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# Murine Coronavirus-Induced Apoptosis in 17Cl-1 Cells Involves a Mitochondria-Mediated Pathway and Its Downstream Caspase-8 Activation and Bid Cleavage

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Mouse hepatitis virus (MHV) infection in murine 17Cl-1 cells results in apoptotic cell death. Inhibition of MHV-induced apoptosis by the pancaspase inhibitor Z-VAD-FMK promoted virus production late in infection, indicating that apoptosis could be a host response to limit the production of viral progeny. Activation of the mitochondria-mediated apoptotic pathway was indicated by the activation of caspase-9 and delay of apoptosis by Bcl-2 overexpression. Analyses of the subcellular distribution of cytochrome c, procaspase-9, and Apaf-1 suggested an aberrant apoptosome formation in the vicinity of the mitochondria, which could be a cell type-specific event. An increase in the amount of Fas (APO-1/CD95), caspase-8 activation, caspase-8-mediated Bid cleavage, and subsequent translocation of truncated Bid to mitochondria, all of which relate to the Fas-mediated pathway, also occurred in MHV-infected 17Cl-1 cells, whereas the formation of the death-inducing signaling complex, a direct indication of the activation of Fas-mediated pathway, was undetectable. Caspase-8 and Bid activation appeared to be downstream of mitochondria, because Bcl-2 overexpression suppressed both events, suggesting that infected 17Cl-1 cells might have activated a receptor-mediated "type II" signaling pathway, in which primary and low levels of receptor-mediated pathway activation lead to the activation of the mitochondria-mediated pathway. All our data indicate that a mitochondria-mediated pathway played a major regulatory role in apoptosis in MHV-infected 17Cl-1 cells.

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## INTRODUCTION

Viral infection may trigger a variety of host cellular responses, and one outcome of virus–host interaction is the activation of an innate cell death machinery, called programmed cell death, or apoptosis; many viruses and viral proteins are capable of inducing apoptosis (reviewed by O'Brien, 1998; Teodoro and Branton, 1997). The induction of apoptosis upon viral infection has been hypothesized to be a host-defense response, since early death of virus-infected cells would prevent viral replication. Many viruses have developed a countermeasure(s) that effectively blocks or delays cell death by expressing antiapoptotic proteins to maximize the production of viral progeny (reviewed by Hardwick, 1998; O'Brien, 1998; Teodoro and Branton, 1997). On the other hand, apoptosis induction at the end of the viral replication cycle might assist in viral dissemination, while attenuating an inflammatory response. Apparently viruses have evolved to regulate apoptosis, which may directly or indirectly contribute to viral pathogenesis.

Apoptosis usually involves the activation of a family of cysteine proteases named caspases, which cleave a

variety of cellular substrates, and the cleavage of certain important protein targets leads to detrimental biochemical and morphological changes and eventual cell destruction (reviewed by Earnshaw *et al.*, 1999; Nicholson, 1999). Caspases are synthesized as proenzymes, which require cleavage for their activation. Initiator caspases, such as caspase-8 and -9, undergo self-cleavage and activation upon receiving apoptotic signals. These upstream caspases then activate effector caspases, such as caspase-3, -6, and -7, which execute the cleavage of other cellular substrates. Caspase cascades can be activated by two major pathways. In one pathway, different stimuli converge death signals to mitochondria, resulting in mitochondrial damage and the release of cytochrome c to the cytoplasm, where it forms apoptosome complexes with Apaf-1 and procaspase-9 in the presence of dATP, leading to the activation of caspase-9 (Li *et al.*, 1997; Saleh *et al.*, 1999; Zou *et al.*, 1999). The other pathway initiates from cell surface death receptors, such as Fas (CD95/APO-1) and TNF-R1. Engagement of these receptors by their cognate ligands results in the aggregation of intracellular death domains and the recruitment of FADD and procaspase-8, forming the death-inducing signaling complex (DISC), where caspase-8 is activated (Kischkel *et al.*, 1995; Medema *et al.*, 1997).

Bcl-2 family proteins play an important role in regulating apoptotic signaling at the mitochondrial level (re-

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viewed by Adams and Cory, 1998; Gross *et al.*, 1999). Antiapoptotic members Bcl-2 and Bcl-X<sub>L</sub> inhibit cytochrome c release, while proapoptotic members Bax, Bak, Bid, and Bik promote cytochrome c release (Finucane *et al.*, 1999; Jurgensmeier *et al.*, 1998; Kluck *et al.*, 1997; Shimizu and Tsujimoto, 2000; Yang *et al.*, 1997). Bid, a 22-kDa "BH3-domain-only" member, is cleaved by caspase-8, and the 15-kDa truncated Bid fragment (p15 tBid) translocates to mitochondrial membranes, where it triggers the homooligomerization of Bak and Bax, resulting in the release of cytochrome c from mitochondria (Eskes *et al.*, 2000; Wei *et al.*, 2000, 2001). Hence Bid plays a role in amplifying receptor-mediated death signals by activating the mitochondrial pathway (Li *et al.*, 1998; Luo *et al.*, 1998).

Coronaviruses are enveloped RNA viruses that cause gastrointestinal and upper respiratory tract illnesses in animals and humans (Perlman, 1998; Wege *et al.*, 1982). Among coronaviruses, mouse hepatitis virus (MHV) is one of the best characterized in terms of its pathogenesis and molecular biology. MHV causes various diseases, including hepatitis, enteritis, and encephalitis, in rodents (Compton *et al.*, 1993; Wege *et al.*, 1982). In addition, infection with certain strains of MHV causes demyelination in rodents, and MHV-induced demyelination has been used as an excellent model system for human demyelinating diseases, such as multiple sclerosis (Bailey *et al.*, 1949; Houtman and Fleming, 1996; Wege *et al.*, 1982). It has been reported that coronaviruses, including MHV (An *et al.*, 1999; Belyavsky *et al.*, 1998), transmissible gastroenteritis coronavirus (TGEV) (Eleouet *et al.*, 1998, 2000), and infectious bronchitis virus (IBV) (Liu *et al.*, 2001) can cause caspase-dependent apoptotic cell death in infected cultured cells. TGEV infection may lead to apoptosis via cellular oxidative stress (Eleouet *et al.*, 1998), and TGEV N protein is a substrate for caspase-6 and -7 (Eleouet *et al.*, 2000). Some coronavirus proteins, such as a 58-kDa protein encoded by IBV open reading frame 1b and MHV E protein, trigger apoptosis when they are overexpressed (An *et al.*, 1999; Liu *et al.*, 2001).

MHV replication in murine fibroblast 17Cl-1 cells induces apoptosis (An *et al.*, 1999). The present study characterized the molecular mechanisms leading to the activation of apoptotic pathways in MHV-infected 17Cl-1 cells. Analyses of subcellular localization of cytochrome c, Apaf-1, and caspase-9 suggested an unusual event of apoptosome formation in association with mitochondria in infected 17Cl-1 cells. Characterization of the effect of overexpressed Bcl-2 and the status of various caspases and Bid demonstrated that mitochondria act as a critical regulator in MHV-induced apoptosis.

