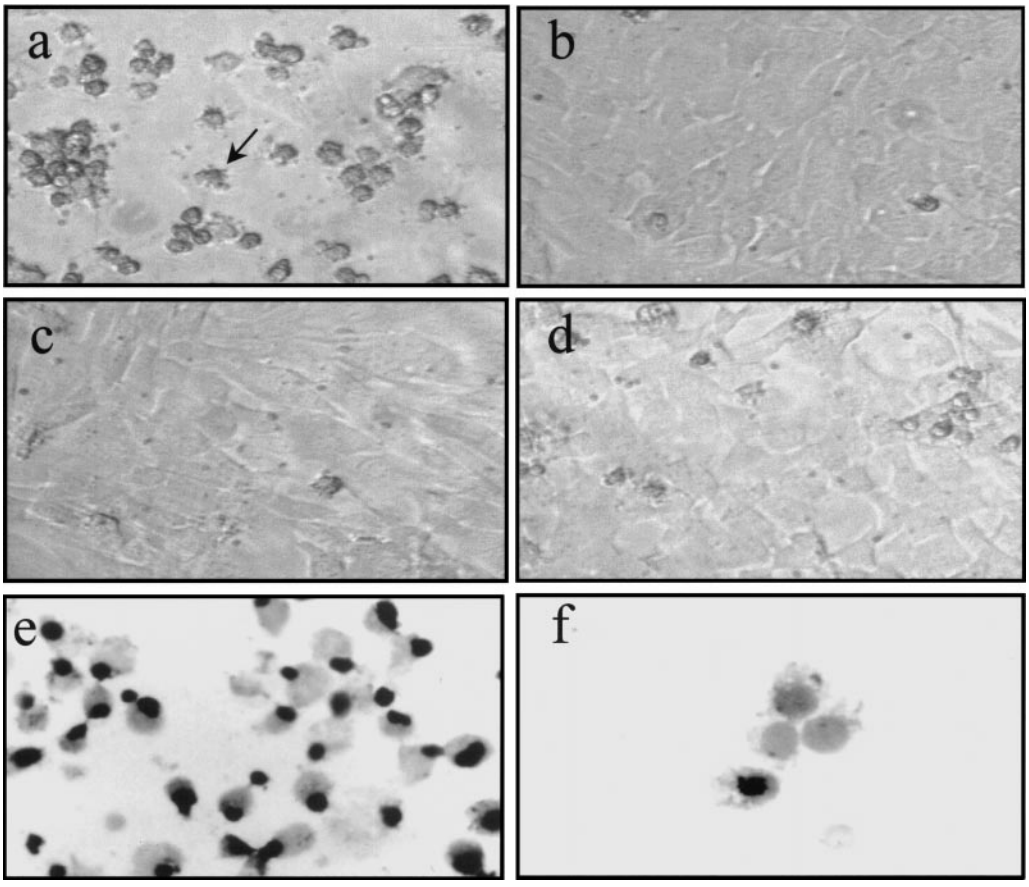

Fig. 2. Protection from TNF-α induced apoptosis. Cell clones were visualized by phase contrast microscopy following 8 h treatment of monolayers with 500 pg/ml TNF-α. **a** negative control line W.1 containing vector only. Note high frequency of detached cells, frequently with plasma membrane blebbing (arrowed); **b**–**d** M11 expressing clones 3.4, 3.1 and 3.11 respectively; **e**, **f** TUNEL analysis of cytospin preparations of detached cells following TNF-α treatment. **e** negative control clone W.1. Note TUNEL positive nuclei containing condensed chromatin; **f** M11 expressing clone 3.4

previously [1]. The nuclei of most detached cells were TUNEL positive (Fig. 2e), confirming apoptosis. M11 expressing clones 3.1 and 3.4 showed almost complete protection from apoptosis (Fig. 2b,c). Few cells detached and were TUNEL positive (Fig. 2f). Clone 3.11 was also protected, but the level was less than that for lines 3.1 and 3.4 (Fig. 2d).

To quantify the degree of apoptotic protection, cell clones were treated with 100 pg/ml TNF-α for 18 h. The frequency of surviving cells was determined microscopically and related to the number seen when treated with cycloheximide alone. Few control W.1 cells remained adherent (Fig. 3). Most cells detached and were apoptotic (TUNEL positive; not shown). The three M11-expressing clones all demonstrated significant survival compared to W.1. These results demonstrated that MHVBcl-2 protected cells against TNF-α induced apoptosis.

