Aeromonas hydrophila Cytotoxic Enterotoxin Activates Mitogen-activated Protein Kinases and Induces Apoptosis in Murine Macrophages and Human Intestinal Epithelial Cells*

Cristi L. Galindo‡, Amin A. Fadl‡, Jian Sha‡, Celso Gutierrez, Jr.§, Vsevolod L. Popov¶, Istvan Boldogh‡, Bharat B. Aggarwall, and Ashok K. Chopra**

From the Departments of §Microbiology & Immunology and ¶Pathology, University of Texas Medical Branch, Galveston, Texas 77555-1070 and the **Department of Immunotherapy, University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030-4095

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A cytotoxic enterotoxin (Act) of Aeromonas hydrophila possesses several biological activities, induces an inflammatory response in the host, and causes apoptosis of murine macrophages. In this study, we utilized five target cell types (a murine macrophage cell line (RAW 264.7), bone marrow-derived transformed macrophages, murine peritoneal macrophages, and two human intestinal epithelial cell lines (T84 and HT-29)) to investigate the effect of Act on mitogen-activated protein kinase (MAPK) pathways and mechanisms leading to apoptosis. As demonstrated by immunoprecipitation/kinase assays or Western blot analysis, Act activated stress-associated p38, c-Jun NH$_2$-terminal kinase (JNK), and extracellular signal-regulated kinase 1/2 (ERK1/2) in these cells. Act also induced phosphorylation of upstream MAPK factors (MAPK kinase 3/6 (MKK3/6), MKK4, and MAP/ERK kinase 1 (MEK1)) and downstream effectors (MAPK-activated protein kinase-2, activating transcription factor-2, and c-Jun). Act evoked cell membrane blebbing, caspase-3 cleavage, and activation of caspases 8 and 9 in these cells. In macrophages that do not express functional tumor necrosis factor receptors, apoptosis and caspase activities were significantly decreased. Immunoblotting of host whole cell lysates revealed Act-induced up-regulation of apoptosis-related proteins, including the mitochondrial proteins cytochrome c and apoptosis-inducing factor. However, mitochondrial membrane depolarization was not detected in response to Act. Taken together, the data demonstrated for the first time Act-induced activation of MAPK signaling and classical caspase-associated apoptosis in macrophages and intestinal epithelial cells. Given the importance of MAPK pathways and apoptosis in inflammation-associated diseases, this study provided new insights into the mechanism of action of Act on host cells.

Aeromonas species (spp.) are significant human pathogens that have been isolated from freshwater, salt water, and a variety of foods, and are frequently isolated from patients with diarrhea (1–3). Among Aeromonas spp., Aeromonas hydrophila is most commonly associated with human infections, leading to intestinal and non-intestinal diseases (4). Furthermore, increased resistance of this organism to antibiotics and chlorination in water presents a significant threat to public health (2, 3, 5–12). As a result, the Environmental Protection Agency has placed this organism on the “Candidate Contaminant List,” and the monitoring of United States water supplies for this organism began in 2002 (4).

A. hydrophila produces an array of virulence factors, one of the most significant of which is a 52-kDa cytotoxic enterotoxin (Act) (5). Our laboratory demonstrated that Act possessed several biological activities, including hemolysis, cytotoxicity, enterotoxicity, and lethality to mice, and induced acute inflammatory responses both in vitro and in vivo (9). Additional studies by our laboratory revealed that Act significantly altered intracellular signaling in murine RAW 264.7 macrophages, which could lead to an inflammatory response in host cells (14–16). The pattern of Act-induced gene expression changes, based on our recent microarray studies, strongly suggested that Act might activate mitogen-activated protein kinase (MAPK) pathways (16).

MAPKs are activated by phosphorylation on both a threonine and tyrosine residue, which is accomplished by dual phosphorylation enzymes, called MAP/ERK kinases (MEKs) or MAPK kinases (MKKs). MEKs are themselves activated via phosphorylation by MAPK kinases (MEKs) or MAPK kinase kinases (MKKs). MEKs are themselves activated via phosphorylation by MEK kinases (MEKs) or MAPK kinase kinases. There are four major MAPK cascades that lead to altered gene expression: ERK1/2, JNK, p38 kinase, and ERK5. ERK1 and ERK2 are activated via phosphorylation by MEK1 and MEK2, respectively (17).

The two MAPK pathways most frequently associated with

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** To whom correspondence should be addressed: Dept. of Microbiology and Immunology, Medical Research Building, 301 University Blvd., University of Texas Medical Branch, Galveston, TX 77555-1070. Tel.: 409-747-0578; Fax: 409-747-8689; E-mail: schopra@utmb.edu.

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immune responses are JNK and p38 kinases. The JNK signaling pathway is a stress-activated MAPK pathway involved in the regulation of cell proliferation and apoptosis (18). The p38 MAPK signaling pathway is associated with cell growth, differentiation, and death, and is activated by a variety of cytokines, including interleukin-1 (IL-1) and tumor necrosis factor (TNF-α) (17, 19). Three MAPK proteins (MEKK3, MKK4, and MKK6) can phosphorylate and activate p38 kinase (17). The activation of p38 leads to the production of several cytokines, including IL-1β, TNF-α, IL-6, and IL-8, as well as cyclooxygenase-2 and inducible nitric-oxide synthase enzymes, and phosphorylation of phospholipase A2 (PLA2) (20–25). The ERK5 pathway is the most recently described MAPK pathway and is primarily associated with response to stress (26, 27). ERK5 is activated via MEK5, which is itself activated by MEKK3 (28).

In the current study, we investigated the effects of Act on MAPK signaling in RAW 264.7 cells, murine peritoneal macrophages, and human intestinal epithelial cells (T84 cells) to determine the role of MAPK signaling in host cells and ultimately dissect the definitive role of signaling molecules that lead to the disease state during Aeromonas infection. Because we previously demonstrated that Act caused apoptosis of RAW 264.7 murine macrophages (16), we also investigated mechanisms associated with Act-induced apoptosis in murine peritoneal (primary) macrophages, TNF receptor (TNFR) knockout (KO) murine bone marrow-derived and transformed macrophages (29), and human intestinal epithelial cells. Taken together, the data clearly demonstrated for the first time that Act-induced host cell signaling involves activation of ERK1/2, p38, and JNK pathways. Furthermore, we delineated the mechanisms of apoptosis in Act-treated host cells.

