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A Proapoptotic Peptide Derived from Reovirus Outer Capsid Protein μ1 Has Membrane-Destabilizing Activity

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The reovirus outer capsid protein μ1 is responsible for cell membrane penetration during virus entry and contains determinants necessary for virus-induced apoptosis. Residues 582 to 611 of μ1 are necessary and sufficient for reovirus-induced apoptosis, and residues 594 and 595 independently regulate the efficiency of viral entry and reovirus-induced cell apoptosis, respectively. Two of three α-helices within this region, helix 1 (residues 582 to 611) and helix 3 (residues 644 to 675), play a role in reovirus-induced apoptosis. Here, we chemically synthesized peptides representing helix 1 (H1), H1:K594D, H1:I595K, and helix 3 (H3) and examined their biological properties. We found that H1, but not H3, was able to cause concentration- and size-dependent leakage of molecules from small unilamellar liposomes. We further found that direct application of H1, but not H1:K594D, H1:I595K, or H3, to cells resulted in cytotoxicity. Application of the H1 peptide to L929 cells caused rapid elevations in intracellular calcium concentration that were independent of phospholipase C activation. Cytotoxicity of H1 was not restricted to eukaryotic cells, as the H1 peptide also had bactericidal activity. Based on these findings, we propose that the proapoptotic function of the H1 region of μ1 is dependent on its capacity to destabilize cellular membranes and cause release of molecules from intracellular organelles that ultimately induces cell necrosis or apoptosis, depending on the dose.

MATERIALS AND METHODS

Cells. L929 cells were maintained in suspension in Joklik’s modified minimal essential medium supplemented with 4% fetal bovine serum, 2 mM glutamine, 100 U ml−1 penicillin, and 100 μg ml−1 streptomycin. CV-1 cells were grown in Ham’s F-12 medium (CellGro) supplemented with 10% fetal bovine serum (HyClone), 100 U ml−1 penicillin, 100 μg ml−1 streptomycin, 1 mM sodium pyruvate, and nonessential amino acids (CellGro). CHO-K1 cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U ml−1 penicillin, and 100 μg ml−1 streptomycin.

Peptide synthesis and CD. Peptides were synthesized by FlexPeptide technology, purified by high-performance liquid chromatography (HPLC) and confirmed by mass spectrometry at GenScript (Piscataway, NJ). Circular dichroism (CD) was used to analyze the secondary structure of the synthetic peptides. Initially, the peptides were analyzed at 50 μM in trifluoroethanol or in the presence of 10% phosphatidylcholine (PC) liposomes (prepared as described below). Spectra were recorded with a model 400 AVIV CD spectrometer (Lake-wood, NJ) in a 1-cm-path-length cuvette at room temperature (RT). Structural analysis was performed using the Delta epsilon (DE) calculation method using K2D CD spectra Deconvolution software (http://www.embld.de/~andre/k2d.html).

Cytotoxicity assay. We used a fluorescence-based cytotoxicity assay (CytoTox-Fluor, Promega, Madison, WI) to quantify cytotoxicity induced by incubation with the synthetic peptides. L929 cells were seeded in 96-well plates and incubated with the indicated concentrations of each peptide for 1 h at 37°C in 100 μl of DMEM, and then the levels of cytotoxicity were measured following incubation with the fluorescent substrate (100 μl) for an additional 30 min at 37°C according to the manufacturer’s instructions. Fluorescence was measured by stabilizing membranes, and this region of μ1 was previously suggested to be involved in membrane penetration during virus entry (20). Based on these previous findings, we hypothesized that one mechanism by which μ1 induces apoptosis is by direct destabilization of intracellular membranes. Such destabilization could induce a cellular stress response or lead to direct leakage of proapoptotic molecules from mitochondria or the endoplasmic reticulum (ER).

Here, we examined the capacity of peptides representing residues 582 to 611 of μ1 to perturb artificial and cellular membranes.
spectrofluorimetry (excitation wavelength [λem] = 485 nm; emission wavelength [λem] = 520 nm), and cytotoxicity was calculated by the following equation: cytotoxicity = (mean for treated cells – mean for untreated cells)/standard deviation (SD) for cell-free controls. Maximum cytotoxicity (100%) was set as the fluorescence measured in cells treated with 30 μg mL−1 digitonin for 1 h at 37°C.

