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Ricin A-chain requires c-Jun N-terminal kinase to induce apoptosis in nontransformed epithelial cells

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Abstract

Ricin is a toxin isolated from castor beans that has potential as a weapon of bioterrorism. This glycoprotein consists of an A-chain (RTA) that damages the ribosome and inhibits protein synthesis and a B-chain that plays a role in cellular uptake. Ricin activates the c-Jun N-terminal kinase (JNK) and p38 signaling pathways; however, a role for these pathways in ricin-induced cell death has not been investigated. Our goals were to determine if RTA alone could activate apoptosis and if the JNK and p38 pathways were required for this response. Comparable caspase activation was observed with both ricin and RTA treatment in the immortalized, nontransformed epithelial cell line, MAC-T. Ribosome depurination and inhibition of protein synthesis were induced in 2 to 4 h with 1 µg/ml RTA and within 4 to 6 h with 0.1 µg/ml RTA. Apoptosis was not observed until 4 h of treatment with either RTA concentration. RTA activated JNK and p38 in a time- and concentration-dependent manner that preceded increases in apoptosis. Inhibition of the JNK pathway reduced RTA-induced caspase activation and poly (ADP-ribose) polymerase cleavage. In contrast, inhibition of the p38 pathway had little effect on RTA-induced caspase 3/7 activation. These studies are the first to demonstrate a role for the JNK signaling pathway in ricin-induced cell death. In addition, the MAC-T cell line is shown to be a sensitive in vitro model system for future studies using RTA mutants to determine relationships between RTA-induced depurination, ribotoxic stress, and apoptosis in normal epithelial cells.

Keywords

Ricin; Apoptosis; Ribosome inactivating protein; Ribotoxic stress response; c-Jun N-terminal kinase

Introduction

Ricin is a type II ribosome inactivating protein (RIP) found in castor beans, which are the seeds of the castor plant, *Ricinus communis*. Due to its high toxicity and ready availability, ricin has

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been listed as a Category B Select Agent by the National Institutes of Health and the Centers for Disease Control and Prevention (Audi et al., 2005). Ricin is a heterodimeric glycoprotein composed of a catalytically active 32 kDa A-chain (RTA) linked by a disulfide bond to a 34 kDa B-chain (RTB), a galactose- and N-acetylgalactosamine-specific lectin. The molecule enters the cells through endocytosis and undergoes retrograde translocation to the Golgi apparatus/endoplasmic reticulum. At this point, the subunits dissociate and a portion of the RTA reaches the cytosol where it inactivates ribosomes by depurinating a single adenine nucleotide (Lord et al., 2005; Watson and Spooner, 2006). This depurination prevents binding of elongation factors, which leads to the inhibition of protein synthesis. However, recent evidence in yeast suggests that ribosome depurination may not by itself cause cell death (Li et al., 2007). Ricin also activates stress-activated protein kinase (SAPK) signaling pathways that are induced by ribosome damage (ribotoxic stress) (Iordanov et al., 1997) and induces apoptosis (Higuchi et al., 2003; Rao et al., 2005; Wu et al., 2004). However, the role of SAPK pathways in ricin-induced apoptosis has not been well delineated.

While the RTB subunit is thought to enhance the entry of ricin into cells, several studies have shown that the RTA subunit can enter the cell on its own and induce cytotoxicity (Casellas et al., 1984; Svinth et al., 1998; Vago et al., 2005; Wales et al., 1993). These studies have mainly focused on protein synthesis inhibition as the endpoint of cytotoxicity. The ability of RTA to induce apoptosis directly and the role of cell signaling cascades in this response have not been reported. The goals of the present study were to determine if RTA alone could induce both protein synthesis inhibition and cell death in mammalian cells and to determine if specific SAPK signaling cascades are required for the apoptotic response.

Materials and methods

Reagents

Ricin A-chain (RTA) and ricin holotoxin were purchased from Sigma-Aldrich and Vector Laboratories, respectively. Chemical inhibitors SP600125 or SB239063 were obtained from Calbiochem.