EXPERIMENTAL PROCEDURES

Cell Culture—The murine macrophage (RAW 264.7) and human intestinal epithelial (T84 and HT-29) cell lines were purchased from the American Type Culture Collection (Manassas, VA). WT, double KO murine bone marrow- derived and transformed macrophages (deficient in both TNFR-1 and TNFR-2), and TNFR1/TNFR2 single KO macrophages were graciously provided by Dr. Aggarwal (29). RAW 264.7 and HT-29 cells were cultured at 37 °C and 5% CO2 in Dulbecco’s minimal essential medium (Life Technologies, Inc., Gaithersburg, MD) containing 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). T84 cells were cultured similarly, but in Dulbecco’s minimal essential media/F-12 (Invitrogen) and 5% fetal bovine serum. WT and TNFR KO macrophages were cultured in RPMI (Invitrogen) and 5% fetal bovine serum. For each experiment, 5 × 105 cells/ml were plated in 35-mm dishes and allowed to attach overnight. The medium was removed, and fresh medium containing the stimulant (6 ng/ml (unless otherwise stated) lipopolysaccharide (LPS)-free Act) was added (14). After each time point, cells were lysed using 100 µl/well of 1× Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA), sonicated twice for 15 s, and centrifuged at 10,000 × g for 10 min.

Peritoneal Macrophage Isolation—Adult 25–30 g Swiss Webster female mice (6–8 weeks old) were purchased from Taconic Farm (Germantown, CA). Mice were housed in the specific pathogen-free animal facility at the University of Texas Medical Branch, Galveston, and given free access to food and water prior to experiments. A 1-ml solution of 3% thioglycolate into the peritoneal cavity of mice to elicit macrophage migration into the cavity. Mice were allowed to rest for 3 days and then sacrificed by an overdose of isoflurane followed by cervical dislocation. A partial midline incision was made to remove the peritoneum and expose the parietal peritoneum lining. Peritoneal exudate cells were harvested by flushing the peritoneal cavity with 7 ml of RPMI medium (Invitrogen) using a 10-ml syringe fitted with a 21-gauge, 1½-inch needle. Exudate cells were centrifuged at 2 × 106 cells in 35-mm tissue culture dishes containing RPMI supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were allowed to adhere for 1 h, and then non-adherent cells were washed from the cultures. Complete media was added to the cultures and Act (12 or 20 ng/ml) was added. Cells were incubated for the appropriate times before being lysed for subsequent Western blot analysis.

Western Blot Analysis—The antibody for β-tubulin was purchased from Santa Cruz Biochemical Corp. (Santa Cruz, CA). All other antibodies and Jurkat T cell control extracts were purchased from Cell Signaling Technology, and Western blot analysis was performed by established procedures (30) with slight modifications according to specifications of the antibody manufacturer. Briefly, equal amounts of total protein were loaded and separated on SDS, 10% polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were blocked with 5% milk and washed in 1× Tween (0.1%), Tris-buffered saline three times for 5 min each. Primary antibodies diluted 1:1000 in Tris-buffered saline (preincubated 1 h, Tris-buffered saline) were allowed to incubate overnight at 4 °C. After washing, horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) was diluted 1:2000 in 5% milk and applied to membranes. Subsequently, membranes were washed and a chemiluminescence substrate (Pierce, Rockford, IL) was applied and allowed to incubate at room temperature for 5 min.

Immunoprecipitation/Kinase Assay—Kinase assay kits for p38 and JNK were purchased from Cell Signaling Technology, and the procedure was performed as described by the manufacturer. Whole cell lysates were incubated with immobilized phospho-p38 antibody or c-Jun fusion protein overnight at 4 °C. Following microcentrifugation and washing, the pellet was suspended in kinase buffer supplemented with 200 µM ATP and 2 µg of ATF-2 fusion protein (for p38 kinase activity) or 100 µM ATP (for JNK activity) and incubated for 30 min at 37 °C. The reaction was terminated with SDS sample loading buffer and the samples subjected to Western blot analysis.

Gel Shift Analysis—Gel shift assays were performed as previously described (14, 16). Briefly, consensus oligonucleotide for CCAAT/enhancer-binding protein-β (C/EBP-β) was labeled using T4 polynucleotide kinase (Promega, Madison, WI) according to the manufacturer. New DNA binding reaction mixtures were assembled. We used unlabelled AP-1 consensuse oligonucleotide as a specific competitor, unlabelled AP-2 or SP-1 consensus oligonucleotides as nonspecific competitors (Oncor), and nuclear extracts from specific time points of Act treatment of RAW cells. Nuclear extracts were prepared using NE-PER kit (Pierce) as described by the manufacturer. The reaction mixtures were incubated at room temperature for 10 min, followed by addition of 20,000 cpm of 32P-labeled transcription factor consensus oligonucleotide, and incubation at room temperature for 20 min. Subsequently, 1 µl of gel loading 1× buffer (Promega) was added to each reaction mixture and samples (5 to 20 µg) were loaded on a nondenaturing 4% polyacrylamide gel. The gel was pre-run in 0.5× Tris borate-EDTA buffer for 30 min at 100 V before loading the samples. After completion of the run, the gel was transferred to Whatman 3MM paper, dried at 80 °C for 5 h, and exposed to x-ray film overnight at 48 h.

Light Scanning Confocal Microscopy—RAW 264.7 (T84 and T84 cells (RAW 264.7 and T84) were grown in cover glass chamber slides (Nalge Nunc International, Rochester, NY) and observed on a Zeiss 510 UV meta confocal microscope (Carl Zeiss, Inc., Thornwood, NY). To view membrane blebbing, Act was added (20 ng/ml), and images were taken every 30 s for 6 h. To examine mitochondrial membrane potential, Act-treated RAW 264.7 cells were stained with 10 µM MitoTracker red CM-HXRos dye (Molecular Probes, Eugene, OR) for 30 min at 37 °C, followed by washing with phosphate-buffered saline (PBS). All observations were made using a plan-neofluar ×40/0.85 objective lens with an electronic zoom of 2. The Nomarski differential interference contrast images were stored on an optical disk and analyzed with Zeiss LSM software.