## RESULTS

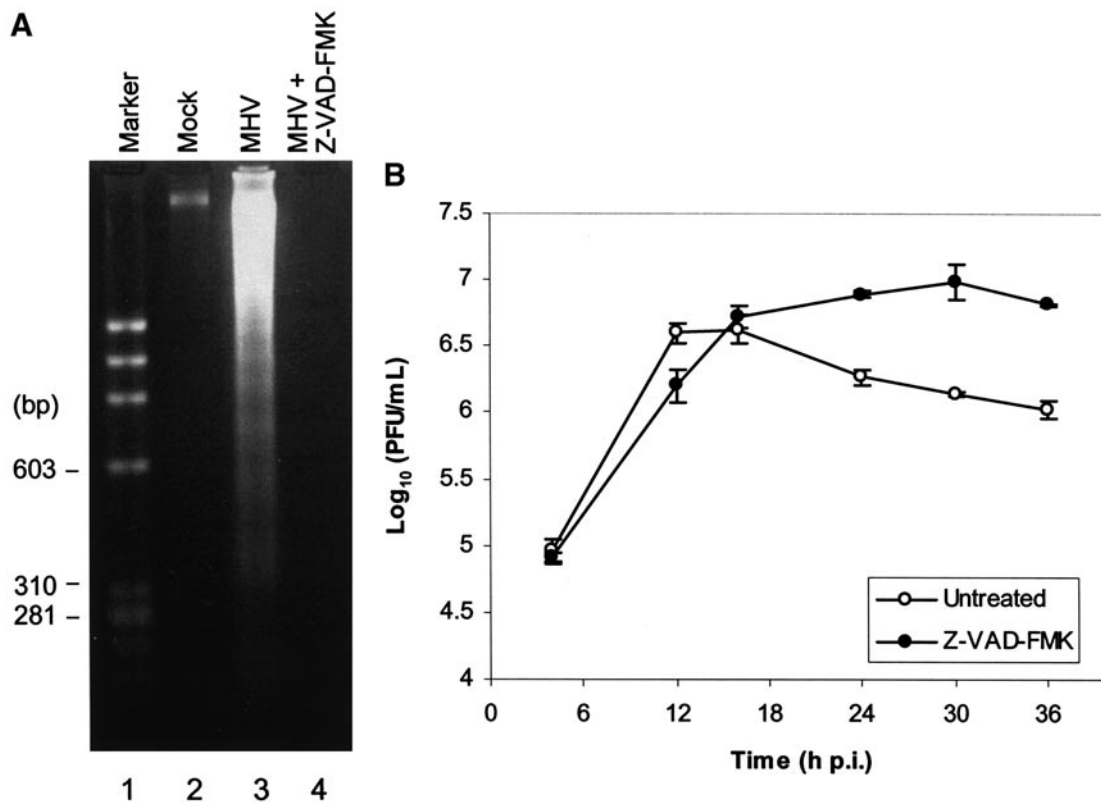
### Effect of the pancaspase inhibitor Z-VAD-FMK on MHV-induced apoptosis and virus growth

To discover the biological significance of MHV-induced apoptosis in 17Cl-1 cells, we examined the effect of the broad-range caspase inhibitor Z-Val-Ala-Asp-fmk (Z-VAD-FMK) on MHV-induced apoptosis and MHV replication. Addition of 150  $\mu$ M Z-VAD-FMK to 17Cl-1 cells that were infected with the A59 strain of MHV (MHV-A59) inhibited the production of apoptotic DNA fragments (Fig. 1A). MHV-A59 infection causes extensive cell fusion in 17Cl-1 cells (An *et al.*, 1999); almost all fused cells detach from the culture dish after 20 h postinfection (p.i.). MHV infection still caused syncytia in the presence of Z-VAD-FMK, yet most of the fused cells remained adherent on the culture dish for up to 48 h, the end point of the experiments. These data demonstrated that MHV-A59-induced apoptosis was executed by caspase-dependent mechanisms, and suppression of apoptosis affected MHV-induced cytopathogenicity.

We next examined the effect of apoptosis on MHV production. As shown in Fig. 1B, in the absence of the caspase inhibitor, the highest virus titer in the culture fluid of infected 17Cl-1 cells was detected at 12 h p.i., and the titer gradually decreased. In contrast, in the presence of Z-VAD-FMK, infected 17Cl-1 cells continuously produced a higher MHV titer from 16 to 36 h p.i. (at 24, 30, and 36 h p.i., Student's *t* test,  $P < 0.001$ ). To eliminate the possibility that MHV was stabilized in the presence of Z-VAD-FMK, culture fluid of MHV-infected cells, which was obtained at 20 h p.i., was incubated with 150  $\mu$ M Z-VAD-FMK or without Z-VAD-FMK at 37°C for another 44 h. A similar MHV titer was obtained in both samples (data not shown), demonstrating that Z-VAD-FMK did not affect the stability of MHV. These data demonstrated that when apoptosis was suppressed, the virus titer remained at a high level, even at the late stage in infection.

### Bcl-2 overexpression delays MHV-induced apoptosis

The antiapoptotic Bcl-2 protein has been known to block apoptosis by maintaining the integrity of mitochondrial membranes and preventing the release of apoptosis-activating factors, such as cytochrome c and apoptosis-inducing factor (AIF), from mitochondria (Kluck *et al.*, 1997; Susin *et al.*, 1999b; Yang *et al.*, 1997). To determine whether the mitochondria-mediated pathway was activated during MHV-induced apoptosis, we examined the effect of Bcl-2 overexpression on MHV-induced apoptosis. Three 17Cl-1 cell lines that stably overexpressed Bcl-2 (17Cl-1/bcl-2) and two control 17Cl-1 cell lines that stably expressed the neomycin phosphotransferase gene (17Cl-1/neo) were established. These cells were infected with MHV-A59 or mock infected. MHV-infected 17Cl-1/neo and 17Cl-1/bcl-2 cells showed syncytia for-



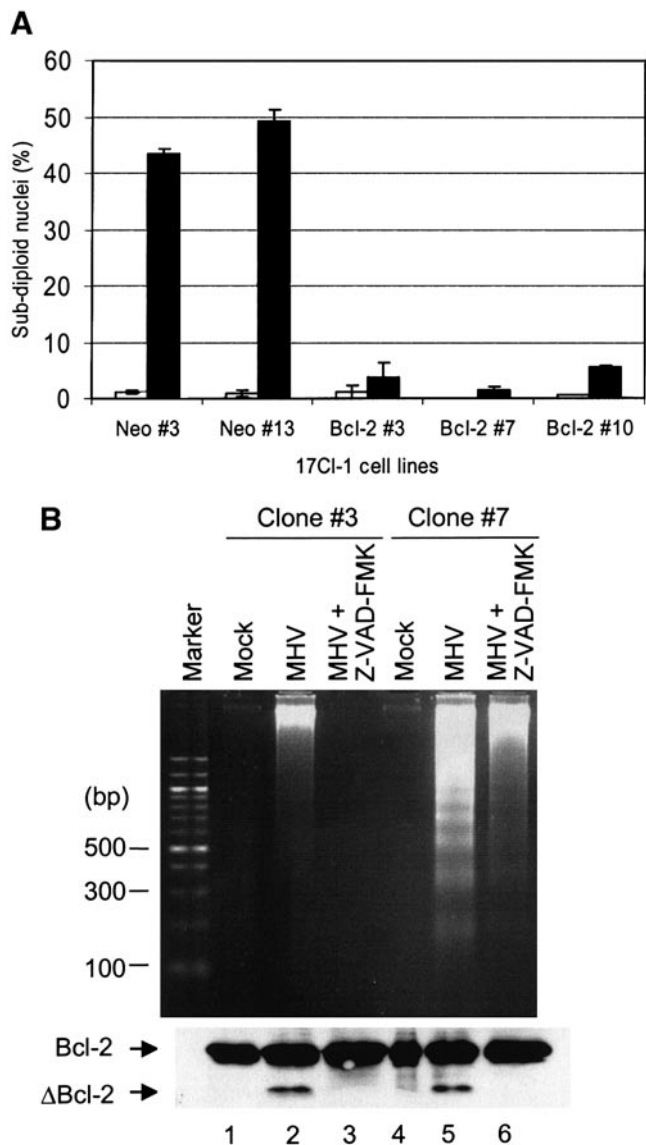
**FIG. 1.** Effect of Z-VAD-FMK on MHV-induced apoptosis (A) and MHV yield (B). (A) The 17Cl-1 cells were mock infected (lane 2) or infected with MHV at a multiplicity of infection (m.o.i.) of 5 in the presence (lane 4) or the absence (lane 3) of Z-VAD-FMK. At 24 h p.i., low-molecular-weight DNA was extracted and separated in a 2% agarose gel; lane 1, DNA size markers. (B) The 17Cl-1 cells were infected with MHV at an m.o.i. of 5 in the presence (filled circles) or the absence (open circles) of Z-VAD-FMK. Culture fluids were collected at indicated times p.i., and MHV titers were determined by plaque assays. The results are expressed as the means of four independent experiments.

