Journal of Virology

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J. Virol. **2011, 85(1):296. DOI: 10.1128/JVI.01982-10.**
Published Ahead of Print 27 October 2010.

JOURNAL OF VIROLOGY, Jan. 2011, p. 296–304 Vol. 85, No. 1
0022-538X/11/$12.00 doi:10.1128/JVI.01982-10

Reovirus Infection or Ectopic Expression of Outer Capsid Protein μ1 Induces Apoptosis Independently of the Cellular Proapoptotic Proteins Bax and Bak[▽]

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Received 17 September 2010/Accepted 18 October 2010

Mammalian orthoreoviruses induce apoptosis *in vivo* and *in vitro*; however, the specific mechanism by which apoptosis is induced is not fully understood. Recent studies have indicated that the reovirus outer capsid protein μ1 is the primary determinant of reovirus-induced apoptosis. Ectopically expressed μ1 induces apoptosis and localizes to intracellular membranes. Here we report that ectopic expression of μ1 activated both the extrinsic and intrinsic apoptotic pathways with activation of initiator caspases-8 and -9 and downstream effector caspase-3. Activation of both pathways was required for μ1-induced apoptosis, as specific inhibition of either caspase-8 or caspase-9 abolished downstream effector caspase-3 activation. Similar to reovirus infection, ectopic expression of μ1 caused release into the cytosol of cytochrome *c* and smac/DIABLO from the mitochondrial intermembrane space. Pancaspase inhibitors did not prevent cytochrome *c* release from cells expressing μ1, indicating that caspases were not required. Additionally, μ1- or reovirus-induced release of cytochrome *c* occurred efficiently in Bax$^{-/-}$Bak$^{-/-}$ mouse embryonic fibroblasts (MEFs). Finally, we found that reovirus-induced apoptosis occurred in Bax$^{-/-}$Bak$^{-/-}$ MEFs, indicating that reovirus-induced apoptosis occurs independently of the proapoptotic Bcl-2 family members Bax and Bak.

The mammalian orthoreoviruses (reoviruses) are nonenveloped viruses that contain a segmented double-stranded RNA (dsRNA) genome. Reoviruses induce apoptosis in infected cells, both *in vivo* and *in vitro* (reviewed in references 7 and 12). Reovirus-induced apoptosis is a primary determinant of neural and cardiac injury in mice (reviewed in reference 7), and mice treated with inhibitors of apoptosis prior to infection have reduced tissue damage in the central nervous system and heart (3, 14). Type 3 reovirus strains induce apoptosis in tissue culture and *in vivo* to a greater extent than type 1 strains, and this phenotypic difference is determined by genetic differences in the S1 and M2 genome segments (30, 31). The S1 genome segment encodes the viral attachment protein σ1 and a small nonstructural protein, σ1s. The M2 gene segment encodes μ1, a major outer capsid protein (31). Ectopic expression of the ϕ domain of μ1 induces apoptosis (9). Moreover, recombinant ϕ mutant viruses have decreased capacity to induce apoptosis *in vitro* and are less neurovirulent *in vivo* (11).

The extrinsic and intrinsic apoptotic pathways that are activated during reovirus infection have been elucidated in some detail (22). The extrinsic pathway is activated during reovirus infection as demonstrated by release of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), upregulation of cell surface death receptors (DR) 4 and 5, and activation of caspase-8 with subsequent cleavage of Bcl-2 family member Bid (8, 22). Evidence that the intrinsic apoptotic pathway is activated during reovirus infection includes cytosolic release of mitochondrial cytochrome *c* and smac/DIABLO and activation of caspase-9 (22, 23). Specific inhibition of either extrinsic or intrinsic apoptotic pathways prevents reovirus-induced apoptosis (22). It has been proposed that reovirus infection activates the extrinsic apoptotic pathway prior to activation of the intrinsic pathway (22). In this model, activated caspase-8 cleaves the BH3-only Bcl-2 protein Bid to generate t-Bid, which is then recruited to the outer mitochondrial membrane, where it activates the proapoptotic Bcl-2 proteins Bax and Bak to permeabilize the outer mitochondrial membrane. Subsequent release of cytochrome *c* from the mitochondrial intermembrane space would induce oligomerization of Apaf-1, assembly of the apoptosome, and activation of caspase-9 (1); release of smac/DIABLO would inhibit the action of cellular inhibitors of apoptosis (IAP), allowing activation of effector caspases to proceed (reviewed in reference 28). In support of this model, reovirus-induced apoptosis is blocked in Bid-deficient MEFs (13).

Here we investigated the apoptotic pathways activated by ectopic expression of μ1 and the roles played by Bax and Bak in release of cytochrome *c* and induction of reovirus-induced apoptosis. Our findings indicate that μ1 and reovirus infection induce apoptosis in cells by a novel mechanism that does not require the proapoptotic Bcl-2 proteins Bax and Bak to cause release of mitochondrial intermembrane space proteins into the cytosol.

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▽ Published ahead of print on 27 October 2010.