Cell culture

The bovine mammary epithelial cell (MEC) line MAC-T was established from primary bovine MECs by immortalization with the Simian virus 40 large T antigen (Huynh et al., 1991). These cells are immortalized but not transformed, as evidenced by their inability to form tumors in nude mice and to grow in soft agar. In addition, when supplied with appropriate substratum and hormones they can be induced to differentiate similar to primary MECs. MAC-T cells were routinely maintained as previously described (Grill et al., 2002). HeLa and Vero cells were kindly provided by Dr. Tom O'Brig (University of Virginia, Charlottesville, VA). HeLa cells were routinely maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS), 20 U/ml penicillin, 20 µg/ml streptomycin, and 50 µg/ml gentamicin. Vero cells were maintained in the same media with the exception that the FBS was not heat-inactivated. For experiments, cells were plated in complete media and grown to confluence, washed twice with phosphate buffered saline (PBS), and incubated in serum-free media supplemented with 0.2% bovine serum albumin (BSA) and 30 nM sodium selenite. Following a washout period, spent media were aspirated and replaced with media without additives ± treatments for various times. For small interfering (si) RNA transfection, MAC-T cells were plated at 3×10^4 cells/cm² in complete media lacking phenol red, insulin, and antibiotics. The next day, cells were transfected in serum-free media with 88 to 172 nM siRNA (Dharmacon) using GeneEraser (Stratagene) according to the manufacturer's protocol. After 2.5 days, cells were serum-deprived for 2 h and treated with RTA. After 6 h, cell lysates were collected and analyzed by western blotting or caspase 3/7 activation was measured as described below.

Ribosomal RNA depurination assay

Ribosomal RNA (rRNA) depurination was analyzed by dual-primer extension analysis as previously described (Parikh et al., 2002). Briefly, both control- and toxin-treated cells were lysed in Trizol (Invitrogen) and stored at -80°C until analysis. Total RNA was purified using RNeasy columns (Qiagen). Total RNA (2 μg) was initially hybridized with two primers end-labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Perkin-Elmer) using T4 kinase (Invitrogen). The depurination primer (5'-AACAGATGGTAGTTTCACCCC-3') annealed 64 nucleotides (nt) 3' of the depurination site on the 28S rRNA and the 28S control primer (5'-TTCACCTCACCGTTACTGAGG-3') annealed 99 nt 3' of the 5' end on the 28S rRNA. SuperScript II Reverse Transcriptase (Invitrogen) was then used in the dual primer extension assay. Extension products for the depurination and control fragments (64 nt and 99 nt, respectively) were separated on a 7 M urea-5% polyacrylamide denaturing gel. Bands were visualized and quantified by PhosphorImager.

Methionine incorporation assay

Confluent serum-starved MAC-T cells were washed two times with methionine-free media (Invitrogen) and incubated in methionine-free media for 45 min prior to treatment with RTA. Fifteen μCi $[\text{}^{35}\text{S}]\text{methionine}$ (MP Biomedicals) were added to each sample during the last hour of RTA treatment. Cells were washed twice with PBS and scraped into 5% trichloroacetic acid (TCA). Samples were centrifuged for 15 min at 3600 rpm. Pellets were washed three times with ice-cold 5% TCA and resuspended in 0.1 M NaOH. Radioactivity was determined by scintillation counting (Beckman Coulter).

Caspase 3/7 assay

Caspase 3/7 activation was measured using the SensoLyte Homogeneous AMC Caspase 3/7 Assay kit according to the manufacturer's protocol (AnaSpec).