mation, as seen in MHV-infected parental 17Cl-1 cells. The infected 17Cl-1/neo cells started to detach from the culture dish after 14 h p.i., whereas the majority of infected 17Cl-1/bcl-2 cells remained attached to the dish for up to 24 h p.i. At 18 h p.i., cells were collected and suspended in staining solutions containing propidium iodide. Nuclear DNA was stained by propidium iodide and the samples were applied for flow cytometric analysis, in which the proportion of nuclei containing subdiploid DNA contents, i.e., apoptotic nuclei, was determined. As shown in Fig. 2A, MHV-induced apoptosis was blocked in all three 17Cl-1/bcl-2 cell lines, but not in both 17Cl-1/neo cell lines. However, when the cultures were incubated further, infected 17Cl-1/bcl-2 cells became detached from the culture dish late in infection (data not shown). Consistent with the observed cytopathogenicity, accumulation of apoptotic DNA fragments occurred in infected 17Cl-1/bcl-2 cells at 32 h p.i. (Fig. 2B, top panel, lanes 2 and 5), indicating that MHV-infected 17Cl-1/bcl-2 cells eventually died by apoptosis. Western blot analysis of Bcl-2 protein revealed the appearance of an additional band with an approximate molecular weight of 23 kDa ( $\Delta$ Bcl-2) in infected 17Cl-1/bcl-2 cells, but not in uninfected cells (Fig. 2B, bottom panel). It is known that Bcl-2

can be cleaved by caspase-3, producing a 23-kDa proteolytic fragment (Cheng *et al.*, 1997). Treatment of Z-VAD-FMK blocked both apoptosis and Bcl-2 cleavages (Fig. 2B, lanes 3 and 6), suggesting that certain caspases, probably caspase-3, cleaved Bcl-2 late in infected 17Cl-1/bcl-2 cells. The finding that overexpression of Bcl-2 delayed MHV-induced apoptosis indicated the involvement of the mitochondria-mediated pathway in MHV-induced cell death in 17Cl-1 cells.

#### Activation of the mitochondrial pathway in the absence of cytochrome c release in MHV-infected 17Cl-1 cells

To further demonstrate the activation of the mitochondria-mediated pathway, we analyzed two hallmarks of this pathway, cytochrome c release from mitochondria and caspase-9 activation (Kluck *et al.*, 1997; Li *et al.*, 1997; Yang *et al.*, 1997; Zou *et al.*, 1997). Etoposide, a topoisomerase II inhibitor, induces apoptosis by activating the mitochondria-mediated pathway (Kluck *et al.*, 1997; Yang *et al.*, 1997); hence etoposide-treated 17Cl-1 cells served as positive controls. Western blot analysis showed that procaspase-9 was processed into a 35-kDa



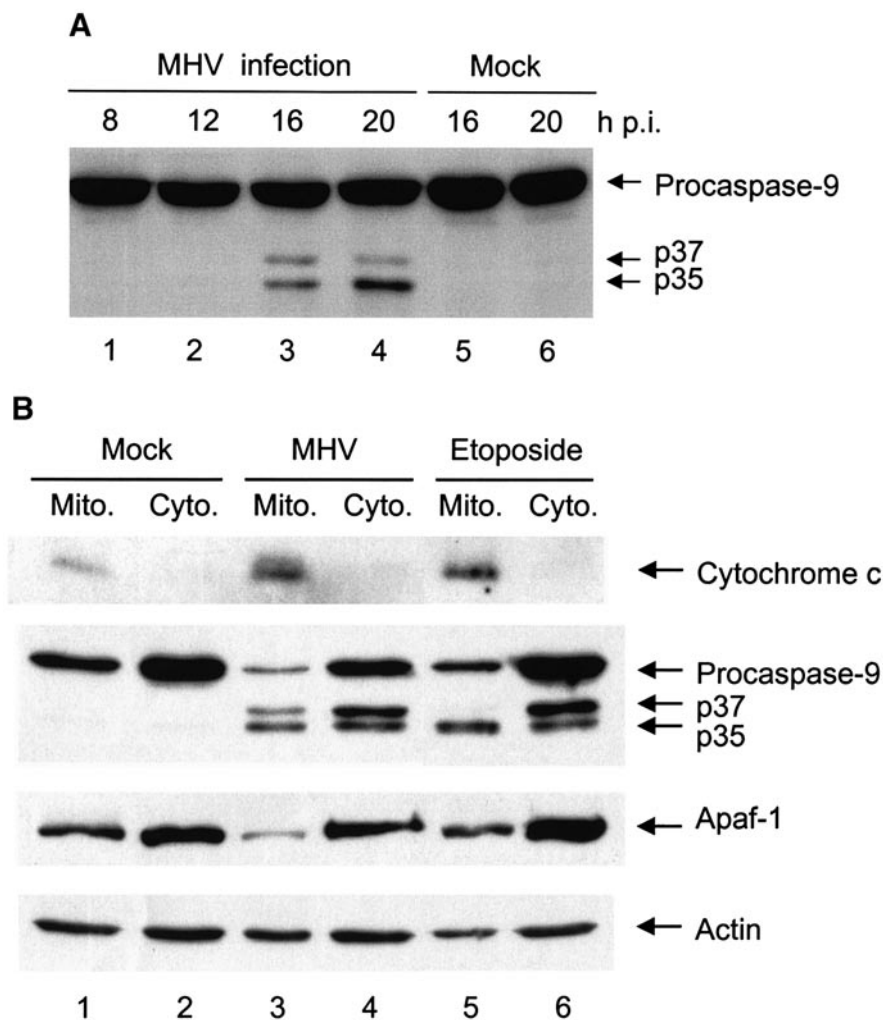
**FIG. 2.** Effect of Bcl-2 overexpression on MHV-induced apoptosis. (A) The 17Cl-1/bcl-2 and 17Cl-1/neo cell clones were mock infected (blank bars) or infected with MHV (filled bars) at an m.o.i. of 10. Cells were collected at 18 h p.i., and the populations of apoptotic nuclei with subdiploid DNA contents were determined as described under Materials and Methods. The results are expressed as the means of two independent experiments. (B) Two 17Cl-1/bcl-2 cell clones (clones 3 and 7) were mock infected (lanes 1 and 4) or infected with MHV at an m.o.i. of 10 in the presence (lanes 3 and 6) or the absence (lanes 2 and 5) of Z-VAD-FMK. At 32 h p.i., low-molecular-weight DNA was extracted and analyzed by electrophoresis in a 2% agarose gel (top), and total cell lysates were subjected to Western blot analysis and probed for Bcl-2 (bottom).

fragment (p35) and a 37-kDa fragment (p37) at 16 and 20 h p.i. (Fig. 3A). No procaspase-9 cleavage occurred in mock-infected cells. p35 is known to be produced by autocleavage of procaspase-9 when the apoptosome complex is formed, while p37 is produced by caspase-3-mediated cleavage of procaspase-9 (Srinivasula *et al.*,

1998). The appearance of p35 in infected cells demonstrated activation of the mitochondrial pathway.