MATERIALS AND METHODS

Cells and viruses. CHO-K1 cells were grown in Ham's F-12 medium (CellGro); HeLa cells were grown in modified Eagle's medium (MEM) with Earle's salt; both were supplemented with 10% fetal bovine serum (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and nonessential amino acids (CellGro). CHO-S suspension cells were grown in CHO-S-SFM II medium (Gibco) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Simian virus 40 (SV40)-transformed wild-type (WT), $Bax^{-/-}$, $Bak^{-/-}$, and $Bax^{-/-}Bak^{-/-}$ double-knockout (DKO) mouse embryonic fibroblasts (MEFs) were a gift (from Stanley Korsemeyer and Nika Danial) and were grown in Dulbecco's modified Eagle's medium (DMEM high glucose; Cellgro) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, nonessential amino acids, and 0.001% β-mercaptoethanol. Virus isolates used were laboratory strains of T3/Human/Ohio/Dearing/1955 and T1/Human/Ohio/Lang/1953 reoviruses obtained from Max Nibert. Viruses were plaque isolated and amplified in murine L929 cells in Joklik's modified essential medium supplemented with 4% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Antibodies and reagents. Mouse monoclonal antibodies (MAbs) to reoviral proteins μ1 (10F6, 10H2, 4A3), σ3 (5C3), and λ2 (7F4) have been previously described (29, 32). Polyclonal rabbit anti-virion (T1L) serum was a kind gift from Barbara Sherry. Mouse MAbs to cytochrome *c* and smac/DIABLO were from Pharmingen, and rabbit polyclonal immunoglobulin G (IgG) fraction against cleaved caspase-3 was from Cell Signaling Technologies. Secondary antibodies for immunofluorescence (IF) microscopy or flow cytometry were goat anti-mouse IgG and goat anti-rabbit IgG conjugated to Alexa 488, Alexa 594, or Alexa 647 (Invitrogen) or subtype 1, 2a, or 2b goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC), allophycocyanin (APC), or phycoerythrin (PE) (Jackson Labs). Zenon antibody labeling kits (Invitrogen) were used to directly label mouse MAbs with Alexa 488 or Alexa 594. The Image-iT LIVE Green caspase-8 detection kit, the Vybrant FAM caspase-3 and -7 assay kit, the Vybrant FAM caspase-8 assay kit, and 4′,6′-diamidino-2-phenylindole (DAPI) were obtained from Invitrogen. The FAM FLICA caspase-9 assay kit was obtained from Axxora. Broad-spectrum caspase inhibitor Q-VD-OPh (Kamiya Biomedical Company) was reconstituted and used according to the manufacturer's directions at a final concentration of 20 μM. Caspase-8 and caspase-9 inhibitors Z-IETD-FMK and Z-LEHD-FMK, respectively (R&D Systems, Inc.), were reconstituted according to the manufacturer's directions and used at a final concentration of 25 μM. Camptothecin (CPT) (Sigma) in dimethyl sulfoxide (DMSO) was used at a final concentration of 20 μM. Etoposide (Sigma) in DMSO was used at a final concentration of 100 μM. Cells were incubated for 24 h at 37°C after treatment with either CPT or etoposide.

Plasmids. The reovirus M2 genes derived from the type 1 Lang (T1L), type 3 Dearing-Cashdollar, and type 3 Dearing-Nibert strains were expressed using the mammalian expression vector pCI-neo (Promega). Unless otherwise stated, pCI-M2 derived from T1L was used for experiments. In-frame truncation mutants of T1L were as previously described (9). In-frame fusions of T1L M2(582-708) to enhanced green fluorescent protein were also previously described (9). In-frame fusion of T1L M2(1-708) with pEGFP-C1 (Clontech) was performed (details available upon request).

Transfections. For IF microscopy, CHO-K1 cells were seeded at 10^5 cells per well, and HeLa cells and the MEFs were seeded at 2.5×10^5 cells per well in six-well plates which contained 18-mm glass coverslips. Cells were transfected using FuGENE 6 transfection reagent (Roche) or Lipofectamine (Invitrogen) according to the manufacturer's instructions and viewed at 24 or 48 h posttransfection (p.t.). For flow cytometry, CHO-S cells were resuspended at a density of 5×10^5 to 10×10^5 cells per ml and transfected with FuGENE 6 or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and analyzed at 24 h p.t.

Immunofluorescence (IF) microscopy. Cells on coverslips were rinsed with phosphate-buffered saline (PBS), fixed in 2% paraformaldehyde in PBS at room temperature for 10 min, and then washed in PBS. Cells were permeabilized with 0.15 to 0.2% Triton X-100 in PBS for 15 min at room temperature and then blocked for 30 to 60 min with either 2% bovine serum albumin (BSA) or 10% normal goat serum (NGS) in PBS at room temperature (15, 16). All antibody incubations were carried out for 30 to 60 min at room temperature in 10% NGS/PBS (when using Zenon antibody labeling kits) or PBS with 1% BSA and 0.1% Triton X-100 (PBSA-T). Cell nuclei were labeled with 300 nM 4′,6′-diamidino-2-phenylindole (DAPI); coverslips were mounted using Prolong (Molecular Probes). Images were obtained with an inverted microscope (Nikon TE2000) equipped with fluorescence optics through a 60× 1.4-numerical-aperture (NA) oil objective with 1× optical zoom. Images were collected digitally with a Coolsnap HQ charge-coupled-device camera (Roper) and Openlab software (Improvision) and then prepared for publication using Photoshop and Illustrator software (Adobe).

Flow cytometry. After incubation, cells were harvested and resuspended in medium (when using caspase staining kits) or PBS. Caspase staining kits were used according to the manufacturer's protocol; then, cells were permeabilized for antibody staining with 0.135% Triton X-100, 1.8% BSA, 10% NGS in PBS for 20 to 30 min at room temperature. Cytochrome *c* release was determined by first incubating the cells in 250 mM sucrose, 20 mM HEPES (pH 7.2), 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM EDTA, 8 mM dithiothreitol (DTT) with 150 μg/ml digitonin for 10 min at 4°C to permeabilize the plasma membrane only (method adapted from references 34 and 35). Cells were then fixed with 1.85% formaldehyde in PBS, and all membranes were permeabilized with 0.135% Triton X-100, 1.8% BSA, 10% NGS in PBS as indicated above. Antibody incubations were carried out at room temperature for 1 to 3 h or at 4°C overnight in PBS with 1% BSA and 0.1% Triton X-100 (PBSA-T). After antibody incubations, cells were washed in PBS and then resuspended in 1% paraformaldehyde in PBS. Flow cytometry data were collected with a FACSCalibur (BD) and analyzed with CellQuest (BD) and FlowJo (Tree Star, Inc.) software. Data from over 10,000 cells was analyzed; cells expressing viral protein were gated. Images were prepared for publication using Photoshop and Illustrator software (Adobe).

Statistical analysis. Statistical analysis was performed using the software JMP 7 (SAS). Dunnett's multicomparison test was used to compare experimental values to the negative control. The drug-treated positive control was significantly different from the negative control in all of the figures in which statistical analyses were performed ($P < 0.0001$). Student's *t* test was used to compare pCI-M2(1-708) derived from different reovirus strains and to compare the pCI-M2(1-708)-transfected cells that were treated with caspase inhibitors to those that were treated with DMSO.