Western immunoblotting

Cells were plated as described above in 60 mm tissue culture dishes. After toxin treatment, cell lysates were collected in lysis buffer as described (Grill et al., 2002). Total protein content was determined using a Bradford protein assay (Bio-Rad Laboratories). Proteins were separated by SDS-PAGE and transferred to nitrocellulose (0.2 μm ; Bio-Rad Laboratories) or polyvinylidene fluoride (0.45 μm ; Millipore) membranes. Antibodies against poly (ADP-ribose) polymerase (PARP), JNK, p38, phospho-p38, cleaved caspase 3, cleaved caspase 7, and phospho-c-Jun (Cell Signaling Technology), phospho-JNK (Santa Cruz Biotechnology), actin (Calbiochem) and HSP60 (Abcam) were used to detect the corresponding proteins. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG and anti-mouse IgG; GE Healthcare and Millipore, respectively) and peroxidase activity was detected by enhanced chemiluminescence (Pierce) followed by autoradiography.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining

Cells were plated at a concentration of 1×10^4 cells/ cm^2 on glass chamber slides (BD Falcon). Confluent cells were serum-starved for 16 h and then treated with RTA. After toxin treatment, cells were fixed with 4% paraformaldehyde and permeabilized using 0.1% sodium citrate and 0.1% Triton X-100. Cells were stained using the In Situ Cell Death Detection kit (Roche) according to the manufacturer's protocol. After counterstaining with Hoechst 33342 (Invitrogen), slides were examined under a Nikon Eclipse E800 fluorescent microscope. Images were captured with ACT-1 software.

Statistical Analysis

Data were analyzed by one-way ANOVA with Dunnett's Multiple Comparison posttest or two-way ANOVA (with repeated measures where appropriate) with Bonferroni posttests using GraphPad Prism Software.

Results

MAC-T cells are more sensitive to RTA than Vero or HeLa cells

To identify an epithelial cell culture model that is sensitive to RTA alone, we compared the ability of RTA to induce apoptosis in Vero and HeLa cells with its ability to induce apoptosis in the non-transformed MEC line MAC-T. The MAC-T cell line has been used in our lab to study stress-induced apoptosis (Leibowitz and Cohick, 2009), while Vero and HeLa cells represent cell lines of epithelial origin that are often used to study the actions of RIPs in vitro. As shown in Fig. 1A, treatment of MAC-T cells with RTA induced 12- to 15-fold increases in apoptosis within 4 to 6 h. The maximal response observed with Vero cells was approximately 2-fold, although greater responses were observed when cells were treated with RTA for more than 18 h (data not shown). HeLa cells exhibited a delayed response to RTA in comparison to MAC-T cells, with increases in caspase activation of 2-fold at 4 h and 7-fold at 6 h of treatment (Fig. 1B). The response to RTA was significantly lower in HeLa cells relative to MAC-T cells at both 4 and 6 h. Therefore, we chose to continue our studies with MAC-T cells since they represented the more sensitive cell line.

RTA and ricin induce similar maximal increases in caspase activation in MAC-T cells

To determine if RTA was as effective as ricin at inducing apoptosis, we treated MAC-T cells with increasing concentrations of each agent for 4 h and then measured caspase activation. As shown in Fig. 2, ricin was at least 1000-fold more sensitive in activating caspase 3/7 than RTA alone, with approximate EC_{50} s of 0.1 ng/ml and 15 ng/ml observed for ricin and RTA, respectively. Ricin was more effective than RTA at inducing caspase activation at concentrations below 0.1 μ g/ml. However, both RTA and ricin induced similar maximal increases in caspase activation of 10- to 12-fold.

RTA depurinates the α -sarcin/ricin loop of 28S rRNA and inhibits protein synthesis

The ability of RTA to induce ribosome depurination was determined using a dual-primer extension assay (Fig. 3A), as previously described for pokeweed antiviral protein (PAP) (Parikh et al., 2002). With the lower concentration of RTA (0.1 μ g/ml), approximately 50% of the large ribosomal subunit was depurinated by 4 h of treatment, with maximal effects of greater than 80% observed by 6 h (Fig. 3B). With 1 μ g/ml RTA, depurination was detectable by 2 h of treatment and reached maximal levels by 4 h. Depurination remained elevated through 10 h of treatment with either concentration of RTA (data not shown). A similar temporal pattern was observed for protein synthesis inhibition (Fig. 4), with 1 μ g/ml RTA inhibiting protein synthesis approximately 50% within 2 h of treatment and maximally inhibiting it between 3 and 4.5 h of treatment. Maximal inhibition of protein synthesis did not occur until the latest time point with the lower concentration of RTA. At each time point, the higher concentration of RTA inhibited protein synthesis to a greater degree relative to the lower concentration.