Hemolysis assay. Hemolysis assays were performed as described previously (34). In brief, washed citrated chicken red blood cells (RBCs) in Alsever solution (Colorado Serum Co., Denver, CO) were suspended in cold phosphate-buffered saline (PBS) containing 2 mM MgCl2 (PBS-Mg) at a concentration of 5% (vol/vol) with different concentrations of the indicated peptides. After incubation for 30 min at 37°C, the RBCs were pelleted by centrifugation (380 × g for 5 min) and released hemoglobin in the supernatant was measured by absorbance at 415 nm. Supernatant collected from RBCs incubated with 0.1% Triton X-100 was used to assess complete release of hemoglobin, and RBCs incubated with PBS were used as a negative control. Data shown are the means ± SD of results from a minimum of three independent experiments.

Preparation of liposomes and lipidic leakage assays. Phosphatidylcholine (PC) in chloroform (18:1; Avanti Lipids) was dried under nitrogen, rehydrated at 1 mg mL−1 in PBS, pH 7.4, containing 12.5 mM amino phosphatidolphosphonate acid (ANTs; Fluka) and 45 mM phosphatidylethanolamine (PE; Fluka) as described in the text. For lower concentrations of cardiolipin, the lipid mixture was dried and resuspended in PBS and incubated with 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM CaCl2, 0.6 mM Na2HPO4, 0.4 mM KH2PO4, 5 mM glucose, 20 mM HEPES, pH 7.4, resuspended in HBSS containing 2.5 mM probenecid (Sigma), and further concentrated by centrifugation (380 × g for 15 h at 4°C). Liposomes were harvested from the top of the gradient and then reequilibrated in PBS by gel filtration on a Sephadex G-25 PD10 column (Amersham Biosciences). Dequenching of ANTS in response to lipidic leakage was assayed by spectrofluorimetry (Cary Eclipse spectrophotometer; Varian, Inc.) (λex = 355 nm; λem = 520 nm). The percent leakage was calculated using the baseline fluorescence emission of ANTS (E0) in buffer only (F0), the sample fluorescence (F1), and the 100% leakage fluorescence (F2) (liposomes were lysed with 0.2% Triton X-100 as control). For treated cells, percent leakage was calculated using the baseline fluorescence emission of ANTS (E0) in buffer only (F0), the sample fluorescence (Ft), and the 100% leakage fluorescence (F2) (liposomes were lysed with 0.2% Triton X-100). F0 and Ft were measured in cells treated with 30 μg mL−1 digitonin for 1 h at 37°C.

Measurement of intracellular free Ca2+ concentration. We assessed changes in intracellular free Ca2+ levels in CHO cells. Spiderman culture L929 cells (approximately 3 × 107 cells) were harvested, washed in PBS buffer, incubated for 1 h at 37°C in the dark in 6 mL of serum-free Joklik’s modified minimal essential medium containing 3 μM fura-2-acetoxymethyl ester (fura-2-AM; Invitrogen) and 8 μM pluronic F-127 (Invitrogen), then washed twice with Hanks balanced salt solution (HBBS) buffer (130 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1.0 mM MgCl2, 0.6 mM NaHPO4, 0.4 mM KH2PO4, 5 mM glucose, 20 mM HEPES, pH 7.4), resuspended in HBBS containing 2.5 mM probenecid (Sigma), and further incubated for 20 min at RT in the dark to allow esterification of fura-2-AM. Following fura-2 loading, the cells were washed three times and resuspended in 3 mL of HBBS containing 1 mM EGTA immediately prior to measurements. Cell suspensions (400 μL) were placed in a quartz cuvette with a magnetic stirrer. Experimental reagents, including peptides, Triton X-100, carbachol, or phospholipase C (PLC) inhibitor (UT-73122; Sigma), were added at the time points indicated in Results. Fluorescence was measured by spectrofluorimetry (λex = 340 and 380 nm; λem = 510 nm). The ratio of the fura-2-AM-Ca2+ complex (λem = 340 nm; λem = 510 nm) to free fura-2-AM (λem = 380 nm; λem = 510 nm) was generated using the Grynkiewicz equation to calculate internal calcium concentration (10).