RESULTS

Ectopic expression of μ1 activates the intrinsic apoptotic pathway. Ectopic expression in cells of μ1 derived from type 1 Lang (T1L) reovirus induces apoptosis, and the φ fragment of μ1 is sufficient for this activity (Fig. 1A) (9). To determine if the intrinsic apoptotic pathway was activated by μ1 expression, we assessed the release of cytochrome *c* (Fig. 1C and D) and smac/DIABLO (Fig. 1E) from mitochondria in CHO-K1 cells transfected with plasmids expressing full-length μ1, pCI-M2(1-708), or a C-terminally truncated form of μ1 [μ1δ; pCI-M2(1-582)] that does not induce apoptosis (9). Using fluorescence microscopy, we found that treatment of CHO-K1 cells with 2 μM staurosporine caused cytosolic dispersion of cytochrome *c* with loss of the normal mitochondrial tubulovesicular distribution (Fig. 1B). Expression of μ1, but not μ1δ, in CHO-K1 cells similarly caused cytosolic release of cytochrome *c* (Fig. 1C) and smac/DIABLO (data not shown). Approximately 85% of cells expressing full-length μ1 or μ1C and 70% of cells that expressed enhanced green fluorescent protein (EGFP)-φ showed release of cytochrome *c* into the cytosol in CHO-K1 cells, which was significantly different from the result for pEGFP-C1-expressing cells ($P < 0.0001$) (Fig. 1D). We found similar results with HeLa cells (data not shown). Release of smac/DIABLO from mitochondria into the cytosol promotes apoptosis by repressing the inhibitory action of cellular inhibitor of apoptosis proteins on caspases (15). Again we found that the φ fragment of μ1 was sufficient to induce the release of smac/DIABLO from mitochondria (Fig. 1E). We conclude that cellular expression of full-length μ1 or portions of μ1 that contain the φ region cause release of cytochrome *c* and smac/DIABLO from mitochondria.

Caspase-9 is an initiator caspase that is activated by assembly of the apoptosome following release of cytochrome *c* (reviewed in reference 26). To test if caspase-9 was activated in