Caspase 3/7 cleavage, PARP cleavage and TUNEL staining indicate that RTA effectively induces apoptosis

Once we had established that the MAC-T cell line was responsive to RTA in terms of depurination, protein synthesis inhibition and caspase activation, the ability of RTA to induce apoptosis in MAC-T cells was evaluated more extensively. As shown in Fig. 2, 0.1 and 1 μ g/ml RTA induced activation of caspases 3 and 7 at 4 h of treatment. This fluorometric assay

cannot discriminate between caspase 3 and caspase 7 as both have substrate specificity for the amino acid sequence Asp-Glu-Val-Asp. To determine if each of these caspases was activated by RTA, we performed western immunoblotting using specific antibodies for their cleaved products. As shown in Fig. 5A, both caspases were activated in a similar concentration- and time-dependent fashion, with cleavage products of each caspase appearing by 4 h of treatment.

The ability of 1 μ g/ml RTA to induce PARP cleavage, a late-stage apoptotic event, was also monitored, as shown in Fig. 5B. Significant PARP cleavage did not occur until 4 hr after exposure to RTA. For this experiment, cells were exposed to RTA for the entire treatment interval, as in previous experiments, or RTA was removed after 1 h, cells were rinsed, and media were replaced with serum-free media (designated by R* in Fig. 5B). Interestingly, similar effects were observed when cells were only exposed to RTA for 1 h.

To determine what fraction of total cells was undergoing apoptosis, TUNEL staining was performed (Fig. 6). Very little TUNEL staining was observed, and nuclei were intact in cells that had been cultured in serum-free media overnight and exposed to 1 μ g/ml RTA for 2 h. TUNEL staining and disintegrated nuclei were observed after 4 h exposure to 1 μ g/ml RTA, with most of the cells exhibiting TUNEL staining by 6 h.

The JNK signaling pathway plays a major role in RTA-induced apoptosis

As shown in Fig. 7A, ricin activated JNK1, JNK2 and p38 by 1 h of treatment, whereas RTA alone did not activate these signaling molecules within this time frame. However, RTA did activate these signaling pathways beginning at 2 h of treatment (Fig. 7B). Both MAPK pathways remained activated through 8 h of treatment, which was the longest treatment interval examined.

To determine if either of these pathways was specifically involved in RTA-induced caspase activation, chemical inhibitors were used to knock down specific signaling molecules. As shown in Fig. 8A, a chemical inhibitor of the JNK pathway (SP600125) knocked down c-jun phosphorylation by approximately 50%, while the p38 inhibitor SB239063 almost completely inhibited activation of p38. Inhibition of JNK1/2 activity by SP600125 significantly reduced RTA-induced caspase 3/7 activation (Fig. 8B). In addition, knock down of the JNK pathway reduced the ability of RTA to induce PARP and caspase 3 cleavage as measured by western blot (Fig. 8C). In contrast, inhibition of the p38 pathway by SB239063 did not significantly decrease RTA-induced caspase 3/7 activation or caspase 3 cleavage (Fig. 8B and 8C). Interestingly, SB239063 did significantly inhibit PARP cleavage induced by RTA (Fig. 8C).

Specific signaling pathways were also blocked using siRNA technology. Transfection with siRNA specific for JNK2 or p38 α substantially decreased both total and phosphorylated levels of each protein (Fig. 9A). As shown in Fig. 9B and 9C, knock down of JNK2 significantly decreased the ability of RTA to induce caspase 3/7 activation as well as PARP cleavage. However, knock down of p38 α had little effect on either biological endpoint. Together, the chemical inhibitor and siRNA data indicate a greater role for the JNK pathway in mediating RTA-induced apoptosis relative to the p38 pathway.