We next examined whether cytochrome c was released from mitochondria to cytoplasm in MHV-infected 17Cl-1 cells. Mitochondrial and cytosolic fractions were prepared by subcellular fractionation and probed for cytochrome c by Western blot analysis. To our surprise, most of the cytochrome c remained in the mitochondria at 16 h p.i., and no significant amounts of cytochrome c were released into the cytosol (Fig. 3B, lanes 3 and 4); at this time postinfection, caspase-9 activation was evident (Fig. 3A, lane 3; Fig. 3B, lanes 3 and 4). The same phenomenon was also observed in etoposide-treated 17Cl-1 cells (Fig. 3B, lanes 5 and 6). In more than 20 repetitions of the experiments with numerous experimental modifications, we did not obtain clear evidence for the release of cytochrome c into cytosol. Immunofluorescence analysis of infected cells using anti-cytochrome c antibodies also failed to demonstrate the release of cytochrome c into the cytosol (data not shown). Hence, we concluded that the release of cytochrome c from mitochondria into the cytosol did not occur in either MHV-infected or etoposide-treated 17Cl-1 cells.

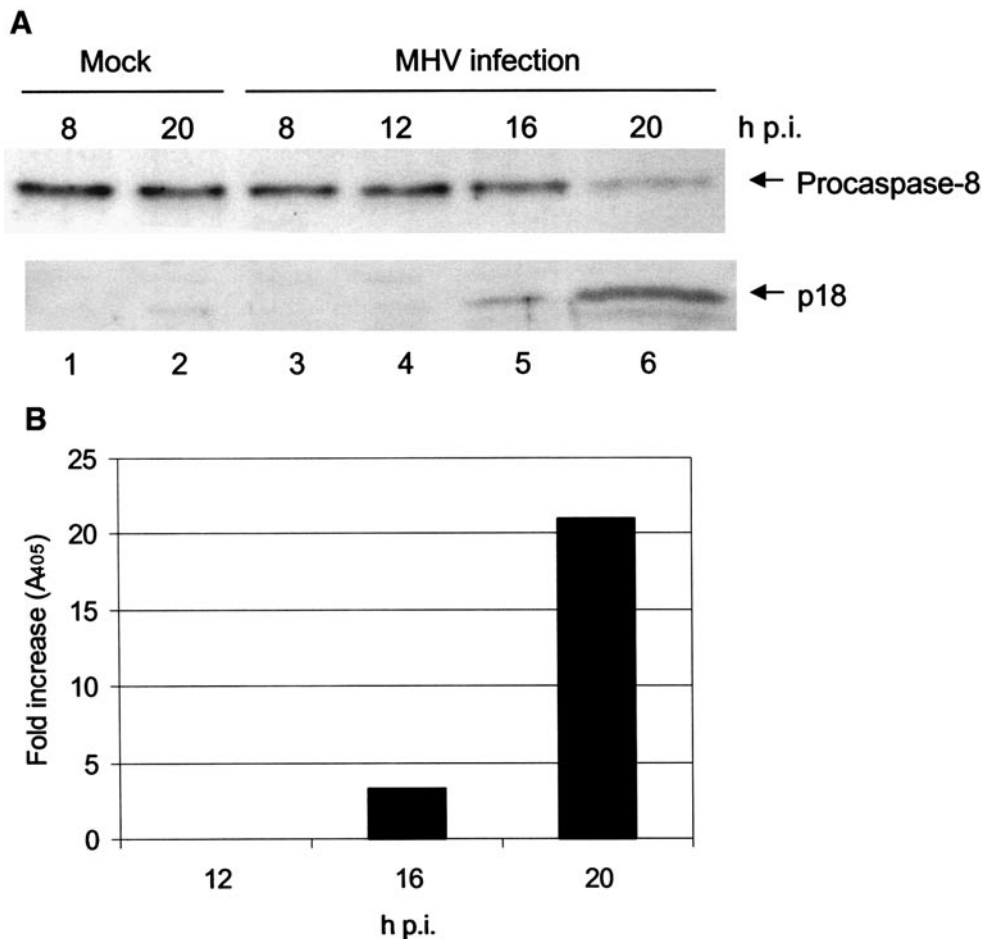
Association of cytochrome c with mitochondria led us to explore a possibility that the apoptosome complex may be formed in the vicinity of mitochondria in apoptotic 17Cl-1 cells. As shown in Fig. 3B, procaspase-9 was found in both mitochondria and cytoplasm in mock-infected cells (Fig. 3B, lanes 1 and 2). Notably, in both MHV-infected and etoposide-treated 17Cl-1 cells, the majority of mitochondria-associated procaspase-9 underwent cleavage, and the autocleavage fragment, p35, was detected in the mitochondrial fractions (Fig. 3B, lanes 3 and 5), suggesting that apoptosome might have been formed in a location associated with mitochondria. The caspase-9 cleavage fragment p37 was also present in the mitochondria of MHV-infected 17Cl-1 cells (Fig. 3B, lane 3), indicating that caspase-3-mediated cleavage of procaspase-9 occurred in the mitochondria. If apoptosome is formed in a location associated with mitochondria, then Apaf-1, another component of the apoptosome complex, should be detected in the mitochondrial fraction. In mock-infected nonapoptotic 17Cl-1 cells, a significant amount of Apaf-1 was detected in the mitochondrial fraction (Fig. 3B, lane 1), while there was a clear decrease in the amount of mitochondrial Apaf-1 in MHV-infected cells (Fig. 3B, lane 3). The decrease of mitochondrial Apaf-1 in infected 17Cl-1 cells could be due to caspase-3-mediated cleavage, as reported previously (Lauber *et al.*, 2001). Although we did not examine the subcellular localization of caspase-3, it is known that procaspase-3 has a cytosolic and mitochondrial distribution in nonapoptotic cells (Mancini *et al.*, 1998). It is possible that the mitochondrial apoptosome formation may directly lead to the activation of mitochondria-associated procaspase-3 in MHV-infected 17Cl-1 cells. In



**FIG. 3.** Activation of the mitochondrial pathway in the absence of mitochondrial cytochrome c release in MHV-infected 17Cl-1 cells. (A) Caspase-9 activation in MHV-infected 17Cl-1 cells. The 17Cl-1 cells were mock infected (lanes 5 and 6) or infected with MHV at an m.o.i. of 10 (lanes 1–4). Cell lysates were collected at the indicated times p.i., and equal amounts of protein (40  $\mu$ g) from each sample were subjected to Western blot analysis and probed for caspase-9. (B) Subcellular localization of cytochrome c, caspase-9, and Apaf-1 in 17Cl-1 cells infected with MHV or treated with etoposide. The 17Cl-1 cells were mock infected (Mock), infected with MHV (MHV), or treated with etoposide (Etoposide). At 16 h p.i. and 18 h after etoposide treatment, cells were collected and subjected to subcellular fractionation. Twenty-five micrograms of protein from each of the mitochondrial (Mito.) or cytosolic (Cyto.) fractions was subjected to Western blot analysis and probed for cytochrome c, caspase-9, Apaf-1, and actin.

contrast, active caspase-3 appeared to be absent in mitochondria of etoposide-treated 17Cl-1 cells, because the mitochondrial fraction lacked caspase-9 cleavage fragment p37 (Fig. 3B, lane 5) and cleavage of Apaf-1 was not evident in the mitochondrial fraction of etoposide-treated 17Cl-1 cells. Detection of p37 in the cytoplasm of etoposide-treated 17Cl-1 cells suggested that active caspase-3 was present in the cytoplasm (Fig. 3B, lane 6). The absence of caspase-9 cleavage fragment p37 in the mitochondrial fraction of etoposide-treated cells (Fig. 3B, lane 5) demonstrated that the contamination of cytosolic proteins in the mitochondrial fractions was negligible under our experimental conditions. It is unclear why mitochondria-associated caspase-3 appeared to be activated in MHV-infected 17Cl-1 cells, but not in etoposide-treated 17Cl-1 cells.