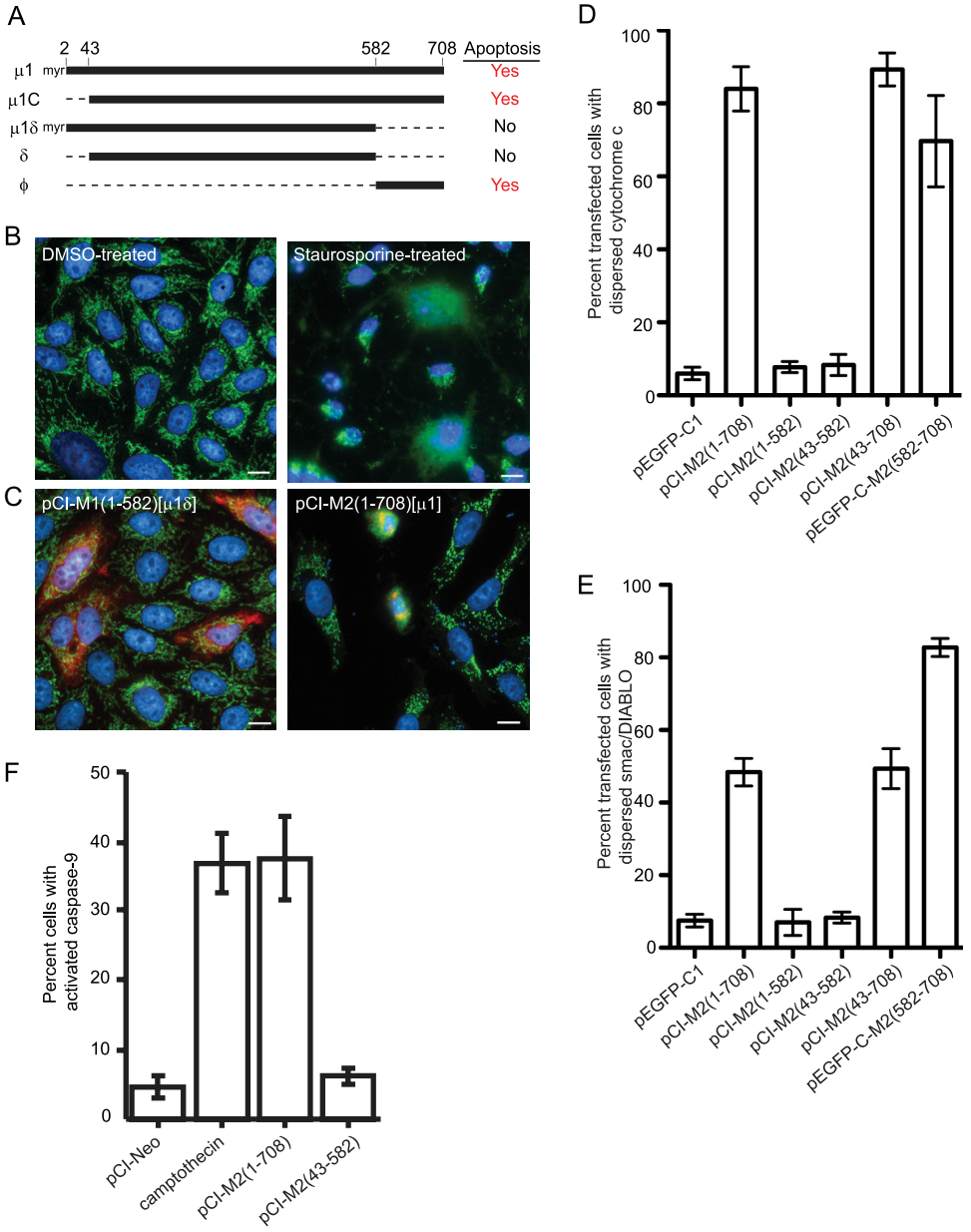

FIG. 1. Ectopic expression of μ1 induces release of cytochrome *c* and smac/DIABLO from the mitochondrial intermembrane space and activates caspase-9. (A) Cartoon illustrating fragments of μ1 generated by proteolysis during virus entry. The capacity of each fragment to induce apoptosis when expressed ectopically is indicated. (B and C) Immunofluorescence (IF) images showing the distribution of cytochrome *c* in CHO-K1 cells treated with DMSO or 2 μM staurosporine for 24 h (B) or expressing μ1δ or μ1 (C). Cells were fixed and immunostained using anti-μ1 MAb (10F6) and anti-cytochrome *c* polyclonal serum followed by Alexa 594-conjugated goat anti-mouse IgG and Alexa 488-conjugated goat anti-rabbit IgG. Nuclei were stained with DAPI. Scale bar, 10 μm. (D) Release of cytochrome *c* or smac/DIABLO (E) by expression of μ1 requires the C-terminal residues 582 to 708 (ϕ). The percentage of CHO-K1 cells expressing μ1 and showing cytosolic dispersion or loss of cytochrome *c* or smac/DIABLO at 48 h posttransfection was quantified. Smac/DIABLO was detected with an anti-smac MAb; μ1 was detected with an anti-μ1 MAb directly conjugated with Texas Red. A minimum of 100 transfected cells were counted. Results are shown as mean ± standard deviation (SD); $n = 3$ experiments. (F) CHO-S cells were transfected with vector control (pCI-Neo), pCI-M2(1-708)[μ1] or pCI-M2(43-582)[δ] or treated with 20 μM CPT. Cells were harvested 24 h posttransfection and then stained for activated caspase-9 using FLICA. Cells were fixed and then stained with anti-μ1 MAb followed by Alexa 647-conjugated goat anti-mouse IgG. Cells were analyzed by flow cytometry. A minimum of 10,000 cells were analyzed. Data shown represent the mean ± SD; $n \geq 3$.

μ1-expressing cells, we monitored activation of caspase-9 by flow cytometry in CHO-S cells expressing pCI-M2(1-708)[μ1], pCI-M2(43-582)[δ], or pCI-Neo (vector control) (Fig. 1F). As a positive control for activation of the intrinsic apoptotic pathway, cells were treated with 20 μM camptothecin. Compared to δ-expressing or vector-only-transfected control cells, significantly more μ1-expressing and CPT-treated positive-control cells had activated caspase-9 ($P < 0.0001$). We conclude from

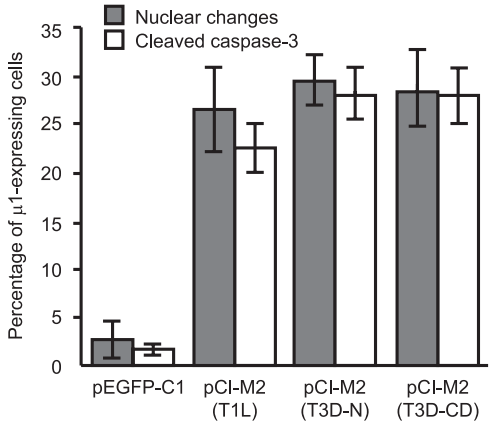

FIG. 2. μ1-induced apoptosis is strain independent. CHO-K1 cells were transfected with vector control (pEGFP-C1) or plasmids expressing μ1 derived from the type 1 Lang [pCI-M2(T1L)], type 3 Dearing-Nibert [pCI-M2(T3D-N)], or the type 3 Dearing-Cashdollar reovirus [pCI-M2(T3D-CD)] strains. Cells were fixed at 48 h posttransfection and immunostained using anti-μ1 MAb and anti-cleaved caspase-3 rabbit polyclonal Ab followed by Alexa 488-conjugated goat anti-mouse IgG and Alexa 594-conjugated goat anti-rabbit IgG. Nuclei were stained with DAPI. Transfected cells were scored based on the appearance of nuclear changes and the presence of cleaved caspase-3. One hundred cells were counted per sample. Data shown represent the mean ± SD; $n = 3$.

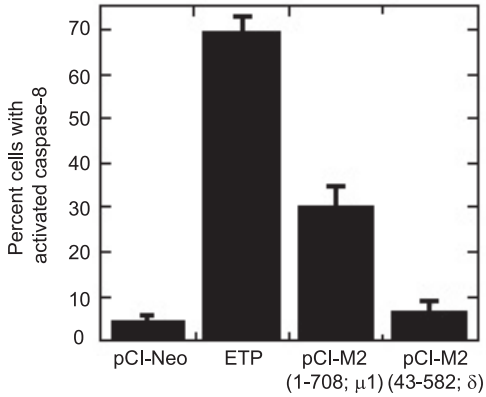

FIG. 3. Ectopic expression of μ1 activates caspase-8. CHO-S cells were transfected with plasmids pCI-Neo, pCI-M2(1-708)[μ1] and pCI-M2(43-582)[δ] or treated with 100 μM etoposide. At 24 h posttransfection, caspase-8 activity was detected by FLICA. Cells were then fixed, immunostained with anti-μ1 MAb followed by Alexa 647-conjugated goat anti-mouse IgG, and then analyzed by flow cytometry. At least 10,000 cells expressing μ1 or δ were analyzed for caspase-8 activity. Data shown represent the mean ± SD; $n \geq 3$.

these findings that ectopic expression of μ1 activates the intrinsic apoptotic pathway with release of cytochrome *c*, smac/DIABLO, and downstream activation of caspase-9.

Induction of apoptosis by μ1 is not strain dependent. Type 3 reovirus strains induce apoptosis in significantly more infected cells than type 1 strains. This difference is in part genetically determined by differences in the M2 genome segment which encodes μ1 (31). To determine if μ1 derived from type 3 and type 1 reovirus strains induce apoptosis to different extents in cells, we compared the capacity to induce apoptosis in CHO-K1 cells of μ1 derived from reovirus strains type 1 Lang, type 3 Dearing-Nibert, and type 3 Dearing-Cashdollar. Compared to the control (pEGFP-C1)-transfected cells, a significantly greater percentage of cells expressing μ1 showed nuclear changes and caspase-3 cleavage, indicating apoptosis induction ($P < 0.0001$) (Fig. 2). However, we found no significant difference in the capacity of μ1 proteins derived from type 1 or type 3 reovirus strains to induce apoptosis. We conclude that the genetic differences in the M2 genome segment responsible for differences in the capacity of types 1 and 3 reoviruses to induce apoptosis do not correlate with the capacity of the encoded μ1 proteins to induce apoptosis.

Ectopic expression of μ1 activates the extrinsic apoptotic pathway in CHO-S cells. Activation of both the extrinsic and intrinsic apoptotic pathways is required for reovirus-induced apoptosis (22). We therefore determined if caspase-8, the initiator caspase downstream of cell surface death receptors (2), was activated in cells expressing μ1. As a positive control we treated CHO-S cells with 100 μM etoposide, which has been shown to activate multiple caspases, including caspase-8 (19, 33). We found that ~30% of CHO-S cells expressing pCI-M2(1-708)[μ1] had activated caspase-8 at 24 h posttransfection. In contrast, only ~5% of δ-expressing cells or vector control-transfected cells had activated caspase-8 (Fig. 3). We conclude that μ1 expression, but not δ expression, causes activation of caspase-8, indicating that μ1 expression activates the extrinsic apoptotic pathway.