Caspase Activity Assay—Colorimetric activity assay kits were purchased from BioVision Inc. (Mountain View, CA), and caspase activity assays were performed as described by the manufacturer. Briefly, reaction mixtures were assembled as follows: 50–200 µg of protein (whole cell lysates from Act-treated RAW 264.7 cells or from TNFR-1 and -2 cytokine control extracts, double KO macrophage control, or Act-treated microphages containing 10 µM dithiothreitol), and 5 µl of peptide substrate (N-acetyl-Leu-Glu-Asp-p-nitroaniline (LEHD-pNA) for caspase 9 assays, N-acetyl-Ile-Glu-Thr-p-nitroaniline (IETD-pNA) for caspase 8 assays, or N-acetyl-Asp-Glu-Val-Asp-4-p-nitroaniline (DEVD-pNA) for
caspase 3 assays). Reactions were incubated at 37 °C for 1–2 h and then read at 405 nm in an enzyme-linked immunosorbent assay reader. Whole cell lysates from camptothecin-treated Jurkat T cells (BioVision Inc.) were used as positive controls.

Flow Cytometry—For detection of apoptosis, variously stimulated HT-29 cells (1 × 10⁶) were stained with annexin V-conjugated to fluorescein isothiocyanate (FITC) and propidium iodide (PI) by using the annexin-V-FITC staining kit (BioVision Inc.) according to the manufacturer’s instruction. Briefly, cells were suspended in 500 μl of annexin V binding buffer and incubated with 5 μl of annexin V and 5 μl of PI for 5 min at room temperature in the dark. At least 20,000 cells were acquired in a FACSCalibur flow cytometer using CellQuest 3.0.1 software (BD Biosciences). Percentages of cells undergoing apoptosis were determined by dual-color analysis. This staining allowed us to distinguish 3 subsets of cells: viable cells (annexin V negative and PI negative), early apoptotic cells (annexin V negative and PI positive), and late apoptotic or necrotic cells (annexin V positive and PI positive). Immediately after staining, the cells were analyzed on a flow cytometer using 488-nm excitation and a 525-nm band pass filter for FITC and a 620-nm filter for PI detection.

For detection of mitochondrial membrane potential, variously stimulated RAW 264.7 cells (1 × 10⁶) were stained for 10 min at 37 °C with 10 μg/ml of the mitochondrial membrane potential sensor dye, 5,5′,6′,6′-tetrafluoro-1,1′,3′,3′-tetrachlorobenzimidazolocarbocyanine iodide (JC-1) (Molecular Probes). JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from 530 to 585 nm. At normal mitochondrial potential, fluoroscent JC-1 aggregates predominate (585 nm). However, as mitochondrial potential drops, the aggregates separate, and the fluorescent monomeric form (530 nm) predominates. JC-1-stained cells were analyzed for relative 585 (FL2) to 530 nm (FL1) fluorescence, as described above.

Apoptotic Protein Screens—Whole lysate protein samples (350 μg) from Act-treated (20 ng/ml) RAW 264.7 cells were analyzed by Kineworks™ KAPS-1.0 Apoptosis Screens (Kinexus Bioinformatics Corp., Vancouver, British Columbia, Canada) as previously described (view the Kinexus website for details and publication links). The Kineworks™ analysis involved resolution of a single lysate sample by SDS-PAGE and subsequent immunoblotting with panels of up to 3 primary antibodies per channel in a 20-lane Immunetics Multiblotter. The antibody mixtures were carefully selected to avoid overlapping cross-reactivity with target proteins. Normalized trace quantity units (CPM) were arbitrary based on the intensity of ECL fluorescence detection for target immunoreactive proteins recorded with a Fluor-S Multimager and quantified using Quantity One Software (Bio-Rad).

Isolation of Cytosolic and Mitochondrial Lysates—RAW 264.7 cells, treated with 20 ng/ml Act for 0, 2, 4, and 6 h, were washed with PBS and centrifuged at 1,000 × g for 5 min. Cell pellets were washed twice with PBS and suspended in ice-cold buffer containing 20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 μg/ml aprotinin, 10 μg/ml pepstatin A, and 250 mM sucrose. Cells were subsequently incubated on ice for 15 min and then homogenized by 15 passages though a 22-gauge needle. Homogenates were centrifuged at 1,000 × g for 5 min at 4 °C, and the resulting supernatants were centrifuged twice at 12,000 × g for 15 min at 4 °C. Supernatants (cytosolic fractions) were subsequently subjected to Western blot analysis. All of the experiments were performed at least in triplicate and representative data are presented.

RESULTS

Act Induces Phosphorylation/Activation of p38 Kinase in RAW 264.7 Cells, Murine Peritoneal Macrophages, and Human Intestinal Epithelial (T84) Cells—RAW 264.7 cells, thioglycolate-induced murine peritoneal macrophages, or T84 cells were treated with 6, 12, or 20 ng/ml of Act for various time points and whole cell lysates were subjected to electrophoresis and Western blot analysis. LPS-treated RAW 264.7 cells (100 ng/ml for 30 min) were used as a positive control in these experiments and other MAPK Western blot analyses. LPS has been shown to induce ERK, p38, and JNK pathways in RAW 264.7 cells (32). Subsequent blots were probed with antibodies specific for the phosphorylated form of p38 (p-p38) or total p38α. As shown in Fig. 1A, Act (6 ng/ml) induced an increase in a 43-kDa sized band, consistent with the phosphorylated form of p38, by 1 h. This increase was observed up to 8 h but disappeared by 12 h. Western blot analysis on samples from RAW 264.7 cells treated...
with a higher dose (12 ng/ml) of Act (for up to 8 h) resulted in similar results, with the earliest induction of p38 phosphorylation observed at 30 min (data not shown). Increased phosphorylation of p38 observed upon Act treatment was not because of an increase in the protein concentration of total p38, as evidenced by similarly sized bands for each time point when blots were probed with an antibody that recognized both the phosphorylated and non-phosphorylated forms of p38/H9251 (Fig. 1B). Act-treated murine peritoneal macrophages exhibited a similar pattern of p38 phosphorylation for both concentrations of Act that were used (12 and 20 ng/ml): Act induced phosphorylation of p38 by 1 h (Fig. 1C), but no increase was observed for total p38/H9251 (Fig. 1D). Likewise, p38 phosphorylation was observed for Act-treated (12 ng/ml) T84 cells (Fig. 1E), with no increase in total protein concentration (Fig. 1F). JNK phosphorylation in T84 cells occurred in the absence of total protein up-regulation (Fig. 1F).

To demonstrate that increased phosphorylation of p38 in Act-treated cells was biologically significant, we examined p38 kinase activity in Act-treated RAW cells. Whole cell lysates from Act-treated (12 ng/ml) RAW 264.7 cells, murine peritoneal macrophages, or T84 cells were subjected to electrophoresis and Western blot analysis using antibodies specific for either the phosphorylated form of JNK or total JNK protein. As shown in Fig. 2A, two bands consistent with phosphorylated JNK were apparent by 30 min (earliest time point tested), which increased ~3.5-fold by 1 h. All immunoprecipitated samples contained the same amount of protein, and equal concentrations of ATP and ATF-2 substrate were used. Western blot analysis using an antibody specific for total ATF-2 demonstrated bands of similar size and intensity for each time point and served as a positive control (data not shown).