Taken together, our results suggested that activation of the mitochondrial pathway in 17Cl-1 cells occurred without the release of cytochrome c from mitochondria to cytoplasm. Mitochondrial pathway activation in 17Cl-1 cells appeared to involve an unusual formation of apoptosome complex in the vicinity of the mitochondria, possibly in the intermembrane space or on the outer membrane of mitochondria. This unique phenotype of mitochondrial pathway activation seems to be cell type specific, rather than stimuli specific. We have tried to further characterize this phenomenon by treating 17Cl-1 cells with staurosporine, a broad-range protein kinase inhibitor that induces apoptosis in other cell types through the activation of the mitochondrial pathway (Kluck *et al.*, 1997; Yang *et al.*, 1997). However, treatment of 17Cl-1 cells with 5  $\mu$ M staurosporine for 24 h did not



**FIG. 4.** Caspase-8 activation in MHV-infected 17CI-1 cells. (A) The 17CI-1 cells were mock infected (lanes 1 and 2) or infected with MHV at an m.o.i. of 10 (lanes 3–6). Cell lysates were collected at the indicated times p.i., and equal amounts of protein (40  $\mu$ g) from each sample were subjected to Western blot analysis and probed for caspase-8. (B) Cell lysates were collected at indicated times p.i. and subjected to a colorimetric caspase-8 activity assay as described under Materials and Methods. The results are expressed as the means of two independent experiments.

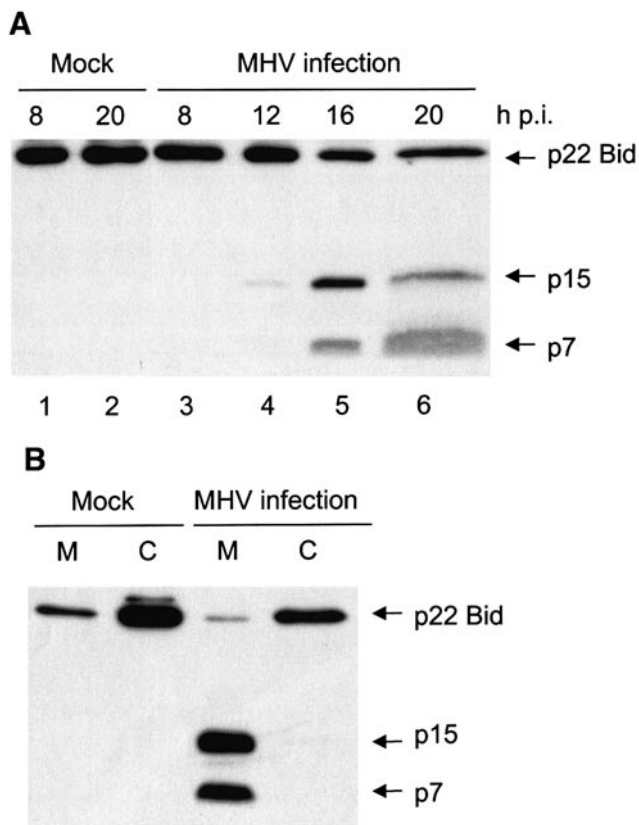
cause cell death (data not shown). Another apoptosis inducer, dexamethasone, also failed to induce apoptosis in 17CI-1 cells (data not shown). Our results indicate that 17CI-1 cells, a spontaneously transformed cell line derived from BALB/c 3T3 cells, have aberrant responses to apoptotic stimuli.

#### Caspase-8 activation and Bid cleavage during MHV-induced apoptosis

Although Bcl-2 overexpression delayed MHV-induced apoptosis, caspase-dependent Bcl-2 cleavage and apoptosis eventually occurred in MHV-infected 17CI-1/bcl-2 cells (Fig. 2). These data suggested that either the accumulation of proapoptotic signals overcame the antiapoptotic function of Bcl-2 later in infection or a Bcl-2-insensitive pathway, i.e., other than the mitochondrial pathway, might also have been activated, leading to caspase-3 activation and subsequent Bcl-2 cleavage. Since receptor-mediated apoptosis is Bcl-2-insensitive, we then investigated whether any receptor-mediated pathway was

activated during MHV-induced apoptosis. We first examined the activation of caspase-8, an apical caspase that is activated in death receptor-mediated apoptosis. As shown in Fig. 4A, procaspase-8 was cleaved into the p18 fragment late in infection. Furthermore, a colorimetric substrate assay for caspase-8 also demonstrated the activation of caspase-8 (Fig. 4B).

Bid, the "BH3-domain-only" pro apoptotic Bcl-2 member, can be cleaved by caspase-8 (Li *et al.*, 1998; Luo *et al.*, 1998), and the p15 tBid targets mitochondria, causing mitochondrial damage and activating the mitochondria-mediated pathway (Eskes *et al.*, 2000; Wei *et al.*, 2000, 2001); hence we next examined the presence of Bid cleavage in infected cells. As shown in Fig. 5A, p15 tBid started to appear at 12 h p.i., and the amounts of p7 and p15 tBid fragments increased later in the course of the infection. Characterization of the subcellular localization of tBid clearly demonstrated that the majority of both p15 and p7 tBid fragments localized in the mitochondrial fraction (Fig. 5B), which is consistent with previous find-



**FIG. 5.** Bid cleavage and translocation of tBid to mitochondria in MHV-infected 17Cl-1 cells. (A) The 17Cl-1 cells were mock infected (lanes 1 and 2) or infected with MHV at an m.o.i. of 10 (lanes 3–6). Cell lysates were collected at the indicated times p.i., and equal amounts of protein (40  $\mu$ g) from each sample were subjected to Western blot analysis and probed for Bid. (B) Twenty-five micrograms of mitochondrial (M) and cytosolic (C) proteins prepared for experiments in Fig. 3B were subjected to Western blot analysis and probed for Bid.

ings by others that p7 and p15 fragments form a tight, noncovalent complex after cleavage by caspase-8 (Chou *et al.*, 1999; Zha *et al.*, 2000), and myristoylated p15 inserts into mitochondrial membranes (Zha *et al.*, 2000). These data indicated that the translocation of tBid to mitochondria was one of the mechanisms that activated the mitochondrial pathway in MHV-infected 17Cl-1 cells. Furthermore, caspase-8 activation and Bid cleavage suggested the activation of a cell surface death receptor(s) during MHV-induced apoptosis.

#### Bcl-2 overexpression suppresses caspase-8 activation and Bid cleavage in infected 17Cl-1 cells

To know whether the receptor-mediated pathway was activated in MHV-infected 17Cl-1 cells, a multiprobe RNase protection assay (mAPO-3 template set, Ribo-Quant multiprobe RNase protection assay system, BD Pharmingen) was used to examine the expression of a panel of genes whose products can regulate receptor-mediated apoptosis. The amount of mRNAs for caspase-8, Fas ligand, FADD, FAP-1, FAF-1, TRAIL, TN-

FRp55, TRADD, and RIP did not change during the course of MHV replication in 17Cl-1 cells, whereas a significant increase (about 20-fold) in the amount of Fas mRNA and a moderate increase (about 3-fold) in the amount of Fas protein occurred from 12 to 24 h p.i. (data not shown), suggesting a possible activation of the Fas-mediated pathway in MHV-infected 17Cl-1 cells. However, anti-Fas antibody (Jo2) failed to coimmunoprecipitate FADD from MHV-infected 17Cl-1 cell extracts (data not shown), indicating that either DISC was not formed or DISC formation was too weak to be detected.