Discussion

In the present study, we have identified a nontransformed epithelial cell culture system that is very sensitive to exogenous RTA. A cellular response was demonstrated by depurination of 28S rRNA, inhibition of protein synthesis, and induction of apoptosis. In addition, while both p38 and JNK MAP kinase signaling pathways were shown to be activated by RTA, the JNK pathway appeared to be more important in inducing the apoptotic response.

While RTA possesses the enzymatic activity required for ribosome depurination, RTB promotes transport of the ricin molecule into cells by binding to cell surface moieties that possess terminal galactose residues (Watson and Spooner, 2006). In the present study, RTA effectively entered MAC-T cells in the absence of RTB, albeit with a much lower efficiency compared to intact ricin, in agreement with previous reports for other cell lines (Casellas et al., 1984; Svinth et al., 1998; Vago et al., 2005; Wales et al., 1993). The exact mode of RTA entrance into the cell in the absence of RTB is unknown, but transport has been proposed to occur via fluid-phase endocytosis (Wales et al., 1993). Svinth et al. (1998) showed that fluid-phase uptake of RTA is similar to that of pokeweed antiviral protein (PAP), a single-chain RIP with no specialized mechanism to cross cell membranes. Mannose receptor-mediated uptake of RTA has also been demonstrated in macrophages (Simmons et al., 1986), although whether or not MAC-T cells possess such receptors has not been determined. In the present work, we show significant variability between cell types in their responsiveness to RTA. Likewise, Wang et al. (2007) reported that RTA alone was not cytotoxic to HEK 293 cells and was only mildly cytotoxic to HeLa cells in the absence of RTB. In endothelial cells, only limited inhibition of protein synthesis and metabolic activity was observed after 48 h with RTA (Lindstrom et al., 1997). In MAC-T cells, higher concentrations of RTA were required to elicit the same biological effects observed with lower concentrations of ricin. However, RTA inhibited protein synthesis and induced ribosome depurination by more than 90% and was as effective as ricin in activating apoptosis. Therefore, the requirement for higher concentrations of RTA is most likely related to the reduced ability of RTA to enter the cell in the absence of RTB.

Studies examining the biological activity of RTA have focused on its use as an immunotoxin for cancer therapy (Wang et al., 2007) or on studies of intracellular trafficking (Liu et al., 2006; Simpson et al., 1996; Svinth et al., 1998; Vago et al., 2005). These studies have primarily measured protein synthesis inhibition as an endpoint of cytotoxicity, although a few have also measured cell viability by MTT assay or trypan blue staining (Liu et al., 2006; Wang et al., 2007). However, we did not identify any studies that examined the effects of RTA specifically on apoptosis *in vitro*.

Previous studies with ricin have shown a correlation between depurination/protein synthesis inhibition and activation of the SAPK/JNK signaling pathway in macrophages (Higuchi et al., 2003; Iordanov et al., 1997; Korcheva et al., 2005) and primary human airway epithelial cells (Wong et al., 2007b). Similarly, we found that RTA alone also activated JNK and p38 pathways in a time frame that corresponded with these parameters. The activation of SAPKs in response to damage to the α -sarcin/ricin loop of the 28S ribosomal RNA is referred to as the ribotoxic stress response. It has been proposed that RTA binding to specific nucleotide sequences of this region both inhibits translation and, independent of this function, stimulates activation of SAPK (Iordanov et al., 1997). A variety of RIPs initiate SAPK signaling via interaction with the α -sarcin/ricin loop, including RTA, α -sarcin, Shiga toxin 1, onnamide A, and theopederin B (Iordanov et al., 1997; Lee et al., 2005; Smith et al., 2003). The antibiotics anisomycin and blasticidin S, as well as ultraviolet radiation, also initiate the ribotoxic stress response (Iordanov et al., 1997; Iordanov et al., 1998; Iordanov and Magun, 1999). The SAPK pathways p38 and JNK1/2 are involved in activating the proinflammatory response as well as apoptosis—two physiological responses that mediate ricin toxicity (Korcheva et al., 2007). Ricin treatment induces the expression of proinflammatory cytokines and chemokines such as TNF- α , interleukin (IL)-1, IL-6, and IL-8 (Gonzalez et al., 2006; Korcheva et al., 2007; Wong et al., 2007a; Yamasaki et al., 2004). The use of specific chemical inhibitors of the SAPK pathways indicates that the JNK and p38 pathways differentially regulate the expression of cytokines and downstream transcription factors in ricin-treated RAW 264.7 macrophages (Korcheva et al., 2005). For example, inhibiting the p38 pathway almost completely blocked IL-1 α and - β expression in response to ricin, while inhibiting the JNK pathway actually increased expression of these cytokines. In contrast, blockage of either pathway equally attenuated the ability of