Membrane and microinjection. Biotin-conjugated H1 and H1:19595K peptides were synthesized, dissolved in PBS, and incubated for 30 min at RT with CV-1 cells on 18-mm glass coverslips. The cells were washed extensively in PBS, then fixed with 2% paraformaldehyde in PBS for 10 min, incubated with Alexa488 Fluor-conjugated streptavidin (Invitrogen), and washed again in PBS, and then the coverslips were mounted on glass slides with Prolong Gold plus DAPI (4′,6-diamidino-2-phenylindole; Invitrogen). Images were obtained with an inverted microscope (Nikon TE2000) equipped with fluorescence optics through a 60× 1.4-numerical-aperture (NA) objective with 1.5× 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).

To assess the distribution of biotinylated H1 in cells, CHO-K1 cells seeded on 50-mm MatTek gridded dishes (MatTek, Ashland, MA) were preincubated with 500 nM MitoTracker CMXRos (Invitrogen) in growth medium for 45 min at 37°C. The labeling medium was replaced with medium lacking phenol red, and the cells were microinjected with ~6 nl of sample using an InjectMan N2 micropipette and a Femtoject micromanipulator (Eppendorf, Hauppauge, NY). Micropipette needles were prepared with a P-97 micropipette puller (Sutter Instrument Co., Novato, CA). Samples were injected using 50 μM biotin-conjugated H1 that was preincubated with Alexa488-conjugated streptavidin or, as a control, Alexa488-conjugated streptavidin. Samples were prepared in PBS buffer and injected into cells, and the cells were incubated at 37°C on a stage warmer (World Precision Instruments, Sarasota, FL). Fluorescence and phase-contrast images were collected using a Nikon TE300 microscope and a Hamamatsu Orca ER charge-coupled-device camera. Images were collected using Simple PCI software (Hamamatsu, Sewickley, PA) and contrast and brightness adjusted with Photoshop (Adobe, San Jose, CA).

To assess the release of cytochrome c from cells microinjected with the H1, H1:19595K, or H3 peptide, CHO-K1 cells maintained at 37°C were microinjected as described above with a 1:1 mixture of Alexa594 bovine serum albumin (BSA) (Invitrogen) and the indicated peptides (final concentration, 50 μM). Twenty minutes after injection, cells were fixed in 4% paraformaldehyde in PBS, washed in PBS, permeabilized with 0.1% Triton X-100 in PBS plus 1% BSA, and then immunostained with anti-cytochrome c monoclonal antibody (MAB) (clone 6H2B4; BD Biosciences) followed by Alexa594-conjugated goat anti-mouse IgG. Fluorescence and phase-contrast images were collected and processed as described above.

Peptide bacterial activity. The bactericidal activity of peptides against Escherichia coli and Brevibacillus subtilis. THH61 was assayed by colony inhibition. Bacterial cultures were grown to early log phase at 37°C in Luria-Bertani (LB) broth and then diluted to 2 × 105 CFU/ml in 10 mM phosphate buffer (pH 7.4) containing 0.03% LB broth. Equal volumes of peptide-containing solution and bacterial culture (25 μl of each) were mixed and incubated at 37°C for 3 h, after which the bacterium-peptide mixture was diluted 100-fold or 1,000-fold with cold phosphate buffer and inoculated on LB agar plates, which were incubated at 37°C overnight. The number of bacterial colonies on each plate was counted the following day and expressed as a percentage of the number of colonies obtained when phosphate buffer was mixed with the bacteria instead of peptide.

Preparation of isolated mitochondria and cytochrome c release assay. Cytochrome c released from mitochondria purified from murine L929 cells was assayed as described previously, with some modification (32). Cells in spinner culture were harvested, washed, and homogenized by Dounce homogenization (80 strokes), and nuclei and intact cells were removed by centrifugation (800 × g for 15 min at 4°C). The supernatant was then centrifuged at 10,000 × g for 15 min at 4°C. The mitochondrial pellet was further washed and resuspended in mitochondrial buffer before being used immediately for further experiments. Both the mitochondrion-free supernatant and the mitochondrial pellet were saved for analysis. Isolated mitochondria were incubated with 100 μM the H1 or H3 peptide, treated with 1% Triton X-100, or incubated with an equal volume of PBS for 20 min at RT. After incubation, the samples were returned to ice and the mitochondrial and supernatant fractions were separated by centrifugation (16,000 × g for 15 min at 4°C). The pellet fraction was resuspended in a volume of mitochondrial buffer equal to that of the supernatant. Samples were then incubated in protein sample buffer with 200 mM DTT on ice for 15 min and then at 80°C for 15 min, cooled on ice, and cleared at 16,000 × g for 1 min, and then proteins were separated on 15% SDS-PAGE. Proteins were transferred to nitrocellulose membranes by Semi-Dry Transfer Cell (Bio-Rad). The membranes were blocked with 5% BSA in Tris-buffered saline (20 mM Tris-base, 150 mM NaCl, pH 7.5) for 1 h and then incubated with 1:1,000, 1:400, or 1:100 primary antibodies: rabbit anti-cytochrome c (Upstate); rabbit anti-human mitochondrion-permeable cytochrome c oxidase inhibitor (Santa Cruz); and rabbit anti-human cytochrome c oxidase subunit 1 (Novus). Membranes were then washed and incubated with 1:5,000, 1:2,000, or 1:1,000 secondary antibodies: horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG (1:10,000; Jackson Laboratories). Proteins were detected on autoradiograph film with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).
RESULTS