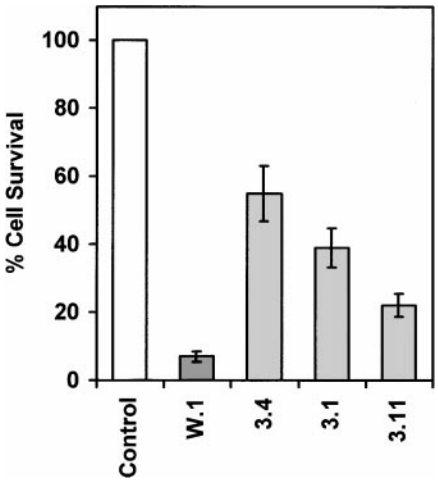

Fig. 3. Quantification of protection from TNF-α induced death in cell clones expressing M11. Monolayer cultures were treated with TNF-α at 100 pg/ml for 18 h and surviving, adherent cells remaining were counted. Control is an arbitrary value representing the initial cell population for each line grown in the absence of TNF-α. Mean ± one SD is given

While this study was in progress, other workers demonstrated that M11 protected HeLa and BHK cells from apoptosis using transient assays [2, 31]. Our results confirm these observations and show that murine epithelial cells that stably express MHVBcl-2 are also protected.

The BH1 and BH2 domains are important not only for dimerization but also for anti-apoptotic activity of the Bcl-2 family of proteins [34]. MHVBcl-2 is unusual in that only a conserved BH1 domain is present in the predicted product [30]. Despite this, MHVBcl-2 has anti-apoptotic activity. Given the highly unusual domain structure of the M11 protein it will be interesting in future to elucidate its mode of action, in particular its pattern of interaction with Bcl-2 family members and other cellular proteins.

Transcription of M11 in vitro: Initially, we evaluated expression by Northern blotting. However, in agreement with recently published work [29] we found that M11 transcription was undetectable. For this reason we used RT-PCR. M11 expression was analysed in relation to the transcription of three other MHV-68 genes; ORF73, ORF72 and gp150. Preliminary Northern data suggested that ORF73 (LANA homolog) was expressed with immediate early (IE) kinetics. ORF72 (cyclin homolog) is transcribed with early (E) kinetics [28]. The gp150 gene is expressed late (L) during infection [20]. C127 cells were infected with MHV-68 at 5 PFU/cell. To distinguish distinct temporal phases of viral gene expression, infection was performed either in the presence or absence of cycloheximide (CHX) and phosphonoacetic acid (PAA) as described previously [21]. RNA was extracted and RT-PCR performed as above using primer sets shown in table 1. β-actin was included to control for mRNA integrity and cDNA synthesis. Control amplifications without reverse transcriptase were uniformly negative. Thus, positive signals were not due to contaminating viral DNA (not shown).

The temporal expression of ORF73, ORF72, M11 and gp150 during lytic infection is shown in Fig. 4. Expression of β-actin was consistently detected indicating that the mRNA was intact and cDNA had been synthesized. ORF73

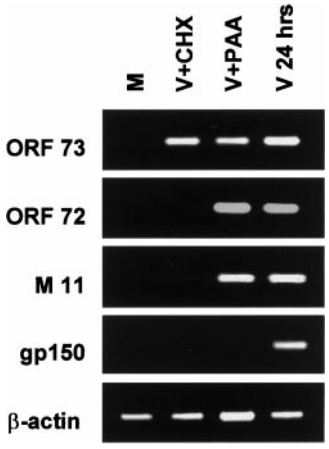

Fig. 4. Expression and temporal regulation of viral transcripts during lytic infection in vitro C127 cells were infected with MHV-68 either alone or in the presence of specific inhibitors of virus gene expression. mRNA was then analysed for transcription of M11, ORF73, ORF72, gp150 and cellular β-actin by RT-PCR. PCR products were analysed by electrophoresis in the presence of ethidium bromide followed by visualization using a UV transilluminator. Images are shown with colours reversed for clarity. *M*, Mock infected; *V + CHX*, infection in the presence of cycloheximide; *V + PAA*, infection in the presence of phosphonoacetic acid; *V 24 hrs*, infection for 24 h in medium alone

transcription was not inhibited by CHX or PAA, ORF72 expression was inhibited by CHX but not by PAA and gp150 expression was inhibited by both CHX and PAA. This shows that ORF73 was expressed with IE, ORF72 with E and gp150 with late kinetics. Thus, RT-PCR was able to differentiate correctly MHV-68 genes of known temporal class. The M11 gene, like ORF72 was inhibited by CHX and not by PAA and was thus expressed with early kinetics.

In agreement with our preliminary results, a recent report failed to detect M11 by Northern analysis [29] pointing to low-level transcription. The temporal expression of MHVBcl-2 is similar to other gammaherpesviruses since transcription of the Bcl-2 homologs of EBV, KSHV and HSV is also a feature of early lytic replication in vitro [7, 9, 16, 17, 23].