Inhibition of caspase activity does not prevent μ1-induced cytochrome *c* release in CHO-S cells. Previous work has suggested that activation of the intrinsic apoptotic pathway during reovirus-induced apoptosis occurs downstream of activation of caspase-8 based on findings that expression of a dominant negative form of FADD, an adaptor protein required for activation of caspase-8, blocked cytochrome *c* release in reovirus-infected cells (22). To determine if caspase activation was necessary for μ1-induced cytochrome *c* release, we pretreated cells with the pancaspase inhibitor Q-VD-OPh or DMSO prior to transfection with plasmids expressing μ1, δ, or vector control. Pretreatment with 20 μM Q-VD-OPh inhibited activation of caspases-3 and -8 (Fig. 4A) as well as caspase-9 (data not shown) in cells expressing μ1. In cells expressing vector alone or the δ fragment of μ1, cytochrome *c* was not released irrespective of whether the cells were pretreated with DMSO or Q-VD-OPh (Fig. 4B). However, cytochrome *c* was released from cells expressing μ1 even when they were pretreated with Q-VD-OPh (79% of Q-VD-OPh-treated μ1-transfected cells released cytochrome *c* compared to 66% of DMSO-treated μ1-transfected cells) (Fig. 4B). We conclude from these findings that activation of caspases is not necessary for μ1-induced release of cytochrome *c*.

Inhibition of upstream initiator caspases prevents activation of caspase-3 in cells expressing μ1. Activation of caspase-8 occurs in reovirus-infected cells and is necessary for reovirus-induced apoptosis (8). Our finding that pancaspase inhibition did not prevent μ1-induced release of cytochrome *c* in cells suggested that upstream activation of caspase-8 might not be required for μ1-induced apoptosis. We therefore examined whether specific inhibitors of initiator caspases-8 and -9 prevented downstream activation of effector caspase-3 in cells expressing μ1. At the time of transfection or treatment with

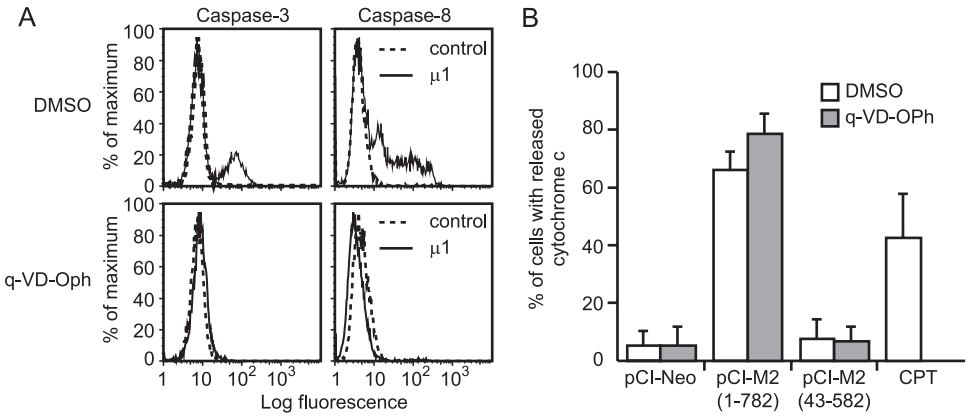

FIG. 4. Pancaspase inhibitor Q-VD-OPH prevents μ1-induced caspase-3 and caspase-8 activation but does not prevent μ1-induced cytochrome *c* release from mitochondria. (A) Q-VD-OPh prevents μ1-induced caspase-8 and caspase-3 activation. Untransfected or CHO-S cells transfected with pCI-M2(1-708)[μ1] were treated with DMSO or 20 μM Q-VD-OPh and stained for caspase-8 activation using FLICA or, for caspase-3 cleavage, using anti-cleaved caspase-3 rabbit polyclonal Ab followed by goat anti-rabbit IgG Alexa 647-conjugated Ab. At least 10,000 cells were analyzed by flow cytometry. Representative histograms are shown. (B) CHO-S cells were transfected with pCI-Neo (vector control), pCI-M2(1-708)[μ1] or pCI-M2(43-582)[δ] or treated with 20 μM CPT. At the time of transfection 20 μM Q-VD-OPh or vol/vol DMSO was added to the cultures. Cells were harvested at 24 h posttransfection and plasma membranes were permeabilized (see Materials and Methods) and then fixed and stained using anti-T1L virion rabbit serum and anti-cytochrome *c* MAb followed by Alexa 647-conjugated goat anti-rabbit IgG and Alexa 488-conjugated goat anti-mouse IgG. At least 10,000 cells expressing μ1 or δ were analyzed by flow cytometry. Data shown represent the mean ± SD; $n \geq 3$.

camptothecin, cells were treated with 25 μM either caspase-8 (Z-IETD-FMK) or caspase-9 (Z-LEHD-FMK) inhibitors or DMSO. At 24 h posttransfection, approximately 51% of cells treated with camptothecin contained activated caspase-3. An average of 25% of μ1-expressing cells were positive for cleaved caspase-3; however, pretreatment with caspase-8 (Z-IETD-FMK) or caspase-9 (Z-LEHD-FMK) inhibitors suppressed the μ1-induced activation of caspase-3 (Fig. 5). We conclude that activation of both initiator caspases-8 and -9 is required for μ1-induced activation of caspase-3.

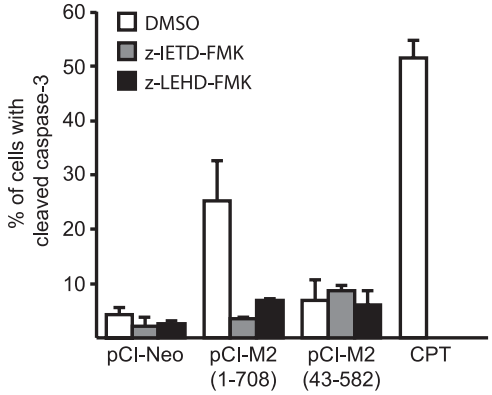

FIG. 5. Specific inhibition of caspase-8 or caspase-9 leads to decreased levels of activated caspase-3 in cells expressing μ1. CHO-S cells were transfected with vector control (pCI-Neo), pCI-M2(1-708)[μ1] or pCI-M2(43-582)[δ] or treated with 20 μM CPT. Simultaneously, a final concentration of 25 μM Z-IETD-FMK or Z-LEHD-FMK or the same volume DMSO diluted in CHO-S-SFM II medium with 1% BSA was added to the cultures. Cells were harvested 24 h posttransfection, fixed, and permeabilized. Cells were then stained using anti-cleaved caspase-3 rabbit polyclonal Ab and anti-μ1 MAb followed by Alexa 647-conjugated goat anti-rabbit IgG and Alexa 488-conjugated goat anti-mouse IgG. At least 10,000 cells expressing μ1 or δ were analyzed by flow cytometry. Data shown represent the mean ± SD; $n \geq 3$.