A synthetic peptide representing residues 582 to 612 of \( \mu.1 \) is \( \alpha \)-helical in solution. We have previously shown that the C-terminal \( \phi \) fragment (residues 582 to 708) of the reovirus outer capsid \( \mu.1 \) protein contains determinants necessary for virus-induced apoptosis. Residues 582 to 611 of \( \mu.1 \) are necessary and sufficient for apoptosis induction (3), and substitutions K594D and I595K within this region in recombinant viruses have reduced apoptosis-inducing capacity in vitro and in vivo (5). In the structure of the \( \mu.1 \) helix, residues 582 to 611 consist of a short loop and an amphipathic \( \alpha \)-helix (Fig. 1A) (19).

Many amphipathic peptides interact with membranes and in some cases destabilize membrane barriers (18). We previously showed that residues 582 to 611 fused to EGFP localize to mitochondria, lipid droplets, and the endoplasmic reticulum in transfected cells (3). Destabilization of mitochondrial and/or ER membranes could in part explain the mechanism by which \( \mu.1 \) induces apoptosis. We therefore examined whether a synthetic peptide representing residues 582 to 611 could destabilize cellular and artificial membranes. Peptides representing residues 582 to 612 of \( \mu.1 \) (peptide H1) and, as a negative control, a peptide representing residues 639 to 675 of \( \mu.1 \) (peptide H3), which does not induce apoptosis when expressed in cells as a fusion with EGFP (3), were chemically synthesized. All of the peptides were soluble in aqueous solutions. We used circular dichroism to identify the secondary structures of the peptides in trifluorethanol (H1 and H3), which is known to stabilize peptide structures. We found that the H1 and H3 peptides were \( \alpha \)-helical in solution (100%) (Fig. 1B). The H1 and mutant peptides (K593D and I595K) had identical spectra, while H3 had deeper and higher peaks, most likely because of the greater length of H3 (Fig. 1B). We observed no significant differences in the secondary structure conformers of the H1 peptide in the presence of liposomes (data not shown).

Peptide H1 (residues 582 to 611) directly permeabilizes liposomes. To examine if the H1 peptide could destabilize membranes, we prepared small unilamellar liposomes (50-nm diameter) consisting of the neutral lipid phosphatidylcholine that contained the fluorophore/quencher pair 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS)–pyridinium (DPX). We then assessed liposome leakage in response to increasing concentrations of H1 by measuring dequenching of ANTS (1). We then assessed liposome leakage in response to increasing concentrations of H1 by measuring dequenching of ANTS (1). We found that H1 induced a concentration-dependent leakage of ANTS from liposomes, with 50% leakage occurring at a concentration of \( \sim 12.6 \mu M \). In contrast, the H3 peptide had no effect on liposome membrane permeability (Fig. 2A). We conclude that H1 peptide has direct membrane-permeabilizing properties.

Mutants of H1 lose the capacity to directly permeabilize liposomes. We previously showed that recombinant viruses or \( \mu.1 \) with the I595K or K594D substitution within the H1 helix were defective in apoptosis induction (5). We therefore examined the capacity of peptides bearing these substitutions to permeabilize PC liposomes. We found that unlike the wild-type H1 peptide, H1:I595K did not induce liposome leakage. The H1:K594D peptide did induce leakage, but \( \sim 2 \)-fold-higher concentrations than those of wild-type H1 were required to induce 50% leakage (Fig. 2A). Thus, the capacity of \( \mu.1, \phi, \) or their I595K and K594D substitution mutants to induce apoptosis when expressed ectopically in cells correlates with the capacities of peptides representing the region that is minimally required for apoptosis induction to permeabilize artificial membranes.