Transcription of M11 in vivo: The spleen and lung are the major organs involved during both acute and persistent MHV-68 infection [22]. We therefore analysed M11 expression in these tissues from mice at two points following infection by RT-PCR. At the first time (14 d), acute infection is resolving and latency is being established. At the second (10 months), mice are persistently infected. BALB/c mice were infected intranasally with MHV-68 (4×10^5 PFU) as previously described [24]. RNA was prepared from spleen and lungs using RNAzol B and transcription analysed by RT-PCR. This was performed as described above except that PCR products were analysed by Southern blotting using [α-^{32}P]-labelled, cloned, gene-specific probes. The sensitivity of the PCRs was checked by limiting dilution of cloned fragment templates and was found to be 1 copy. A series of PCR controls were incorporated. To check for carry over of DNA, PCR was performed on cDNA synthesized in the absence of reverse transcriptase. Controls for false positive signals included PCR analysis of cDNA prepared from mock infected C127 cells and uninfected mice as well as PCR in the absence of cDNA. These control reactions were negative in all cases. M11 transcription was related to the expression of ORF50 and gp150 to control for lytic cycle gene expression. ORF50 (EBV R transactivator homolog) was chosen because it is expressed during all temporal phases of the virus lytic cycle [11, 12, 33] and gp150

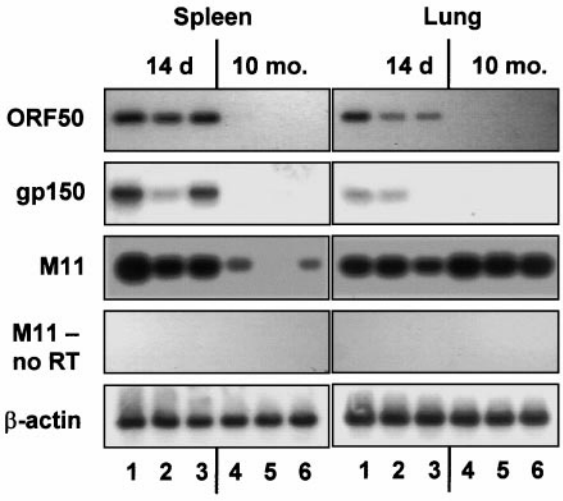

Fig. 5. Expression of M11 in vivo. Mice were infected for either 14 days or 10 months with MHV-68. Spleens and lungs were then sampled and total RNA analysed by RT-PCR followed by Southern blotting and autoradiography. Primers and probes specific for M11, ORF50, gp150 and β-actin were used as indicated. Numbers at the bottom of the figure refer to individual mice

because it is actively transcribed during the late productive cycle [20]. The results are shown in Fig. 5.

Transcription of β-actin was readily detected in all samples indicating the integrity of RNA and completion of cDNA synthesis. Expression of ORF50 was detected in both the spleen and the lung 14 d p.i. in all three mice, but by 10 months expression was negative. Likewise, gp150 RNA was detected in all mice in the spleen and two mice in the lung at 14 d p.i. but in no mice at 10 months p.i. M11 expression was seen in the spleen and lung of all mice at 14 d p.i. By 10 months p.i., M11 was expressed in the spleens of two mice but at a reduced level. However, M11 transcription in the lungs at 10 months p.i. was abundant in all animals. Control PCR amplifications performed on samples without reverse transcriptase (no RT) were negative. Thus, the signals seen were not due to contaminating virus DNA.

At 14 d p.i., productive MHV-68 infection is not detected by biological assay and latently infected cells are found in the spleen [24, 25]. However, the detection at this time of ORF50 and gp150 (productive) RNA shows that lytic cycle gene expression was occurring at a low level in both the spleen and lung. M11 expression was also readily detectable in both organs at this time point. This is therefore more than likely due to expression of M11 by productively infected cells. At 10 months p.i., a persistent infection has been established, with latently infected cells being present in both the lung and the spleen [22]. The lack of ORF50 and gp150 RNA at this time indicates an undetectable level of productive cycle transcription. However, M11 RNA was detected in two of three spleen and all lung samples. Thus, M11 was expressed during persistence. This strongly implicates M11 as a latency-associated transcript. While this study was in progress, other workers identified M11 as a putative latent transcript in the lymphoid system but using intraperitoneal infection of B cell deficient mice [29]. Our results therefore confirm and extend these observations by using intact mice and analysing lungs, a site that we have previously shown to be important for MHV-68 persistence [22].

While RT-PCR is exquisitely sensitive, it gives no information on the nature of the cells expressing M11 during persistence. It is therefore be of critical importance to analyse this question at a single cell level. The expression of M11 during persistence and possibly during latency in vivo is intriguing since in the case of EBV and KSHV the vBcl-2s have only been associated with expression during the virus lytic cycle [7, 23]. Our data argues for a re-evaluation of the expression of EBV and KSHV vBcl-2 proteins in vivo.

The role of virus Bcl-2 homologs: The conservation of such genes by gammaherpesviruses suggests an important role for these proteins. It has been postulated that viral Bcl-2 products prevent apoptosis during infection, allowing increased replication and dissemination of virus [15, 35]. Based on our data, therefore, MHVBcl-2 would be expected to protect against apoptosis mediated by cytotoxic cells during the immune response to acute and persistent MHV-68 infection. This may be important in the lung where there is a constant exposure to cytotoxic factors and immunological challenge, and thus exposure of infected cells to pro-apoptotic signals may be frequent. Our data suggests that this may entail the presence of MHVBcl-2 as a survival factor not only in cells reactivating virus, but also in latently infected cells. The precise role of the MHVBcl-2 remains to be determined by targeted mutagenesis of the gene in a recombinant virus.

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