Proapoptotic Bcl-2 family proteins Bax and Bak are not required for μ1-induced cytochrome *c* release. Members of the Bcl-2 family of proteins regulate activation of the intrinsic apoptotic pathway (6). Bax and Bak are Bcl-2 family members that, following activation, undergo conformational changes which cause them to oligomerize in the outer mitochondrial membrane and form pores through which proapoptotic molecules such as cytochrome *c* pass (21, 24). Our observations thus far indicated that μ1-induced cytochrome *c* release occurred independently of caspase activation. We therefore tested the capacity of μ1 or δ to induce mitochondrial cytochrome *c* release in SV40-transformed WT, $Bax^{-/-}$, $Bak^{-/-}$, and $Bax^{-/-}Bak^{-/-}$ MEFs. We found that WT, $Bax^{-/-}$, and $Bak^{-/-}$ MEFs released cytochrome *c* in response to treatment with the topoisomerase inhibitor etoposide for 24 h; however, as previously reported, $Bax^{-/-}Bak^{-/-}$ DKO MEFs were resistant to etoposide-induced apoptosis and cytochrome *c* was retained in the mitochondrial intermembrane space (36) (see Fig. 7A). Ectopic expression of μ1 caused the cytosolic release of cytochrome *c* in WT, $Bax^{-/-}$, and $Bak^{-/-}$ MEFs (Fig. 6). Unexpectedly, we found that cytochrome *c* was released from ~35% of $Bax^{-/-}Bak^{-/-}$ DKO MEFs expressing μ1 compared to fewer than 5% of DKO MEFs expressing μ1δ or GFP (Fig. 6). We conclude from these findings that ectopic expression of μ1 causes cytochrome *c* release from the intermitochondrial space independently of the action of the proapoptotic Bcl-2 family proteins Bax and Bak.

Bax and Bak are not required for reovirus-induced activation of caspase-3 or cytochrome *c* release. Based on the above findings we hypothesized that reovirus-induced cytochrome *c* release and apoptosis induction are also independent of Bax and Bak. To test this hypothesis we first compared the capacity

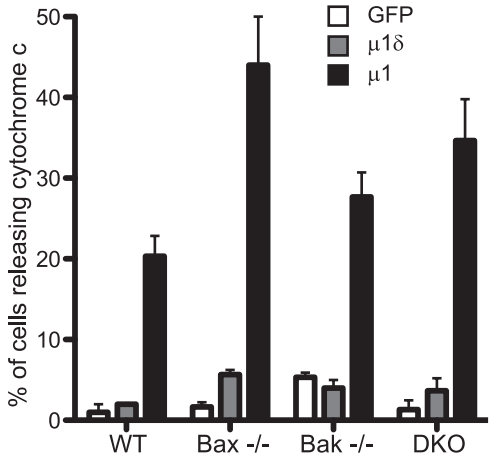

FIG. 6. Proapoptotic Bcl-2 proteins Bax and Bak are not required for μ1-induced cytochrome *c* release. Wild-type (WT), $Bax^{-/-}$, $Bak^{-/-}$, and $Bax^{-/-}Bak^{-/-}$ (DKO) MEFs were transfected with plasmids expressing EGFP, μ1δ (residues 1 to 582), or μ1. At 24 h posttransfection the cells were fixed and immunostained for expression of μ1 and cytochrome *c*. Cells expressing EGFP, μ1δ, or μ1 were scored for release of cytochrome *c* from mitochondria by microscopy. At least 100 cells were counted in each experiment. Data shown represent the mean ± SD; $n \geq 3$.

of T3D and T1L reoviruses to replicate in WT, $Bax^{-/-}$, $Bak^{-/-}$, and $Bax^{-/-}Bak^{-/-}$ MEFs. All of the cell lines were able to support reovirus replication (data for WT and $Bax^{-/-}Bak^{-/-}$ DKO MEFs are shown in Fig. 7A). We then compared the capacity of T3D reovirus to induce apoptosis as assessed by activation of effector caspases-3 and -7. In control experiments, we found that treatment with 100 μM etoposide induced activation of caspases-3 and -7 in ~70% of WT, $Bax^{-/-}$, and $Bak^{-/-}$ MEFs. As previously reported, $Bax^{-/-}Bak^{-/-}$ MEFs were resistant to apoptosis induction by etoposide (Fig. 7B and C). In contrast, there was no significant difference between the MEFs infected with T3D at a multiplicity of infection (MOI) of 10, with 65 to 85% of the cells being apoptotic by 48 h postinfection. As reoviruses are known to activate the extrinsic apoptotic pathway, it was possible that activation of caspases-3 and -7 in the $Bax^{-/-}Bak^{-/-}$ MEFs occurred solely as a consequence of activation of the extrinsic apoptotic pathway. We therefore examined cytochrome *c* release in the MEFs infected with T3D. We found that all the cell lines, including the $Bax^{-/-}Bak^{-/-}$ MEFs, released cytochrome *c* in response to reovirus infection; however, we noted a partial dependence on Bak, as ~50% fewer infected $Bak^{-/-}$ or $Bax^{-/-}Bak^{-/-}$ MEFs released cytochrome *c* (Fig. 7B). We conclude from these findings that Bax and Bak are not required for reovirus-induced apoptosis and mitochondrial release of cytochrome *c*.

DISCUSSION

Previous work has demonstrated a clear link between reovirus-induced apoptosis and viral pathogenesis. In this study, we have shown that expression of reovirus outer capsid protein μ1 activates initiator caspases-8 and -9 and induces cytochrome *c* and smac/DIABLO release from the mitochondrial intermembrane space, mirroring events that occur during reovirus-induced apoptosis (8, 22, 23). These findings provide further evidence that μ1 is the major factor responsible for reovirus-induced apoptosis.