**Fig. 2.** Act-induced phosphorylation of JNK in RAW 264.7 cells, murine peritoneal macrophages, and T84 cells, and increased activity of JNK kinase in Act-treated RAW 264.7 cells. Cells were stimulated with Act for the indicated times, lysed, and Western blot analysis was performed with anti-phospho-JNK (p-JNK) antibody. Blots were probed with anti-JNK (total JNK) antibody as a control for protein loading. Panels A and B, Western blot analysis of Act-treated (12 ng/ml) RAW 264.7 cells. Panels C and D, Western blot analysis of Act-treated (12 ng/ml) murine peritoneal macrophages. Panels E and F, Western blot analysis of Act-treated (12 ng/ml) T84 cells. Panel G, lysates from Act-treated (12 ng/ml) RAW 264.7 cells were immunoprecipitated with immobilized c-Jun fusion protein, kinase assays were performed in the presence of ATP, and Western blot analysis was performed with anti-phospho-c-Jun antibody.
precipitation/kinase assays on whole cell lysates from Act-treated (12 ng/ml) RAW 264.7 cells. Phosphorylated JNK was immunoprecipitated with substrate (c-Jun)-conjugated beads, kinase assays were performed, and the resulting reactions were subjected to electrophoresis and Western blot analysis using an antibody specific for phosphorylated c-Jun. As shown in Fig. 2G, JNK was indeed activated, because phosphorylated c-Jun was detected by 30 min with a 3-fold increase by 2 h (last time point tested). As controls, equal concentrations of whole cell lysates were used, and Western blot analysis was performed using an antibody that recognized both the phosphorylated and non-phosphorylated forms of c-Jun (data not shown).

**Act Induces Phosphorylation of MEK3/6 and MEK4 (but Not MEK7) in RAW 264.7 Cells and Murine Peritoneal Macrophages**—Because MEK3/6 and MEK7 are known to phosphorylate p38 and JNK, respectively, and MEK4 has been shown to activate both the p38 and JNK pathways simultaneously, we examined the phosphorylation status of all three upstream kinases in Act-treated RAW 264.7 cells and murine peritoneal macrophages. Whole cell lysates from Act-treated (12 ng/ml) RAW 264.7 cells or murine peritoneal macrophages were subjected to electrophoresis and Western blot analysis using antibodies specific for phosphorylated or total (phosphorylated and non-phosphorylated) MEK3/6 (Fig. 3). As shown in Fig. 3A, Act induced phosphorylation of MKK3/6 by 1 h in RAW 264.7 cells, which is consistent with the hypothesis that MKK3/6 is responsible for p38 phosphorylation at 1 h in Act-treated macrophages (Fig. 1A). All time points contained similar amounts of total MKK3/6 (Fig. 3B). Likewise, total MKK3/6 concentrations were similar for the untreated control and Act-treated (20 ng/ml) murine peritoneal macrophage samples (Fig. 3D), whereas phosphorylated MKK3/6 was up-regulated by 1 h (Fig. 3C).

Western blot analysis of whole cell lysates from Act-treated (12 ng/ml) RAW 264.7 cells also demonstrated MKK4 phosphorylation by 1 h (Fig. 3E) with no change in total protein concentration (Fig. 3F). Similar results were obtained for Act-treated (20 ng/ml) murine peritoneal macrophages (Fig. 3, G and H). However, Western blot analysis of Act-treated RAW 264.7 cells (12 ng/ml) did not demonstrate any increase in phosphorylated MKK7 (data not shown). Neither was MKK7 phosphorylated in Act-treated (12 or 20 ng/ml) murine peritoneal macrophages (data not shown). On the contrary, LPS at the tested dose (100 ng/ml) and time point (30 min) increased MKK7 levels as expected (data not shown).

**Act Induces Phosphorylation of ATF-2, c-Jun, and p90RSK in RAW 264.7 Cells and Murine Peritoneal Macrophages**—Because Act induced activation of p38 and JNK kinases and their upstream effectors, MKK3/6 and MKK4, we next evaluated the phosphorylation status of downstream factors known to be involved in MAPK signaling (Figs. 4 and 5). ATF-2, which could be phosphorylated by p38 or JNK, was indeed phosphorylated in Act-treated (12 ng/ml) RAW 264.7 cells by 30 min (Fig. 4A) and in murine peritoneal macrophages by 2 h (Fig. 4C). The observed increase in phosphorylation of ATF-2 was not because of an increase in overall protein concentration, as shown in Fig. 4, B and D. We also examined the phosphorylation status of MAPKAPk-2, which is known to be phosphorylated by p38. As shown in Fig. 4E, Act treatment (12 ng/ml) of RAW 264.7 cells did not result in phosphorylation of MAPKAPk-2. Nor was an increase in protein production observed in response to toxin
treatment (Fig. 4F). However, MAPKAPk-2 was phosphorylated in Act-treated (20 ng/ml) murine peritoneal macrophages (Fig. 4G), which was not a result of increased protein production (Fig. 4H). An extra band of 50 kDa in size was observed for all samples used (Fig. 4, E–H), which most likely represented hyperphosphorylation of MAPKAPk-2.

Because we demonstrated Act-induced activation of JNK in this study and of the downstream transcription factor AP-1 in RAW 264.7 cells previously (16), we next investigated whether or not c-Jun, the prototypical subunit of AP-1, was phosphorylated in Act-treated macrophages. As shown by Western blot analysis in Fig. 5A, Act treatment (12 ng/ml) of RAW 264.7 cells did lead to the phosphorylation of c-Jun, which was apparent by 2 h. Because protein levels of c-Jun, as well as other AP1 subunits, are known to be altered by activation of all three major MAPK pathways, we used β-tubulin as the internal control for protein loading (Fig. 5B). Phosphorylation of c-Jun was also observed for Act-treated (12 ng/ml) murine peritoneal macrophages as early as 30 min (Fig. 5C) with equal loading of protein (Fig. 5D), suggesting that AP-1 activation occurs in these cells and perhaps earlier than for RAW 264.7 cells.