ricin to induce TNF- α gene expression (Korcheva et al., 2005). Chemical inhibition of the p38 pathway in the human monocyte/macrophage cell line 28SC blocked ricin-induced IL-8 secretion (Gonzalez et al., 2006). In human airway cells an important role for p38 in mediating ricin-induced expression of multiple proinflammatory genes was demonstrated (Korcheva et al., 2007). Taken collectively, these data indicate that p38 plays a major role in regulating gene expression of proinflammatory signals in response to ricin.

In contrast, less is known about the role of the SAPK pathways in regulating ricin-mediated apoptosis. In ricin-treated RAW 264.7 macrophages, blocking the p38 MAPK pathway inhibited both TNF- α secretion and apoptosis (Higuchi et al., 2003). The role of the JNK pathway in ricin-induced apoptosis was not investigated. Interestingly, a caspase pan inhibitor blocked not only ricin-induced apoptosis but also TNF- secretion in these cells, suggesting a cross-talk between these events. In the present study, the role of the p38 and JNK pathways in RTA-induced apoptosis was investigated. Using chemical inhibitors for each pathway, as well as siRNA for JNK2 and p38, a stronger role for the JNK pathway was identified, based on caspase activation and PARP cleavage. A role for the p38 pathway cannot be completely ruled out as blocking this pathway with a chemical inhibitor did significantly reduce RTA-induced PARP cleavage. However, this finding was not supported by siRNA experiments, warranting further investigation of whether or not this is a specific effect of inhibiting the p38 pathway.

We have previously reported that apoptosis is not induced by TNF- α alone in MAC-T cells but is induced when cells are treated with TNF- α in combination with low concentrations of anisomycin (Leibowitz and Cohick, 2009). We proposed that this is due to the prolonged activation of JNK by anisomycin, in contrast to the transient JNK activation observed with TNF- α . Blocking the JNK pathway with chemical inhibitors or siRNA also reduced TNF- α - and anisomycin-induced apoptosis, suggesting that this pathway is a major component of ribotoxic stress-induced apoptosis in MAC-T cells. However, the exact trigger by which the ribosome signals the cell to activate the SAPK pathways is presently unclear.

Ricin toxicity can occur via aerosolization, injection, or ingestion (Audi et al., 2005). The actual cause of cell death in vivo is complex and likely involves multiple mechanisms. For example, animal models of inhalation have shown that ricin induces an inflammatory response in the lungs that produces local tissue damage that mimics adult respiratory distress syndrome (Korcheva et al., 2005; Griffiths et al., 2007). It is not clear how apoptosis contributes to ricin cytotoxicity. Both apoptotic and necrotic cells have been observed in the intestine following ricin administration via oral gavage (Smallshaw et al., 2007). As discussed above, engagement of apoptotic pathways may be required for the induction of inflammatory genes (Higuchi et al., 2003). Whether ricin-induced apoptosis can be triggered in the absence of protein synthesis inhibition has not been determined in mammalian cells. The type I RIP saporin has been shown to activate apoptosis in the absence of translational inhibition (Sikriwal et al., 2008). The development of effective therapies to combat ricin poisoning will require a thorough understanding of all facets of ricin action.