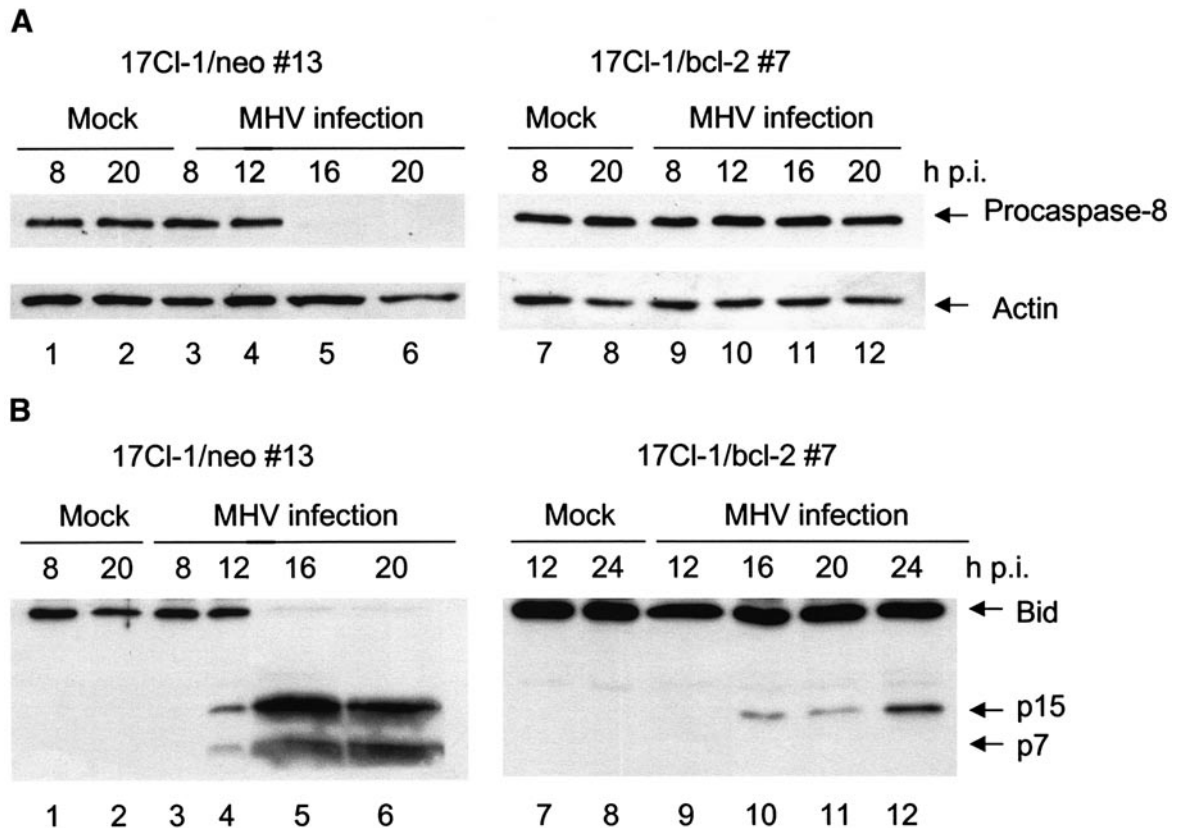
Previous studies by others on anti-Fas antibody-induced apoptosis in Jurkat and CEM cells showed an unusual apoptotic pathway activation (Scaffidi *et al.*, 1998). It has been proposed that in these cells, called type II cells, Fas-mediated pathway activation causes very low levels of DISC formation, which is usually at an undetectable level. Nevertheless, low levels of DISC formation lead to activation of limited amounts of caspase-8, which are yet sufficient to cleave Bid. Truncated Bid fragments then relocate to mitochondria and amplify the apoptotic signals by activating the mitochondrial pathway (Scaffidi *et al.*, 1999). Activation of the mitochondrial pathway subsequently efficiently activates caspase-8, and since the major caspase-8 activation step is downstream of mitochondria in type II cells (Scaffidi *et al.*, 1998), both caspase-8 activation and apoptosis are blocked by overexpression of Bcl-2.

Although we could not detect the formation of DISC in MHV-infected 17Cl-1 cells, it is possible that the upregulated Fas protein activates the Fas-mediated pathway through the same or a similar mechanism that is used in type II cells. If this is the case, overexpression of Bcl-2 should prevent caspase-8 activation. Western blot analysis of procaspase-8 demonstrated that the majority of procaspase-8 was cleaved in infected 17Cl-1/neo cells at 16 h p.i. (Fig. 6A). In contrast, no significant procaspase-8 cleavage occurred in infected 17Cl-1/bcl-2 cells, even at 20 h p.i. Consistent with the magnitude of procaspase-8 cleavage, extensive Bid cleavage occurred only in infected 17Cl-1/neo cells late in infection; infected 17Cl-1/bcl-2 cells showed very low levels of Bid cleavage (Fig. 6B). These data clearly showed that Bcl-2 overexpression suppressed caspase-8 activation and Bid cleavage, and hence the major step of caspase-8 activation occurred downstream of mitochondria. All our data indicate that a mitochondria-mediated pathway played a major regulatory role in apoptosis in MHV-infected 17Cl-1 cells.

## DISCUSSION

We examined biological roles of apoptosis in MHV replication and mechanisms of MHV-induced apoptosis in 17Cl-1 cells. We first showed that MHV induced caspase-dependent apoptosis in this cell line. Subsequent studies showed that there was no significant dif-





**FIG. 6.** Effect of Bcl-2 overexpression on caspase-8 activation (A) and Bid cleavage (B). 17Cl-1/neo clone 13 cells and 17Cl-1/bcl-2 clone 7 cells were mock infected or infected with MHV at an m.o.i. of 10. At the indicated times p.i., total cell lysates were collected and 40  $\mu$ g of protein from each sample was subjected to Western blot analysis and probed for (A) caspase-8, actin, and (B) Bid.

ference in the production of MHV between cells treated with the pancaspase inhibitor and cells not treated before 12 h p.i., whereas MHV titers were higher in the former than the latter from 16 to 36 h p.i. (Fig. 1B). These data demonstrated that pancaspase inhibitor-mediated, prolonged cell survival supported further viral replication. MHV-induced apoptosis in this cell culture system appears to serve as an innate host cell response to limit the production of viral progeny. The highest MHV titer was detected prior to the execution of apoptosis in pancaspase-untreated 17Cl-1 cells (Fig. 1B), suggesting that production of infectious MHV was less efficient in the cells that underwent apoptosis. Probably the cellular environment in apoptotic cells was not optimal for MHV replication.