Because we previously demonstrated Act-induced activation of the transcription factors cyclic AMP-response element-binding protein and nuclear factor-κB (NF-κB) in RAW 264.7 cells (14, 15), and p90RSK has been shown to activate cyclic AMP-response element-binding protein and NF-κB (33–35), we investigated phosphorylation of p90RSK in Act-treated RAW 264.7 cells and murine peritoneal macrophages as early as 30 min (Fig. 5C) with equal loading of protein (Fig. 5D), suggesting that AP-1 activation occurs in these cells and perhaps earlier than for RAW 264.7 cells.

Act Induces Phosphorylation of ERK1/2 in RAW 264.7 Cells, Murine Peritoneal Macrophages, and Human Intestinal Epithelial (T84) Cells—Because p90RSK is activated by ERK kinase family members, we investigated the phosphorylation status of ERK1/2, the upstream ERK1/2 activator MEK-1, and ERK5 in toxin (12 ng/ml)-treated RAW 264.7 cells and murine peritoneal macrophages treated with 20 ng/ml Act. As shown by Western blot analysis, ERK1/2 phosphorylation was increased in response to Act, with up-regulation occurring as early as 15 min and more strongly by 30 min (Fig. 6A). MEK1/2 was also phosphorylated in response to Act in RAW 264.7 cells (Fig. 6B), although induction was detected later (1 h).

Act also caused phosphorylation of both ERK1/2 and MEK1/2 in murine peritoneal macrophages (Fig. 6, C and D, respectively), which was detected by 1 h (earliest time point tested). Total protein levels of ERK1/2 and MEK1 were unchanged in response to Act treatment in both RAW 264.7 cells and murine peritoneal macrophages (data not shown). Additionally, we investigated the phosphorylation status of ERK1/2 in Act-treated T84 cells (12 ng/ml), and as shown in Fig. 6E, an increase in ERK1/2 phosphorylation was observed by 2 h. No increase in
total ERK1/2 concentration (phosphorylated and non-phosphorylated) resulted from Act treatment (data not shown). The two bands observed for ERK1/2 represented the two isoforms: ERK1 (p44) and ERK2 (p42).

**Act Causes Activation of the Transcription Factor C/EBP-β in RAW 264.7 Cells**—Our laboratory previously demonstrated Act-induced up-regulation of the macrophage-specific transcription factor C/EBP-β in RAW 264.7 cells using microarrays and real-time reverse transcriptase-PCR (16). Additionally, Act activated ERK1/2 in both RAW 264.7 cells (Fig. 6A) and murine peritoneal macrophages (Fig. 6C), which have been shown to activate C/EBP-β (36, 37). Act has also been shown to induce the production of IL-1β, IL-6, macrophage inflammatory protein-1α, TNF-α, and inducible nitric-oxide synthase (14–16), all of which are transcription targets of C/EBP-β (38). We therefore performed gel shift assays to detect C/EBP-β activation in RAW 264.7 cells. Fig. 7 clearly demonstrated that Act (6 ng/ml) caused nuclear translocation of a protein capable of binding a
Act activated the C/EBP-β transcription factor in murine macrophages, as determined by gel shift assay. Nuclear extracts from RAW 264.7 cells stimulated with 100 ng/ml LPS for 2 h (lane 1) or Act (6 ng/ml) for various times (0 and 30 min and 2, 4, 6, and 8 h) were mixed with consensus oligonucleotides for C/EBP-β and subjected to nondenaturing 4% polyacrylamide gel electrophoresis (lanes 3–9). Binding reactions were also performed in the absence of nuclear extract and served as a negative control (lane 2). Nuclear extract from RAW cells treated for 4 h with Act was mixed with unlabeled C/EBP-β consensus oligonucleotide (~50-fold excess) before adding the labeled oligonucleotide (lane 9). The gel was dried and subjected to autoradiography.

Fig. 7. Demonstration of C/EBP-β activation in RAW 264.7 cells by gel shift analysis. Act activated the C/EBP-β transcription factor in murine macrophages, as determined by gel shift assay. Nuclear extracts from RAW 264.7 cells stimulated with 100 ng/ml LPS for 2 h (lane 1) or Act (6 ng/ml) for various times (0 and 30 min and 2, 4, 6, and 8 h) were mixed with consensus oligonucleotides for C/EBP-β and subjected to nondenaturing 4% polyacrylamide gel electrophoresis (lanes 3–9). Binding reactions were also performed in the absence of nuclear extract and served as a negative control (lane 2). Nuclear extract from RAW cells treated for 4 h with Act was mixed with unlabeled C/EBP-β consensus oligonucleotide (~50-fold excess) before adding the labeled oligonucleotide (lane 9). The gel was dried and subjected to autoradiography.

As a positive control for C/EBP-β activation, nuclear extracts from LPS-treated macrophages (2 h) were loaded in lane 9/H252 and 8 h) were mixed with consensus oligonucleotides for C/EBP-β (specific) and SP-1 or AP-2 (nonspecific) consensus oligonucleotides. As shown in Fig. 7, addition of the specific competitor abrogated binding (lane 9), whereas the nonspecific competitor did not (not shown), indicating that the band observed was indeed specific for C/EBP-β.

Act Induces Apoptotic Blebbing of Murine Macrophages and Human Intestinal Epithelial (T84) Cells—In addition to the induction of inflammatory cascades, MAPKs have also been shown to mediate apoptosis (39, 40). Recently, our laboratory demonstrated for the first time Act-induced apoptosis of RAW 264.7 cells (16). As further evidence that apoptosis is the mechanism of cell death, we visualized Act-treated RAW 264.7 cells by confocal microscopy. Live cells were grown on microscopy slides, treated with 20 ng/ml Act, and photographed every 15 s. As shown in Fig. 8A, cells were healthy before toxin treatment, and blebbing occurred by 2.5 h (Fig. 8B) and continued past 4 h (Fig. 8C). We also examined cellular morphology in Act-treated (20 ng/ml) RAW 264.7 cells by electron microscopy. As shown in Fig. 8D, Act treatment resulted in classical apoptosis morphology that was apparent by 3 h and even more pronounced by 6 h (data not shown). For instance, Act induced condensation of chromatin starting from the periphery of the nuclei, consistent with apoptosis-associated cleavage of DNA, caused cells to shrink and to become denser, and induced blebbing of fragments of the cytoplasm (Fig. 8D). Untreated cells were used as a negative control and were healthy (data not shown).

Because Act caused extensive intestinal damage in vivo, we also examined cell morphology of Act-treated T84 cells by confocal microscopy. Blebbing of peripheral cells began at ~2 h post-treatment (25 ng/ml of Act) and was clearly apparent by 5 h (Fig. 8F), suggesting that Act caused apoptosis of T84 cells. Fig. 8E depicts the morphology of untreated T84 cells.