Synthetic H1 peptide causes size-selective leakage of FITC-dextran from liposomes. The \( \mu.1N \) fragment of \( \mu.1 \) (Fig. 1A) is required for virus entry but is not involved in \( \mu.1 \)-mediated
apoptosis induction. Zhang et al. showed that a synthetic myristoylated \( \mu \)IN peptide likely forms size-selective pores in artificial membranes (34). To address the possibility that the H1 peptide also forms size-selective pores, we prepared liposomes preloaded with self-quenching concentrations of 10- or 40-kDa fluorescein isothiocyanate (FITC)-dextran polymers. Leakage of the FITC-dextran molecules was assayed by fluorescence dequenching (Fig. 2B). We found that concentrations of H1 that caused release of ANTS also caused release of 10 kDa FITC-dextran; however, the release of 40 kDa FITC-dextran was much less efficient (Fig. 2B). These findings indicate that size-limited pores formed in liposomes treated with the H1 peptide. The H1 peptide permeabilizes cell membranes. (i) H1 is cytotoxic for L929 cells. Based on our finding that the H1 peptide directly permeabilized artificial membranes, we investigated whether incubation of H1 with cells was cytotoxic. Using an assay that quantifies release of protease activity from dead cells (CytoTox-Fluor; Promega), we found that concentrations of H1 of 50 \( \mu \)M or greater were cytotoxic to L929 cells after 1 h of incubation (Fig. 3A). In contrast, similar concentrations of the H3 peptide had no effect on the viability of L929 cells (Fig. 3A). The I595K and K594D mutant H1 peptides were not cytotoxic, even at 200 \( \mu \)M (Fig. 3A).

(ii) H1 causes hemolysis of red blood cells. To further determine if the H1 peptide was directly permeabilizing the plasma membranes of cells, we assayed for leakage of hemoglobin from chicken red blood cells. Unlike for the \( \mu \)IN peptide, where concentrations of 10 \( \mu \)g ml\(^{-1}\) led to 70% hemolysis, we found that only 30% hemolysis occurred when chicken RBCs were incubated with 20-fold-higher concentrations of H1 peptide (Fig. 3B). This difference in efficiency of hemoglobin release (~68 kDa) likely reflects a lower size exclusion limit of pores formed by the H1 peptide than those formed by \( \mu \)IN, which allow release of hemoglobin and 40-kDa FITC-dextran (34).

(iii) H1 causes changes in cytosolic Ca\(^{2+}\) levels. We hypothesized that the cytotoxicity that we observed in L929 cells was caused by increases in cytosolic Ca\(^{2+}\) concentrations. In support of this hypothesis, we found that application of the H1 peptide to fura-2-loaded L929 cells induced substantial and rapid increases in cytosolic [Ca\(^{2+}\)] with 50 \( \mu \)M H1 causing a 3- to 4-fold increase in cytosolic [Ca\(^{2+}\)] by 2 min after addition of the peptide. In contrast, application of 50 \( \mu \)M H3 had no noticeable effect on cytosolic [Ca\(^{2+}\)] (Fig. 3C). As these experiments were carried out with Ca\(^{2+}\)-free buffer containing EGTA, this finding suggested that in addition to having direct membrane permeabilization effects, the H1 peptide might also stimulate phospholipase C-mediated IP3 signaling pathways with release of Ca\(^{2+}\) from intracellular stores. Addition of purified rotavirus NSP4 or a peptide representing NSP4, which elevates cytosolic calcium in Sf9 insect cells, which is blocked by the phospholipase C inhibitor (U-73122) (29). Treatment with U-73122 prevented the increase in Ca\(^{2+}\) induced by 100 \( \mu \)M carbachol, a PLC receptor agonist (Fig. 3D). However, we found that pretreatment of cells with the PLC inhibitor (U-73122) had no effect on H1-induced increases in intracellular Ca\(^{2+}\) (Fig. 3D). Based on these findings, we conclude that the rapid changes in intracellular Ca\(^{2+}\) cannot be attributed to direct permeabilization of the plasma membrane alone. We speculate that the H1 peptide may access the intracellular membranes of the ER or mitochondria, causing the release of calcium stores.