Activation of the mitochondria-mediated pathway results in the formation of an apoptosome complex, in which procaspase-9 undergoes autocleavage to produce the p35 fragment (Srinivasula *et al.*, 1998; Zou *et al.*, 1999). Bcl-2 protein is known to maintain the integrity of mitochondrial membranes and prevent the release of apoptosis-activating factors, such as cytochrome c and AIF, from mitochondria (Kluck *et al.*, 1997; Susin *et al.*, 1999b; Yang *et al.*, 1997). Therefore, production of caspase-9 fragment p35 in MHV-infected cells and sup-

pression of apoptosis in 17Cl-1/bcl-2 cells strongly suggested activation of the mitochondria-mediated pathway in MHV-infected 17Cl-1 cells. Unexpectedly, cytochrome c release from mitochondria, another hallmark of mitochondria-mediated pathway activation, was not evident in infected 17Cl-1 cells. In addition, the other two components of apoptosome, caspase-9 and Apaf-1, were both found to have a mitochondrial distribution in non-apoptotic 17Cl-1 cells. When cells underwent apoptosis after MHV infection, most of the mitochondria-associated procaspase-9 and Apaf-1 were cleaved, and the caspase-9 fragment p35, which is known to be produced in the apoptosome (Zou *et al.*, 1999), was also found in the mitochondrial fraction (Fig. 3B, lane 3). These data suggested that apoptosome formation occurred in mitochondria, probably on the mitochondrial outer membrane or in the intermembrane space, in MHV-infected 17Cl-1 cells. When 17Cl-1 cells were treated with etoposide, a known reagent that activates the mitochondria-mediated pathway (Kluck *et al.*, 1997; Yang *et al.*, 1997), both cytochrome c and caspase-9 fragment p35 were also found to accumulate in the mitochondrial fraction, indicating that the formation of apoptosome in mitochondria was not stimuli specific; rather, it was more likely to be a unique feature of 17Cl-1 cells. Several previous studies

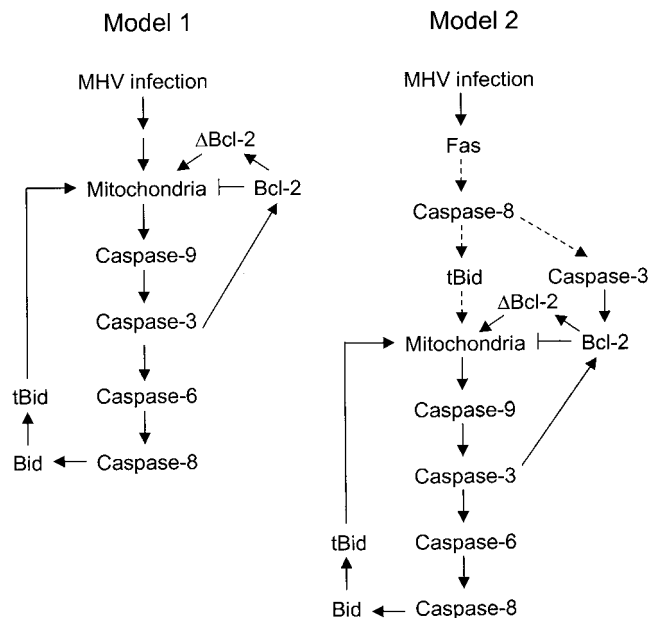


FIG. 7. Two models for the apoptotic pathway activation in MHV-infected 17Cl-1 cells (see text for description).

indicate that cytochrome c accumulation in the cytosol during apoptosis is a cell type- and inducer-dependent phenomenon (Adachi *et al.*, 1998; Chauhan *et al.*, 1997; Tang *et al.*, 1998), and the formation of apoptosome in the mitochondrial intermembrane space was also suggested in a previous report (Adachi *et al.*, 1998). Although cytochrome c, Apaf-1, and procaspase-9 were present in the mitochondria, apoptosome was not formed in untreated 17Cl-1 cells, indicating that these components are probably located in different compartments of the mitochondria. We speculate that in apoptotic 17Cl-1 cells, the mitochondrial structure may undergo certain modifications to allow the apoptosome components to interact and form the complex. It has been shown recently that tBid induces a striking remodeling of the mitochondrial structure with mobilization of the cytochrome c stores in cristae (Scorrano *et al.*, 2002). In MHV-infected cells, caspase-9 cleavage fragment p37 was also detected in the mitochondrial fraction, indicating the presence of mitochondria-associated caspase-3 activity. The presence of procaspase-3 and procaspase-9 in the mitochondria is not unexpected, since both procaspases have been reported to have cytosolic and mitochondrial distributions (Mancini *et al.*, 1998; Susin *et al.*, 1999a).

Based on the data shown in Figs. 2 and 3, we suspect that the integrity of mitochondrial membranes was altered in MHV-infected 17Cl-1 cells; we currently do not know what molecule(s) caused the mitochondrial membrane damage in the first place (see Fig. 7, Model 1). We speculate that the alteration in the integrity of mitochondrial membranes triggered the activation of the mitochondria-mediated pathway. Recent studies indicated that caspase-8 can be activated by caspase-3, probably

via caspase-6 (Slee *et al.*, 1999; Tang *et al.*, 2000). It is possible that a similar or the same caspase activation cascade occurred in MHV-infected 17Cl-1 cells. Namely, activation of the mitochondria-mediated pathway results in the activation of caspase-9 (Fig. 3) and caspase-3 (data not shown), which then may activate the downstream caspase-6 and caspase-8 (Fig. 4). Caspase-8 cleaves Bid to produce tBid fragments, which then relocates to the mitochondria (Fig. 5B) to further disturb mitochondrial membranes; translocation of tBid to the mitochondria amplifies the mitochondrial pathway activation. In this model, the accumulation of proapoptotic signals that cause mitochondrial damage overcomes the antiapoptotic function of overexpressed Bcl-2 in 17Cl-1/bcl-2 cells. Once a low level of mitochondrial pathway activation starts in 17Cl-1/bcl-2 cells, it leads to caspase-3 activation and subsequent Bcl-2 cleavage; cleaved Bcl-2 does not protect the integrity of mitochondria; instead, it has proapoptotic activities (Cheng *et al.*, 1997). A continuous increase in the amount of caspase-3-mediated Bcl-2 cleavage and accumulation of proapoptotic tBid in the mitochondrial membranes overwhelm the antiapoptotic function of Bcl-2, ultimately leading to cell death.

The receptor-mediated apoptotic pathway is another major pathway in activating caspase cascades. In this pathway, DISC formation leads to caspase-8 activation. Activated caspase-8 then activates caspase-3 and Bid. Although caspase-8 activation and Bid cleavage did occur in MHV-infected 17Cl-1 cells (Figs. 4 and 5), it is less likely that these two events were efficiently activated by the direct death receptor activation, because Bcl-2 overexpression efficiently blocked caspase-8 activation and Bid cleavage (Fig. 6). If the receptor-mediated pathway plays the major direct role in caspase-8 activation and Bid cleavage, then both events should have occurred in 17Cl-1/bcl-2 cells; overexpression of Bcl-2 should block only the mitochondria-mediated pathway, but not the receptor-mediated pathway. Caspase-8 activation and Bid cleavage may occur in MHV-infected 17Cl-1 cells through either the same or a similar apoptotic mechanism as that found in type II cells (Scaffidi *et al.*, 1998, 1999) (see Fig. 7, Model 2). In this model, the increased Fas protein in infected 17Cl-1 cells results in a very low level of DISC formation, which we were unable to detect. This putative low level of DISC is able to activate a limited amount of caspase-8. Activated caspase-8 then cleaves caspase-3 and Bid. The p15 tBid relocates to mitochondria and activates the mitochondrial pathway. Once the mitochondrial pathway is activated, subsequent activation of caspase-9, caspase-3, caspase-6, and caspase-8 follows, as discussed above. In 17Cl-1/bcl-2 cells, caspase-3 activated by these mechanisms also cleaves Bcl-2, producing the proapoptotic  $\Delta$ Bcl-2 fragment. If MHV-infected 17Cl-1 cells undergo an apoptotic pathway similar to that in type II cells, then the

increase of Fas late in infection may play an important role in the regulation of apoptosis.