To determine whether Act did indeed cause apoptosis of human intestinal epithelial cells, HT-29 cells were cultured with Act (25 ng/ml) or cycloheximide (100 μg/ml) for 6 h and assayed for annexin V-FITC and PI staining by flow cytometry. As shown in Fig. 8, less than 5% of untreated HT-29 cells were apoptotic (panel G, lower right quadrant). However, HT-29 cells treated with 25 ng/ml Act for 6 h exhibited marked apoptosis (34.0%, quadrant 4, Fig. 8F), which was greater than that observed for cycloheximide-treated positive control cells (23.5%, quadrant 4, Fig. 8F). Considered with membrane blebbing of T84 cells in response to Act (Fig. 8F), the data demonstrated, for the first time, Act-induced apoptosis of intestinal epithelial cells, using two different cell lines.

Act Induces Activation of Caspases in Marine Macrophages and Human Intestinal Epithelial (T84) Cells—To determine whether Act-induced apoptosis of macrophages and intestinal epithelial cells was caspase-dependent, we performed Western blot analysis on whole cell lysates from Act-treated RAW 264.7 cells (20 ng/ml), murine peritoneal macrophages (12 ng/ml), and T84 cells (20 ng/ml). As shown in Fig. 9A, a faint band representing the cleaved/activated form of caspase 3 was apparent by 2 h, with a much stronger band appearing by 4 h and continuing to at least 6 h. Likewise, the cleaved product of caspase 3 was induced by Act treatment of murine peritoneal macrophages (Fig. 9B), although activation occurred later, beginning at 4 h and stronger by 8 h. Act treatment of T84 cells also resulted in cleavage of caspase 3, as evidenced by the lower-molecular weight band clearly visible by 4 h (Fig. 9C).

Extracts from untreated Jurkat T cells were used as negative controls, and extracts from cytochrome c–treated Jurkat T cells were used as positive controls (Fig. 9). Blots were probed with anti-β-tubulin antibody to control for protein loading (Fig. 9D). Taken together, the data suggested that Act did indeed induce caspase-dependent apoptosis of macrophages and intestinal epithelial cells.

To determine which major apoptotic pathway (intrinsic/mitochondrial or extrinsic/death receptor) might be activated by Act, we performed activity assays for caspase 9 (intrinsic-associated) and caspase 8 (extrinsic-associated) in whole cell lysates (80 μg) from Act-treated (20 ng/ml) RAW 264.7 cells (0, 2, 4, and 6 h). As shown, Act induced caspase 9 activation in RAW 264.7 cells by 2 h, which increased with time (Fig. 9E). This suggested that Act might activate the mitochondrial-associated pathway of cell death via release of cytochrome c. To determine whether the death receptor pathway might also contribute to Act-induced apoptosis, we performed activity assays for caspase 8 in Act-treated RAW 264.7 cells. Surprisingly, Act also induced caspase 8 activation by 2 h (Fig. 9E). Whole cell lysates (100 μg) from camptothecin–treated Jurkat T cells were used as a positive control.

Absence of the TNF Receptor Reduces Act-induced Apoptosis and Caspase Activities of Murine Macrophages—Because binding of TNF to its receptor (TNFR) could lead to cleavage of caspase 8, we utilized single and double knockout cells (bone marrow–derived murine macrophages), in which one or both of the two isoforms of the TNF receptor (TNFR-1 and TNFR-2) were deleted (29), to examine Act-induced apoptosis. WT or double KO cells were treated with 20 ng/ml Act for 0, 2, or 4 h, stained with annexin V-FITC and PI, and examined by flow cytometry. As depicted graphically in Fig. 10A, Act caused apoptosis of WT macrophages by 2 h (3.6%), which increased by 4 h (16.1%), compared with untreated controls. However, Act-treated KO macrophages exhibited no increase in apoptosis by 2 h and only a marginal increase (3.4%) by 4 h (Fig. 10A). These results strongly suggested that Act-induced apoptosis of host cells involved engagement of TNFR-1 and/or TNFR-2 and subsequent activation of caspase 8.

To determine whether TNF-α was involved in Act-induced activation of caspases, activity assays for caspases 3, 8, and 9...
were performed on whole cell lysates (200 μg) from Act-treated (20 ng/ml) WT and TNFR double KO macrophages (0, 2.5, and 4 h). As shown in Fig. 10B, Act induced activation of all three caspases in WT macrophages by 2.5 h, and activity was greatly increased by 4 h. However, activation of all 3 caspases was significantly diminished in Act-treated TNFR double KO macrophages (Fig. 10B).

To determine which TNF receptor contributed to caspase
activation, caspase activity assays were performed on whole cell lysates from Act-treated WT, double KO, and single KO (TNFR-1 or TNFR-2) macrophages (0 and 6 h). As shown in Fig. 10C, Act induced activation of all three caspases in WT macrophages at 6 h. Activation of all three caspases in TNFR-2 single KO macrophages was similar to that of WT cells (Fig. 10C), indicating that TNFR-2 was not required for Act-mediated caspase activation. However, caspase activation was nearly absent in TNFR-1 single KO and double KO macrophages. Considered together, the results suggested that TNF-α binding to TNFR-1 contributed significantly to Act-mediated apoptosis and that apoptosis was mediated via activation of caspases 3, 8, and 9.

Act Up-regulates Apoptosis-associated Proteins in RAW 264.7 Cells—To characterize the host cell apoptotic response to Act, we undertook an un-biased proteomics based approach to discover which proteins might mediate Act-induced apoptosis. RAW 264.7 cells were chosen for this study, because Act-induced apoptosis was originally discovered using these cells (16) and because the large volumes of lysates that were needed (350 μl (1 mg/ml) per treatment) could be obtained from this transformed cell line with little difficulty. RAW 264.7 cells were treated with PBS or Act (20 ng/ml) for 6 h and whole cell lysates were applied to Kinetworks™ Apoptosis Screens to analyze the expression profiles of 25 apoptosis-associated proteins. We compared normalized CPM, arbitrarily based on the fluorescence intensity values, and considered a 25% increase or decrease in expression to be significant. Of the 25 proteins screened (data not shown), five were significantly up-regulated and one down-regulated by Act at 6 h (Figs. 11, A–C). Immunoblots including these six proteins are shown in Fig. 11, A (0 h) and B (6 h), for comparison, and the data are graphically represented along with CPM values and percentage increases/decreases in Fig. 11C.