H1 peptide has antibacterial activity for Gram-negative and Gram-positive bacteria. Based on the cytotoxicity of H1 for L929 cells, the membrane-permeabilizing activity of H1, and the propensity of cationic amphipathic helical peptides to have antibacterial activity (33), we tested whether the H1 peptide had antibacterial activity. We found dose-dependent inhibition of the growth of Gram-positive (B. subtilis) and Gram-negative (E. coli) bacteria by the H1 peptide (Fig. 4A), with complete inhibition of colony formation in this assay at concentrations of H1 greater than 25 \( \mu \)M. In contrast, 50 \( \mu \)M the H3 peptide had little effect (Fig. 4B), and 50 \( \mu \)M concentrations of the K594D and I595K substitution mutants had decreased antibacterial...
activity compared to the level for WT H1 (Fig. 4B). We conclude that the H1 peptide has antibacterial properties.

**Effect of lipid composition on H1-induced ANTS leakage.**
Green fluorescent protein (GFP)-fused H1 localizes prominently to mitochondria in transfected cells, causes release of cytochrome c, and induces apoptosis (3, 31). Mitochondrial membranes have increased concentrations of the neutral lipid cardiolipin, and permeabilization of liposomes by the Bcl-2 homology domain 3 (BH3)-only peptides derived from pro-apoptotic proteins Bax and Bak is promoted by increased con-
centrations of cardiolipin (16). We therefore prepared liposomes with lipid contents that approximated those of *Xenopus* mitochondria and examined the effect of the presence or absence of 7 mol% cardiolipin on the efficiency of H1 membrane permeabilization. We found that the efficiency of H1 permeabilization of liposomes was increased when liposomes contained cardiolipin (Fig. 5A). Based on these findings, we tested whether H1 could directly permeabilize the outer mitochondrial membranes of isolated mitochondria and cause release of cytochrome c, which is normally retained in the mitochondrial intermembrane space. We isolated mitochondria from L929 cells and then incubated them with different concentrations of the H1, H3, H1:K594D, and H1:IS95K peptides. To assess permeabilization of the outer mitochondrial membrane, we examined for leakage of cytochrome c from the intermembrane space. Although the H1 peptide localized to mitochon-

![Graph](image1.png)

**FIG. 4.** The peptide H1 has a bactericidal activity on Gram-positive and Gram-negative bacteria. (A) The capacity of different concentrations of the H1 peptide to inhibit bacterial growth was assayed as described in Materials and Methods. (B) The indicated peptides were tested for their bactericidal activity at 50 μM. Results shown are the means ± SD for three independent experiments.

![Graph](image2.png)

**FIG. 5.** H1 preferentially permeabilizes liposomes containing the mitochondrial lipid cardiolipin (A) but does not cause release of cytochrome c from isolated mitochondria (B) or mitochondria in CHO-K1 cells microinjected with H1 (C). (A) ANTS-DPX-loaded liposomes containing a mixture of lipids characteristic of mitochondria from *Xenopus* oocytes were prepared with or without 7 mol% cardiolipin. Leakage of ANTS was quantified as before upon incubation with the indicated H1 concentrations. Means ± SD of results from three independent experiments are shown. (B) Mitochondria isolated from L929 cells were incubated with 100 μM the H1 or H3 peptide or with equal volumes of PBS or 1% Triton X-100 for 20 min at RT. After incubation, mitochondria were pelleted, and cytochrome c present in the pellet (P) and supernatant (S) fractions was assayed by immunoblot analysis. A sample of purified cytochrome c (marked C) was loaded in the first lane. (C) CHO-K1 cells were microinjected with a 1:1 mixture of Alexa594 BSA, and the H1, H1:IS95K, or H3 peptide (final concentration, 50 μM). Cells were incubated for a further 20 min at 37°C and then fixed and immunostained with mouse anti-cytochrome c MAb followed by Alexa488-conjugated goat anti-mouse IgG.
drial membranes (Fig. 6B), we found that incubation of isolated mitochondria with 100 μM the H1 or H3 peptide did not cause cytochrome c release, suggesting that any pores formed by the H1 peptide in mitochondrial membranes do not allow egress of cytochrome c from the mitochondrial intermembrane space (Fig. 5B).