In summary, we have developed a nontransformed epithelial cell culture system that is exquisitely sensitive to RTA alone. Future studies will utilize RTA mutants that differ in their abilities to depurinate the ribosome and induce cytotoxicity to determine the connections between ribosomal events, signal transduction mechanisms, and apoptosis in mammalian cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Comparison of RTA-induced apoptosis in MAC-T, Vero and HeLa cells
(A) Confluent MAC-T and Vero cells were serum-starved for 16 h prior to treatment with 1 $\mu\text{g/ml}$ RTA. **(B)** MAC-T and HeLa cells were serum-starved for 2 h prior to treatment with 1 $\mu\text{g/ml}$ RTA. Apoptosis was measured using the fluorescent SensoLyte Homogeneous AMC Caspase 3/7 Assay kit (AnaSpec). Bars represent mean $\pm$ SE of 3 experiments. Data are relative to caspase 3/7 activity in the absence of RTA. Solid bars, MAC-T cells; striped bars, Vero cells or HeLa cells. Data were analyzed by 2-way ANOVA with repeated measures with Bonferroni posttests. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for Vero or HeLa cells versus MAC-T cells.

Figure 2. Comparison of RTA- and ricin holoenzyme-induced apoptosis

Apoptosis was measured using the fluorescent SensoLyte Homogeneous AMC Caspase 3/7 Assay kit (AnaSpec). Confluent MAC-T cells were serum-starved for 16 h before treatment with RTA or ricin holoenzyme for 6 h. Bars represent mean $\pm$ SE of 4 experiments. Data are relative to caspase 3/7 activity in the absence of RTA. Solid bars, RTA; striped bars, ricin holoenzyme. Data were analyzed by 2-way ANOVA with Bonferroni posttests. * $P < 0.01$; ** $P < 0.001$ for ricin holoenzyme versus RTA.

Figure 3. RTA induces ribosome depurination in a time- and concentration-dependent manner in MAC-T cells

(A) Cells were serum-deprived for 16 h prior to RTA or vehicle treatment (i.e. glycerol). Total RNA was analyzed by a dual primer extension assay with primers designed to recognize sequences downstream of the depurination site and the 5' end of the bovine 28S ribosome.

(B) Depurination was quantified and normalized against total 28S RNA. Solid bars, 0.1 µg/ml RTA; striped bars, 1 µg/ml RTA. Bars represent mean $\pm$ SD of 2 experiments.

Figure 4. RTA inhibits protein synthesis in a time- and concentration-dependent manner

Total protein synthesis was determined by [^{35}S]methionine incorporation. Confluent MAC-T cells were serum-starved for 16 h prior to incubation in methionine-free media 45 min prior to treatment with RTA. [^{35}S]methionine was added to cells during the last hour of RTA treatment. Bars represent mean $\pm$ SE of 3 experiments. Solid bars, 0.1 $\mu\text{g/ml}$ RTA; striped bars, 1.0 $\mu\text{g/ml}$ RTA. Data were analyzed by 2-way repeated measures ANOVA with Bonferroni posttests. * $P < 0.05$; ** $P < 0.01$ for 0.1 $\mu\text{g/ml}$ versus 1.0 $\mu\text{g/ml}$ RTA.

Figure 5. RTA induces cleavage of caspase 3/7 and PARP

(A) MAC-T cells were serum starved for 16 h prior to addition of glycerol (G) or RTA, 0.1 µg/ml or 1 µg/ml for the indicated times. Cell lysates (30 µg total protein) were separated by SDS-PAGE and immunoblotted. Figure is representative of 3 separate experiments. The additional experiments are shown in Supplemental Figure 1. (B) Cells were serum-starved for 18 h prior to addition of glycerol (G), serum-free media (SF), or 1 µg/ml RTA (R*, RTA was removed after 1 hour of treatment and replaced with SF media; R, RTA remained in the culture media the entire treatment time). Cell lysates (50 µg total protein) were separated by SDS-PAGE and immunoblotted. Bands at 119 and 89 kDa represent intact and cleaved PARP, respectively. Figure is representative of 2 separate experiments. The additional experiment is shown in Supplemental Figure 1. The caspase 7 antibody recognizes the 20 kDa subunit, while

the caspase 3 antibody recognizes two cleaved species; the 17 kDa subunit and the 19 kDa subunit which contains the prodomain.