The present study exclusively used 17Cl-1 cells to characterize MHV-induced apoptosis in the cell culture; hence we do not know whether similar results will be obtained when other cell lines are used to study the biological roles of apoptosis in MHV replication and mechanisms of MHV-induced apoptosis. Analysis of MHV-susceptible cell lines, including murine L2 cells (C.-J. Chen and S. Makino, unpublished data), DBT cells (An *et al.*, 1999), and J774.1 cells (S. Banerjee and S. Makino, unpublished data), demonstrated that none of these cell lines showed apoptotic cell death based on DNA fragmentation analysis. Studies using other MHV-susceptible cell lines will be needed to know whether the present data represent features of MHV-induced apoptosis.

## MATERIALS AND METHODS

### Viruses and cells

A plaque-cloned MHV-A59 was used throughout this study. Mouse 17Cl-1 cells (Sturman and Takemoto, 1972) were maintained in Dulbecco's modified Eagle's medium (GIBCO-BRL) containing 10% fetal calf serum. DBT cells (Hirano *et al.*, 1974) were used for propagation of MHV and plaque assays.

### Establishment of Bcl-2-overexpressing 17Cl-1 cell lines

The 17Cl-1 cells at approximately 50% confluence were transfected with either the plasmid pZipBcl-2, which expresses the human bcl-2 gene (Levine *et al.*, 1993), or its parental plasmid, pZipNeo (Cepko *et al.*, 1984), which lacks the bcl-2 gene, using the FuGENE 6 (Roche)-mediated transfection method. The 17Cl-1 cell clones stably transfected with pZipBcl-2 (17Cl-1/bcl-2) or with pZipNeo (17Cl-1/neo) were selected by adding 600  $\mu\text{g/ml}$  of Geneticin (GIBCO-BRL) to the culture medium for 2 weeks.

### DNA fragmentation assay

Low-molecular-weight apoptotic DNA fragments were extracted and analyzed by electrophoresis in 2% agarose gels as previously described (An *et al.*, 1999).

### Subdiploid population detection

Apoptotic nuclei containing subdiploid DNA content were measured by propidium iodide staining and flow cytometry by a previously described method (Yu *et al.*, 1993). Briefly, detached cells and trypsinized adherent cells were pooled, washed with phosphate-buffered saline (PBS), resuspended in a low-salt stain (3% polyethylene glycol 8000, 50  $\mu\text{g/ml}$  propidium iodide, 0.1% Triton X-100, 4 mM sodium citrate, 10  $\mu\text{g/ml}$  of RNase A), and

incubated at 37°C for 20 min. An equal volume of high-salt stain (3% polyethylene glycol 8000, 50  $\mu\text{g/ml}$  propidium iodide, 0.1% Triton X-100, 400 mM sodium chloride) was then added to the cell suspension. Propidium iodide-stained nuclei were stored at 4°C overnight before flow cytometry analysis.

### Measurement of caspase-8 activity

Cellular extracts were assayed for caspase-8 activity using a colorimetric caspase-8 substrate labeled with *p*-nitroaniline (Ac-Ile-Glu-Thr-Asp-pNA; Ac-IETD-pNA). At different times p.i., detached and adherent cells were collected by using a rubber policeman, washed with PBS, and resuspended in chilled lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM  $\text{NaH}_2\text{PO}_4$ , 10 mM  $\text{Na}_2\text{HPO}_4$ , 150 mM NaCl, 1% Triton X-100). After incubation on ice for 10 min, the lysates were centrifuged at 10,000 *g* for 3 min at 4°C, and the supernatants were collected and protein concentrations were determined (DC protein assay, Bio-Rad). Fifty microliters of cell lysates containing 200  $\mu\text{g}$  of protein was mixed with 50  $\mu\text{l}$  of 2 $\times$  reaction buffer (20 mM HEPES, pH 6.8, 200 mM NaCl, 2 mM EDTA, 0.2% CHAPS, 20% glycerol, 10 mM DTT) and 5  $\mu\text{l}$  of 4 mM Ac-IETD-pNA (Biosource International). After incubation at 37°C for 2 h in the dark, the released chromophore was measured by determining the absorbance at 405 nm in a 96-well plate. Background readings from cell lysates and buffers were subtracted, and fold increase in caspase-8 activity was determined by comparing the readings of MHV-infected samples with those of mock-infected samples.

### Total cell lysate preparation

The 17Cl-1 cells were collected at different times p.i. and washed once with PBS. The cell pellets were resuspended in triple detergent lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA) containing protease inhibitors (5  $\mu\text{g/ml}$  each of leupeptin and pepstatin A, 10  $\mu\text{g/ml}$  of aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min. The cell lysates were centrifuged at 14,000 *g* for 10 min at 4°C, and the supernatants were subject to protein concentration measurement (DC Protein Assay, Bio-Rad) and Western blot analysis.

### Western blot analysis

Whole cell lysates prepared from cells at different times p.i. were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose or PVDF membranes (Bio-Rad). The membranes were blocked in blocking solution (0.05% Tween 20 and 5% nonfat dry milk in PBS), incubated with primary and secondary antibodies diluted in blocking solutions for 1 h each, and developed with enhanced chemiluminescence

(ECL, Amersham Pharmacia). The following mouse monoclonal antibodies were used: anti-cytochrome c (clone 7H8.2C12, BD Pharmingen), anti-caspase-9 (clone 5B4, StressGen), anti-procaspase-8 (clone 5D3, MBL), and antihuman Bcl-2 (clone 124, Roche). A rat monoclonal antibody was used to detect Apaf-1 (clone 13F11, Chemicon). A rabbit polyclonal antibody against caspase-8 (H-134, Santa Cruz Biotechnology) was used to detect the 18-kDa cleavage fragment, and a rabbit polyclonal antibody was used to detect Bid (a gift from Stanley Korsmeyer, Harvard Medical School). Actin was detected by a goat antiactin polyclonal antibody (I-19, Santa Cruz Biotechnology). Sheep antimouse IgG (Amersham Pharmacia), goat antirabbit and antirat IgGs, and donkey antigoat IgG (Santa Cruz Biotechnology) were used as secondary antibodies.

### Effect of caspase inhibitors on apoptosis induction and MHV growth

The 17CI-1 or 17CI-1/bcl-2 cells were incubated for 2 h with 150  $\mu$ M Z-VAD-FMK (Enzyme System Products) prior to MHV infection. After MHV infection at an m.o.i. of 5 for 1 h at 37°C, cells were incubated in the presence of 150  $\mu$ M Z-VAD-FMK for 24 h, and the drug was replenished at 24 h p.i. At different times p.i., low-molecular-weight DNA fragments and protein were extracted from the cells and analyzed by agarose gel electrophoresis and SDS-PAGE, respectively. To determine MHV titers, culture fluid samples were collected at different times p.i. and subject to plaque assays.

### Subcellular fractionation

The 17CI-1 cells ( $5 \times 10^7$  cells) were infected with MHV at an m.o.i. of 10 or treated with 50  $\mu$ M etoposide. At different times p.i. or drug treatment, both detached and adherent cells were collected by scraping and centrifugation at 600  $g$  for 10 min at 4°C. The cell pellets were washed once with PBS and resuspended with 5 vol of HIM buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 1 mM dithiothreitol, 10 mM HEPES, pH 7.5) containing protease inhibitors. After 10 min on ice, cells were disrupted with a glass Dounce homogenizer (Wheaton), with 30 strokes of the tight pestle. The homogenates were centrifuged twice at 1000  $g$  for 10 min at 4°C, and the supernatants were further centrifuged at 10,000  $g$  for 15 min at 4°C to collect the mitochondrial pellets. The supernatants of the 10,000  $g$  spin were centrifuged at 100,000  $g$  for 1 h at 4°C, and the resulting supernatants were collected as cytosolic fractions.

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