Reovirus-induced apoptosis is blocked by overexpression of the anti-apoptotic protein Bcl-2, presumably because Bcl-2 overexpression prevents the activation of proapoptotic Bcl-2 family members (20, 27). With few exceptions, the final common pathway to mitochondrial outer membrane permeabilization involves the proapoptotic Bcl-2 family members Bax and Bak (10). Our findings indicate, however, that μ1 can induce permeabilization of mitochondrial membranes independently of Bax and Bak. Furthermore, reovirus infection induced release of cytochrome *c* and apoptosis (as indicated by activation of effector caspases-3 and -7) in $Bax^{-/-}Bak^{-/-}$ DKO with levels of caspase-3 and -7 activation similar to those in WT MEFs. Although reovirus infection appears to induce apoptosis to the same extent in $Bax^{-/-}Bak^{-/-}$ DKO as in WT MEFs, cytochrome *c* release in response to reovirus infection appears to be partially dependent on Bak. Only a few proapoptotic agents can induce apoptosis in $Bax^{-/-}Bak^{-/-}$ DKO MEFs. One such agent, gossypol, was identified in a screen for small molecules that could induce apoptosis in $Bax^{-/-}Bak^{-/-}$ cells. Gossypol appears to act by inducing a conformational change in anti-apoptotic Bcl-2. This in turn is thought to convert Bcl-2 to a proapoptotic protein by allowing its BH-3 helix to insert into the outer mitochondrial membrane (25). Such a mechanism is possible for reovirus-induced apoptosis in $Bax^{-/-}Bak^{-/-}$ cells; however, previous findings have shown that overexpression of Bcl-2 in MDCK cells inhibits reovirus-induced apoptosis (27), whereas gossypol induces apoptosis in cells overexpressing Bcl-2 (25). Danthi et al. recently showed that cleavage of full-length Bid to tBid is required for reovirus-induced apoptosis and that Bid cleavage is dependent on upstream NF-κB activation (13). One possibility is that μ1 together with tBid can substitute for Bax and Bak and directly permeabilize the mitochondrial membrane of infected cells leading to release of cytochrome *c* and smac/DIABLO.

Activation of caspase-8 most commonly occurs after ligation of TNF family death receptors by cytokines, such as TNF, TRAIL, or Fas (10). In reovirus-induced apoptosis, TRAIL binding to death receptors 4 and 5 induces formation of the death-inducing signaling complex and activation of caspase-8; blocking this activation protects cells from apoptosis (8). However, the mechanism by which reovirus infection leads to TRAIL secretion has not been identified. Here we found that ectopic expression of μ1 activates caspase-8, and, similar to the situation in reovirus-infected cells, inhibiting caspase-8 activation through the use of z-IETD-FMK prevents downstream apoptosis induction. These findings suggest that μ1 expression during reovirus infection is at least in part responsible for activation of caspase-8. This suggestion is supported by the finding that ectopic expression of μ1 or truncated versions of μ1 activate NF-κB and the recent finding that NF-κB activation is required for cleavage of BID, which in turn is dependent upon TRAIL-R signaling (12, 13). It has been proposed that during reovirus infection, activation of the intrinsic apoptotic pathway is mediated by caspase-8 cleavage of Bid with subsequent recruitment of tBid to mitochondrial membranes and activation of Bax and Bak. However, we found that the pan-caspase inhibitor Q-VD-OPh did not impair cytochrome *c* release from mitochondria in μ1-transfected cells. In addition,