**Microinjected H1 does not cause release of cytochrome c from mitochondria.** We have previously shown that expression of GFP-H1 causes release of cytochrome c from the mitochondrial intermembrane space at 48 h posttransfection (31). However, we found that purified mitochondria did not release cytochrome c when they were incubated with the H1 peptide (Fig. 5B). To test whether the H1 peptide could cause release of cytochrome c from mitochondria in cells, we microinjected H1 (final concentration, 50 μM) together with BSA fluorescently labeled with Alexa594 into CV-1 cells. After an incubation period of 20 to 30 min, the cells were fixed and immunostained for cytochrome c. We found that, similar to our findings with purified mitochondria, the H1 peptide did not cause release of cytochrome c from mitochondria in cells after a 20-min incubation period (Fig. 5C). As expected, microinjection of the H1:I595K or H3 peptide also did not cause release of cytochrome c. We conclude that although the H1 peptide can permeabilize the plasma membrane and artificial membranes, it does not permeabilize mitochondrial membranes to an extent that will allow release of cytochrome c.

**Biotinylated H1 localizes to mitochondrial membranes and disrupts normal mitochondrial architecture.** We therefore assessed the subcellular distribution of a biotinylated form of H1 complexed with streptavidin-Alexa488 after it was microinjected into CHO-K1 cells. Control microinjections of streptavidin-Alexa488 alone were diffusely distributed throughout the cell (Fig. 6A). However, the biotinylated H1 colocalized with the mitochondrial marker Mitotracker CMXRos (Fig. 6B). In addition, 1 min following microinjection, the mitochondria in several H1-injected cells became punctate and aggregated and differed from the normal tubulovesicular distribution of mitochondria in control injected cells (Fig. 6B, insets). These findings indicate that H1 localizes to mitochondrial membranes and disrupts normal mitochondrial architecture.

**Biotinylated H1 but not H1:I595K peptide forms a filamentous mosaic on the plasma membrane when incubated with cells.** To further investigate the mechanism of H1-induced plasma membrane permeability, we assessed the cell surface distribution of biotinylated H1 and H1:I595K. CV-1 monkey kidney cells were incubated with 50 μM H1 for 30 min at RT and then washed and fixed, and the distribution of biotinylated H1 was detected by the addition of streptavidin-Alexa488. The biotinylated H1 peptide formed a filamentous mosaic-like pattern on the plasma membrane of the CV-1 cells. In addition, larger aggregates of peptide could be seen adherent to the plasma membrane. In contrast, the mutant peptide H1:I595K distributed as punctate spots on the plasma membrane and did not form larger aggregates (Fig. 7). We conclude from these
findings that the H1 peptide can form filamentous arrays upon interaction with cellular membranes perhaps similar to those described for some cell-penetrating peptides upon interaction with artificial membranes (22, 23).

**DISCUSSION**

The reovirus outer capsid protein μ1 is the primary determinant of reovirus-induced apoptosis (3, 5–7). μ1 also has an additional critical role in membrane penetration during virus entry. In this regard, a myristoylated N-terminal fragment of μ1 (μ1N; residues 2 to 42) that is produced by autocatalytic cleavage of μ1 during virus entry has direct membrane-permeabilizing activity (21, 34). The C-terminal φ fragment of μ1, although not functionally required for entry, increases its efficiency and was suggested to have chaperone-like activity (13). In previous work, we showed that ectopic expression of GFP-
fused to residues 582 to 611 of μ1 (within φ) localized to mitochondria and induced apoptosis (3). More recently, we have found that ectopic expression of μ1 or reovirus infection causes release of cytochrome c and apoptosis in cells lacking the proapoptotic BCL-2 family members Bax and Bak (31). We now show that residues 582 to 611 of μ1 also have direct membrane-permeabilizing activity. These findings raise the possibility that one mechanism by which μ1 initiates apoptosis in reovirus-infected cells is by directly permeabilizing cellular membranes. The μ1 protein localizes to mitochondria and the ER in infected cells (3), and we speculate that direct permeabilization of these membranes with release of proapoptotic molecules into the cytosol initiates the apoptotic process in infected cells. Our data show that the H1 peptide directly permeabilizes artificial membranes, causing release of small molecules up to 10 kDa in size, but cannot itself cause release of cytochrome c from the intermembrane space of purified mitochondria. In contrast, ectopic expression of GFP-φ causes release of cytochrome c by 48 h posttransfection (31). It is possible that the release of cytochrome c and smac/DIABLO, which occurs during reovirus infection (14, 15), is a consequence of direct permeabilization of the outer mitochondrial membrane by μ1, as the full-length μ1 protein may be able to oligomerize and form larger pores in intracellular membranes. However, cleaved tBid is required for reovirus-induced apoptosis (8). Therefore, it is possible that μ1 or a fragment of μ1 acts together with cleaved tBid to permeabilize mitochondrial membranes in the absence of Bax and Bak. The activation of calpain during reovirus infection (9) might be explained by leakage of calcium from the ER as a consequence of μ1-induced membrane damage.