As shown in Fig. 11C, two mitochondrial proteins were apparently up-regulated by Act: cytochrome c (65%) and AIF (82%). The nuclear transport cellular apoptosis susceptibility (CAS) factor was up-regulated 284% by Act at 6 h (Fig. 11C). DNA damage-inducible poly(ADP-ribose) polymerase (PARP) and the p53-inducible apoptosis effector related to PMP-22 (PERP) were also up-regulated by Act (61 and 35%, respectively). Only one protein, namely stress-associated superox-
ide dismutase (Cu/Zn), was down-regulated by Act (26%, Fig. 11C).

Act Causes Mitochondrial Release of Cytochrome c and AIF in RAW 264.7 Cells—To determine whether Act-induced increases in cytochrome c and AIF were the result of release from mitochondria, we separated mitochondrial and cytosolic fractions from Act-treated (20 ng/ml) RAW 264.7 cells and performed Western blot analysis. Blots were probed with anti-cytochrome c or anti-AIF antibodies. As a positive control, we used whole cell lysates from cytochrome c-treated Jurkat T cells. As shown in Fig. 11D, Act increased cytosolic levels of cytochrome c and AIF by 2 h, suggesting that Act-induced cell death involved the mitochondrial pathway of apoptosis. Blots were probed with anti-p90RSK to control for equal loading of protein (Fig. 11D).

Mitochondrial Membrane Depolarization Does Not Precede Act-mediated Cell Death in RAW 264.7 Cells—To determine whether Act-induced apoptosis of RAW 264.7 cells involved depolarization of mitochondria, we stained Act-treated cells with the membrane potential sensor dye JC-1 and acquired cells by flow cytometry. As shown in Fig. 12, A and B, there was no shift from JC-1 aggregates (indicative of healthy, respiring cells) to JC-1 monomers (representative of cells with depolarized mitochondrial membranes) in RAW 264.7 cells treated with 20 ng/ml Act (upper right quadrant). As a positive control, cells were treated with the mitochondrial respiratory chain decoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP) (250 μM for 30 min), which did result in a shift toward JC-1 monomers (Fig. 12C, upper right quadrant).

We also examined size and morphology of cell populations, based on forward and size scattering and compared that with JC-1 staining. Based on forward and side scattering measurements, Act treatment resulted in 2 distinct populations of cells (data not shown): cells of normal, healthy size and morphology, which we colored blue, and cells that were shrunken, colored in orange. We then gated the cells, based on these two cell populations (blue and orange), and analyzed the cells for JC-1 staining, so that we could compare cell size/morphology with mitochondrial membrane potential.
FIG. 11. KinetWorks™ KAPS 1.0 apoptotic protein analysis of whole cell lysates from Act-treated RAW 264.7 cells, and presence of cytochrome c and AIF in the cytosol of Act-treated RAW 264.7 cells as determined by Western blot analysis. Cells were stimulated with 20 ng/ml Act for 6 h, lysed, and KAPS 1.0 KinetWorks™ protein screens were performed. Immunoblots from untreated control cells (A) and RAW 264.7 cells treated with Act for 6 h (B) are shown. Bands corresponding to Act-induced up-regulated or down-regulated proteins are labeled and indicated by arrows for comparison. C, graphical representation of Act-induced alteration of the apoptosis-associated proteins shown in A and B. Act-induced alterations in protein levels at 6 h compared with 0 h are shown as percentages above the bars. Normalized CPM values (arbitrarily based on ECL florescence intensity values) are shown in table format below the graph. D, RAW 264.7 cells were stimulated with 20 ng/ml Act for the indicated times, cytosolic fractions were isolated, and Western blot analysis was performed with anti-cytochrome c or anti-AIF antibody. Blots were probed with anti-p90RSK antibody as a control for protein loading. Whole cell lysates from cytochrome c-treated Jurkat T cells were used as a positive control (PC). As shown, both cytochrome c and AIF were present in higher amounts in cytosolic fractions by 2 h.
(Fig. 12). Whereas Act treatment did not result in mitochondrial membrane depolarization, there was a substantial decrease in cell size (blue → orange), indicative of cell death (Fig. 12, A and B).

To confirm the results obtained using JC-1, we viewed Act-treated (20 ng/ml) RAW 264.7 cells by confocal microscopy after staining with MitoTracker red CM-H$_2$XRos. MitoTracker red does not fluoresce until it enters an actively respiring cell, where it is subsequently oxidized and then sequestered in the mitochondria. As shown in Fig. 12E, Act treatment did not result in depolarization of mitochondrial membranes, which was comparable with the untreated control (Fig. 12D). As expected, cells treated with the mitochondrial decoupler CCCP did experience loss in mitochondrial membrane potential, re-
sulting in very low levels of red fluorescence (Fig. 12F). Actively respiring mitochondria, based on strong red fluorescence, were apparent even at 6 h in Act-treated cells that were clearly undergoing cell death, as evidenced by cell shrinkage and membrane blebbing (Fig. 12H) compared with PBS-treated (Fig. 12G) or CCCP-treated RAW 264.7 cells (Fig. 12F), which appeared normal. Considered with JC-1 staining, the data clearly demonstrated that mitochondrial membrane depolarization did not precede Act-induced cell death.

**DISCUSSION**

MAPK cascades have been shown to be intimately involved in cell proliferation, differentiation, apoptosis, and inflammation, and are pivotal during immune responses (41). Furthermore, the importance of MAPK signaling cascades in a variety of human diseases has led to the development of anti-inflammatory drugs that target various MAPK enzymes (42–53). We therefore investigated the effect of Act on MAPKs, in which we demonstrated for the first time Act-induced activation of the p38 (Fig. 1), JNK (Fig. 2), and ERK1/2 (Fig. 6) signaling pathways in a murine macrophage cell line (RAW 264.7), murine peritoneal macrophages, and a human intestinal epithelial cell line (T84).

Because phosphorylation of p38 and JNK is mediated by MKK3/6 and MKK7, respectively, and MKK4 phosphorylates both p38 and JNK, we evaluated activation of these kinases in both RAW 264.7 cells and murine peritoneal macrophages. Whereas Act induced phosphorylation of MKK3/6 and MKK4 (Fig. 3), it did not phosphorylate MKK7 in these cells (data not shown). We therefore believe that Act-induced JNK activation occurs via MKK4. However, activation of p38 might result from phosphorylation by either MKK3/6 or MKK4. It is likely that p38 and JNK act coordinate to induce inflammatory responses observed in Act-treated host cells.