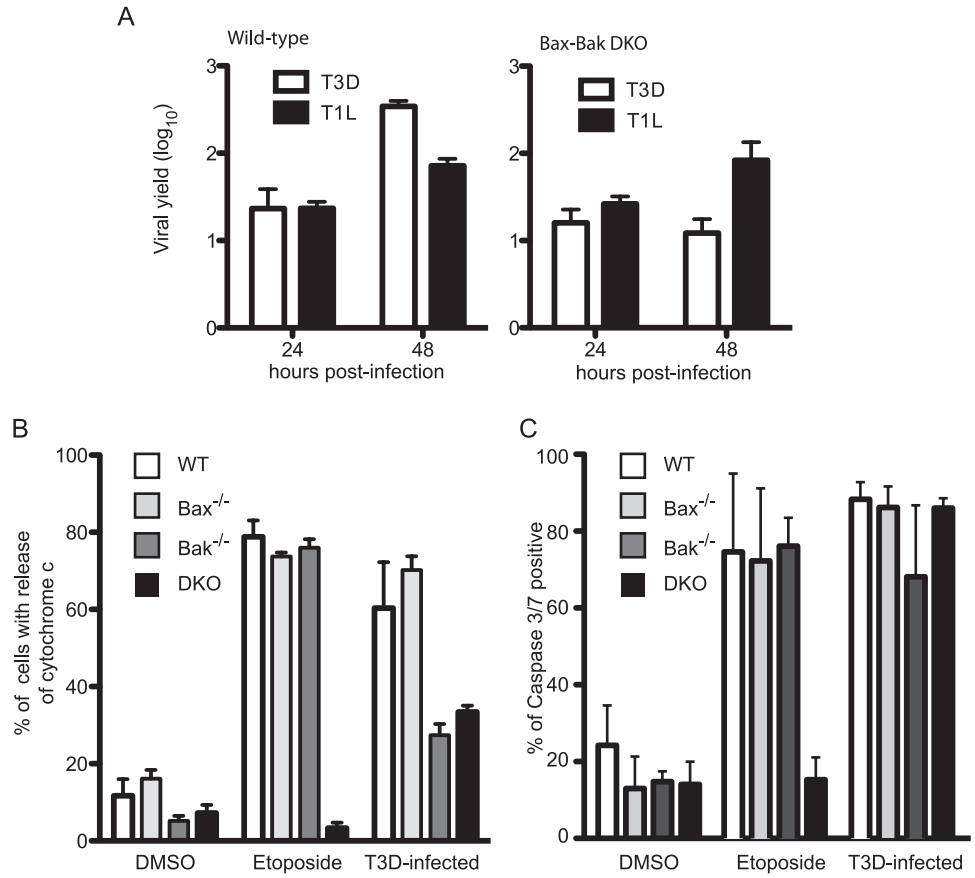

FIG. 7. Reovirus infection induces cytochrome *c* release from mitochondria and activation of caspase-3 in $Bax^{-/-}Bak^{-/-}$ DKO MEFs. (A) Reovirus strains T1L and T3D replicate in WT and DKO MEFs. Wild-type or DKO MEFs were adsorbed with T1L or T3D at an MOI of 5 PFU/cell. Titers of virus in cell lysates at the indicated intervals postinfection were determined by plaque assay. Results are expressed as mean viral yield for three experiments. Error bars indicate SD. (B) Capacity of etoposide (100 μM) or T3D reovirus infection to cause release of cytochrome *c* from mitochondria in WT, $Bax^{-/-}$, $Bak^{-/-}$, and $Bax^{-/-}Bak^{-/-}$ MEFs. Cells were incubated with 100 μM etoposide or DMSO for 24 h or infected or mock infected with T3D at a multiplicity of 5 PFU/cell for 48 h. Cells were harvested, and the plasma membrane was permeabilized with digitonin (see Materials and Methods) and then fixed and immunostained with anti-cytochrome *c* MAb and anti-μNS rabbit serum followed by Alexa 488-conjugated goat anti-mouse IgG and Alexa 647-conjugated goat anti-rabbit IgG. Cells were analyzed by flow cytometry. For infected cells, at least 10,000 μNS-positive cells were gated and scored for loss of cytochrome *c* positivity. Results are expressed as the mean and SD of three replicates from each of at least two experiments. (C) Capacity of etoposide (100 μM) or T3D reovirus infection to cause activation of caspase-3 in WT, $Bax^{-/-}$, $Bak^{-/-}$, and $Bax^{-/-}Bak^{-/-}$ MEFs. Cells were infected as described for panel B and then harvested at 48 h postinfection, and caspase-3 and -7 activity was detected by FLICA. Cells were then fixed and immunostained with anti-μNS serum followed by Alexa 647-conjugated goat anti-rabbit IgG. At least 10,000 cells expressing μNS were analyzed by flow cytometry for caspase-3 and -7 activity. Data shown represent the mean ± SD; $n \geq 3$.

μ1 was capable of causing cytochrome *c* release in MEFs lacking Bax and Bak. These findings suggest that the intrinsic and extrinsic apoptotic pathways can be activated independently in μ1-expressing cells.

Although release of cytochrome *c* from the mitochondrial intermembrane space occurred independently of the activation of initiator caspases in μ1-expressing cells, we found that caspase-8 and -9 activity was required for activation of effector caspase-3. During reovirus infection the extrinsic apoptotic pathway is activated following the release of TRAIL from infected cells and upregulation of death receptors 4 and 5 on the surface of infected cells (8). However, it is not currently known what triggers this activation of the extrinsic apoptotic pathway. One possibility is that virus-induced ER stress is responsible; we have previously shown that μ1 localizes to ER membranes (9). ER stress can activate caspase-8 (18), and recently it was shown that TRAIL-inducible apoptosis is dependent upon Par-4; Par-4 is secreted in response to ER stress and upon binding Grp78 (also secreted from the ER in response to stress) on the cell surface causes activation of the extrinsic apoptotic pathway (5). We are currently examining this possible mechanism of μ1-induced caspase-8 activation.

Reovirus-induced apoptosis does not require caspase-9 activity. However, activation of the intrinsic apoptotic pathway is essential, as release of smac/DIABLO from the mitochondrial intermembrane space is needed to repress the activity of the cellular inhibitor of apoptosis proteins (cIAPs) to allow activation of effector caspases (23). In contrast, we found that caspase-9 activity was required for μ1-induced apoptosis. These findings indicate that reovirus-induced and μ1-induced apoptosis are not mechanistically the same. During reovirus infection, IPS-1 and IRF-3 are activated and augment the

proapoptotic response (17). This is thought to occur as a consequence of release of dsRNA from viral particles or during viral replication. Activation of this additional proapoptotic signaling pathway during reovirus infection may explain why caspase-9 activation is dispensable for reovirus-induced but not for μ1-induced apoptosis.

Genetic studies have shown that strain differences in apoptotic potential map to the μ1-encoding M2 gene (reviewed in reference 7). However, we found that ectopic expression of μ1 derived from the relatively nonapoptogenic T1L and apoptogenic T3D strains led to similar levels of apoptosis induction. These findings suggest that the apoptogenic potential of the μ1 protein does not intrinsically differ between strains. There are several possible explanations for this discrepancy. As reovirus-induced apoptosis occurs subsequent to membrane penetration but prior to transcription, it is possible that the release of the ϕ fragment of μ1 from intermediate subviral particles in apoptogenic reovirus strains is more efficient than that in strains that induce apoptosis less effectively. An alternative explanation is that the strain differences in apoptogenic potential relate to the efficiency of μ1 assembly with σ3. We have previously shown that coexpression of σ3 with μ1 abrogates the capacity of μ1 to induce apoptosis (9).

In summary, our findings that reovirus infection causes release of cytochrome *c* from the intermitochondrial space and activation of effector caspases-3 and -7 in $Bax^{-/-}Bak^{-/-}$ DKO cells show that Bax and Bak are not required for reovirus-induced apoptosis. Rodgers et al. showed that overexpression of Bcl-2 inhibits reovirus-induced apoptosis in MDCK cells (27). In light of our findings, this suggests that additional BH3-only proapoptotic proteins may be involved in reovirus-induced cytochrome *c* release from the mitochondrial intermembrane space. One possibility is that tBid is sufficient to permeabilize mitochondria for release of smac/DIABLO and cytochrome *c* during reovirus infection. The insertion of tBid into the mitochondrial membrane is mechanistically similar to that of Bax, and tBid can permeabilize artificial membranes (4). It is possible that in the presence of μ1, tBid can directly substitute for Bax and Bak and permeabilize mitochondrial membranes, leading to the release of cytochrome *c* and smac/DIABLO. Finally it is also possible that μ1 itself can directly permeabilize the outer mitochondrial membrane. We are currently examining these possibilities.

ACKNOWLEDGMENTS

We thank Hollis Erb for her expert advice on statistical analysis of the data and Karin Hoelzer for assistance in performing the statistical tests.

This work was supported by a Burroughs Wellcome Fund Investigators of Pathogenesis of Infectious Disease award (to J.S.L.P.) and by NIH grants R01 AI063036 (to J.S.L.P.) and T32 AI07618 (to M.L.W.).

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