Figure 6. RTA induces TdT-mediated dUTP nick end labeling (TUNEL) staining in a time-dependent manner

MAC-T cells were plated on glass chamber slides and grown to confluence. Cells were serum-starved overnight and treated with 1 $\mu\text{g/ml}$ RTA for 2, 4, and 6 h. Nuclei were stained with Hoechst 33342 (left panel), and ricin-induced DNA strand breaks were detected using TUNEL staining (right panel).

Figure 7. RTA and ricin holoenzyme activate JNK and p38 in MAC-T cells

(A) Cells were serum-starved for 16 h prior to treatment with glycerol (G), 1 μ g/ml RTA (A) or ricin holoenzyme (R) for the indicated times. (B) Cells were serum starved for 16 h before treatment with 0.1 and 1 μ g/ml RTA for the indicated times. For each experiment, total cell lysates (30 μ g) were immunoblotted with phospho-specific antibodies, then membranes were stripped and reprobed with antibodies for total protein. Blots are representative of 2 to 3 separate experiments. Additional experiments are shown in Supplemental Figure 2.

Figure 8. Effect of chemical inhibition of JNK1/2 and p38 signaling pathways on RTA-induced caspase activation and PARP cleavage
 Confluent MAC-T cells were serum-starved for 16 h before treatment with RTA ± inhibitor for 4 h. **(A)** Total cell lysates (30 µg) were separated by SDS-PAGE, immunoblotted and quantitated by densitometry. Bars represent mean ± SE of 3 separate experiments. A representative blot is shown on the left. **(B)** Apoptosis was measured using the fluorescent SensoLyte Homogeneous AMC Caspase 3/7 Assay kit (AnaSpec). Bars represent mean ± SE of 3 separate experiments for SP600125 (50 µM) and 5 separate experiments for SB239063 (30 µM). Striped bars, DMSO control; solid bars, inhibitor. Data were analyzed by 2-way ANOVA with Bonferroni posttests. * $P < 0.001$; NS = $P > 0.05$ for treatment with inhibitor versus treatment without inhibitor. **(C)** Total cell lysates (30 µg) were separated by SDS-PAGE, immunoblotted, and quantitated by densitometry. Bars represent mean ± SE of 5 separate experiments. Data were analyzed by one-way ANOVA with Dunnett's Multiple Comparison posttest. * $P < 0.05$; ** $P < 0.01$; NS = $P > 0.05$; indicates difference from RTA alone. A representative blot is shown on the left.

Figure 9. siRNA inhibition of JNK2 but not p38 decreases RTA-induced caspase 3/7 activation and PARP cleavage

MAC-T cells were transfected with siRNA and treated with 1 μg/ml RTA for 4 to 6 h. (A) Total cell lysates were separated by SDS-PAGE and immunoblotted. Blots are representative of 2 separate experiments. (B) Apoptosis was measured using the fluorescent SensoLyte Homogeneous AMC Caspase 3/7 Assay kit (AnaSpec). Bars represent mean ± SE of 6 separate experiments. Data were analyzed by 2-way ANOVA with Bonferroni posttests. * $P < 0.05$; ** $P < 0.01$; NS = $P > 0.05$. (C) Immunoblots probed with PARP antibody were quantitated by densitometry and normalized using HSP60. Bars represent the mean ± SE of 3 separate experiments. Data were analyzed by one-way ANOVA with Dunnett's Multiple Comparison posttests. * $P < 0.05$; ** $P < 0.01$; NS = $P > 0.05$; indicates difference from RTA alone.