To further dissect Act-induced activation of p38 and JNK signaling cascades, we investigated factors known to lie downstream of both kinases: ATF-2, MAPKAPk-2, and c-Jun. ATF-2, which can be phosphorylated/activated by either p38 or JNK, is a transcription factor that trans-activates genes containing cAMP response-like elements in their promoters, such as inducible nitric-oxide synthase (54) and TNF-α (55, 56). Act treatment did cause phosphorylation of ATF-2 in RAW 264.7 cells and murine peritoneal macrophages (Fig. 4, A and C), which is consistent with the activation of p38 and JNK signaling cascades. Likewise, c-Jun was phosphorylated in response to Act in both RAW 264.7 cells and murine peritoneal macrophages (Fig. 5, A and C), which strongly suggested that Act-induced AP-1 activation resulted from activation of the JNK pathway.

The potential contribution of other MAPKs, such as p38, to Act-induced AP-1 activation requires further study. Phosphorylation of MAPKAPk-2, which can regulate cytochrome translation via phosphorylation of AU-rich elements in their mRNAs (57), was observed for murine peritoneal macrophages (Fig. 4G) but was not obvious in RAW 264.7 cells (Fig. 4E). MAPKAPk-2 deficiency severely reduces the biosynthesis of TNF-α in response to LPS (58), but because Act induces TNF-α production in RAW 264.7 cells (14), the function of MAPKAPk-2 in response to Act might be redundant.

p90RSK has been shown to activate the transcription factors cyclic AMP-response element-binding protein and NF-κB (33–35), which are both activated by Act in murine RAW 264.7 macrophages (14, 15). We now demonstrated phosphorylation of p90RSK in Act-treated RAW 264.7 cells and murine peritoneal macrophages (Fig. 5, E and G). It is possible that Act-induced phosphorylation of p90RSK in murine macrophages was mediated by ERK1/2, a known activator of p90RSK, because Act also induced phosphorylation of ERK1/2 (Fig. 6, A and C) and the ERK1 activator, MEK1 (Fig. 6, B and D). It is unclear why phosphorylation of MEK1, which is known to phosphorylate ERK1, was detected later than phosphorylation of ERK1/2. It is possible that the sensitivities of the antibodies differed. Alternatively, because signaling cascades are often amplified as they progress downstream, it is plausible that the levels of activated MEK1 were too low to be detected as early as 15 min. Considered together, the data clearly demonstrated Act-induced activation of all three major MAPK pathways (ERK1/2, p38, and JNK) in murine macrophages and human intestinal epithelial cells, which likely contributes to inflammatory host cell responses to the toxin.

MAPKs are responsible for regulating a variety of cellular processes, including apoptosis. It is therefore possible that activation of MAPKs plays a role in Act-induced host cell death. As shown in Fig. 8, Act induced classic hallmarks of apoptosis in both macrophages and intestinal epithelial cells. An interesting observation was that Act-induced blebbing and cell death of T84 cells occurred later than for RAW 264.7 cells and proceeded from the periphery of the epithelial clump toward the center. We confirmed apoptosis as the mechanism of Act-induced intestinal epithelial cell death by annexin V-FITC and PI staining of Act-treated HT-29 cells (Fig. 8, G–I). By utilizing a different epithelial cell line (HT-29 cells in lieu of T84 cells) for this study, we also demonstrated that Act-induced cell death of epithelial cells was not peculiar to the T84 cell line. Whereas Act is known to cause severe intestinal damage in vivo, this study demonstrated for the first time that a potential cause for intestinal damage induced by *A. hydrophila* infection is likely Act-mediated apoptosis.

Act induced caspase 3-cleavage in RAW 264.7 cells by 2 h (Fig. 9A). This correlated with previous findings (that Act caused presentation of phosphatidylserine to the outer membrane layer between 2 and 4 h, DNA laddering by 4 h (16), and membrane blebbing by 2.5 h (Fig. 8B), and strongly suggested that Act-induced apoptosis was mediated, at least in part, by caspase 3-cleavage. Act also induced caspase 3-cleavage in murine primary macrophages by 4 h (Fig. 9B). The later induction was because of the fact that we used a lower dose of the toxin (12 ng/ml for murine primary macrophages compared with 20 ng/ml for RAW 264.7 cells). Indeed, Western blot analysis of murine peritoneal macrophages treated with 20 ng/ml Act demonstrated caspase 3-cleavage, which occurred between 1 and 3 h (data not shown). Finally, examination of Act-treated (20 ng/ml) T84 cells also revealed caspase 3-cleavage by 4 h (Fig. 9C), which was consistent with Act-induced membrane blebbing by 5 h (Fig. 8F).

We also demonstrated caspase 9 activation in Act-treated RAW 264.7 cells (Fig. 9E), which was expected, because Act has been shown to cause production of ROS and activation of DNA repair machinery (15). Caspase 8, which is activated in response to death receptor engagement, was also activated by Act in RAW 264.7 cells (Fig. 9E). It is therefore likely that Act induces apoptosis via activation of both the death receptor-mediated and mitochondrial pathways. TNFR-1 (29) is a strong candidate as the death receptor responsible for caspase 8 activation in response to toxin treatment, because Act-induced apoptosis was inhibited in TNFR-1 double KO macrophages (Fig. 10A). Additionally, Act-induced activation of caspases 3 and 9 were also greatly reduced in TNFR-1 and TNFR double KO cells compared with WT macrophages (Fig. 10, B and C). Caspase activation was not abrogated in TNFR-2 KO cells, which was expected because TNFR-1 contains death domains, whereas TNFR-2 does not (59), and binding of TNF-α to TNFR-2 has been shown to be insufficient for mediating cell death (60, 61). The results strongly suggested that TNF-α was...
largely responsible for Act-induced apoptosis of host cells, which was mediated via engagement of TNFR-1. It is also possible that Act-induced activation of MAPKs involves TNF-α. Both TNFR-1 and -2 are required for TNF-α-induced activation of p38, JNK, and ERK (29). We are currently investigating whether or not Act-induced activation of these kinases is mediated by TNF-α using these TNFR KO macrophages.

Based on a proteomics approach, five apoptotic proteins (cytochrome c, AIF, CAS, PARP, and PERP) were up-regulated and -2 are required for TNF-α-induced activation of p38, JNK, and ERK (29). We are currently investigating whether or not Act-induced activation of these kinases is mediated by TNF-α using these TNFR KO macrophages.

Intracellular Signaling by Act in Macrophages and Epithelial Cells

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