Our data also indicate that the H1 peptide can directly permeabilize the plasma membranes of cells, leading to cytotoxicity that is accompanied by increases in intracellular calcium. Although we initially expected that the increased calcium resulted from influx from the extracellular environment, we found that cytosolic calcium levels significantly increased even when extracellular calcium was absent. We further showed that this increase in cytosolic calcium was not a consequence ofactivation of phospholipase C signaling. Our findings that direct application of biotinylated H1 to cells leads to localization of the peptide to mitochondria suggest that in following plasma membrane permeabilization, H1 can access the cytosol and target mitochondrial and perhaps other intracellular membranes. A recombinant mutant virus bearing the I595K mutation in μ1 has significantly decreased capacity to activate NF-κB and induce apoptosis (5). The efficiency of this mutant in inducing hemolysis of red blood cells and in entering cells was comparable to that of wild-type virus. However, the H1 peptide bearing this mutant was not able to permeabilize cellular membranes. Our findings suggest a strong correlation between H1-induced membrane permeabilization and apoptosis induction but clearly discriminate the capacity of φ to permeabilize membranes from its proposed chaperone role during virus entry.

The H1 peptide had moderate antibacterial effects against Gram-positive and Gram-negative bacteria. Peptides derived from HIV-1 gp41 have also been shown to kill Gram-positive and Gram-negative bacteria (27). The mechanism of the antibacterial action of H1 is likely related to its membrane-destabilizing properties. The action of cationic amphipathic antibacterial peptides such as magainin on Gram-negative bacteria is thought to be due to the capacity of these peptides to displace Mg2+ and Ca2+ cations from lipopolysaccharide (LPS), causing localized destabilization of the bacterial outer membrane. The peptides can then disrupt the cytoplasmic membrane in a process called self-promoted uptake (11, 24).

The formation of filamentous structures on the plasma membrane by H1 indicates that it can self-associate in the presence of membranes to form higher-order oligomeric structures. In contrast, the mutant H1:I595K, which does not destabilize membranes, although able to associate with the plasma membrane, did not form such structures, suggesting that the capacity of H1 to self-associate or aggregate may be required for membrane destabilization. The micellar aggregate model of peptide-mediated membrane destabilization proposes that peptide aggregation within the membrane leads to the formation of pores (12). Furthermore, collapse of such aggregates is predicted to allow translocation of peptides into the cytosol. In support of this mode of action for H1, we have found that some cells incubated with biotinylated H1 have peptide labeling on organelar membranes (data not shown).

We have proposed that when large numbers of viral particles are added to cells, the φ fragment of μ1 may gain access to the cytosol of cells and induce apoptosis (3, 5). This hypothesis would in part explain the capacity of UV-inactivated virions to induce apoptosis (30) and the requirement for disassembly of virions in order to induce apoptosis (4). Our current findings now indicate that the minimal region of φ required for apoptosis induction has direct membrane-destabilizing activity and can target mitochondrial membranes. However, we found that neither incubation of the H1 peptide with purified mitochondria nor microinjection of H1 into cells led to release of cytochrome c from the mitochondrial intermembrane space. Taken together, these findings support a model whereby C-terminal fragments of μ1 produced during virus entry and delivered into the cytosol during membrane penetration or produced in the cytosol as a consequence of the action of cytosolic proteases can target and destabilize mitochondrial and other intracellular membranes. Such destabilization would be predicted to lead to direct release of calcium and to activation of cellular stress response pathways that would in aggregate promote apoptosis. The recent finding that tBid is required for reovirus-induced apoptosis supports this model (8). We have previously noted differences in the localization patterns of μ1 and GFP-φ, with GFP-φ being much more strongly associated with mitochondrial membranes and μ1 more often seen in association with the ER and lipid droplets (3). We propose that targeting of μ1 to the ER leads to local release of ER calcium. Calcium release from the ER could be responsible for activating calpain, which has been shown to be required for reovirus-induced apoptosis (9). Our current findings suggest that reovirus-induced apoptosis may initiate as a consequence of μ1-induced intracellular membrane permeabilization.

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