Ricin, one of type II ribosomal inactivating proteins, inhibits protein biosynthesis by its RNA N-glycosidase activity. By yeast two-hybrid screening, the human BAT3 (HLA-B-associated transcript 2) was isolated as a ricin A-chain interacting protein. A canonical caspase-3 cleavage site, DEQD^1001 was found at the C-terminal region of BAT3. Ricin induced the apoptosis by activating caspase-3 and leading to the cleavage of BAT3 at 4 h after treatment while DNA ladderin at 24 h. The cleavage is completely inhibited by zDEVD-fmk, a caspase-3 specific inhibitor. In addition, cleavage of BAT3 is blocked in caspase-3-deficient MCF-7 cells, indicating that BAT3 is a novel caspase-3 substrate. Evidence indicates that caspase-3 activated by ricin acts on BAT3 at the caspase cleavage site, DEQD^1001, to release a C-terminal fragment designated CTF-131. The CTF-131 induces phosphatidylserine exposure, cell rounding, and chromatin condensation as ricin does. Moreover, silencing expression of endogenous BAT3 concomitantly suppresses ricin-induced apoptosis. Together, our results suggest a model that ricin triggers morphological changes of apoptosis by caspase-3-mediated proteolytic activation of BAT3.

Ricin is a toxic protein, which was originally isolated from the seeds of Ricinus communis (castor beans) (1, 2). It is composed of a toxophoric A-chain (RTA)^3 with RNA N-glycosidase activity and a galactose-binding B-chain (RTB) with lectin activity (3, 4). To enter target cells, ricin utilizes its lectin subunit to interact with the galactose moiety of glycoprotein or glycolipid on the cell membrane to allow its internalization by receptor-mediated endocytosis (5, 6), whereas its toxophoric subunit inhibits protein biosynthesis by depurination of a single adenine residue (A4324) in the 28 S ribosomal RNA. In humans, a Scythe homologue termed HLA-B-associated transcript 3 (BAT3) was cloned by Benerji et al. (17). BAT3 is a proline-rich protein, which is localized in the nucleus. It contains a N-terminal ubiquitin-like domain and two functional C-terminal nuclear localization signals (17, 18). BAT3 and Scythe share 62% amino acid sequence similarity, whereas whether BAT3 also functions as an apoptotic regulator is not yet understood.

Although RTA inhibits translation and activates caspase-3 to promote cell death, however, the precise molecular mechanism is unclear. Using yeast two-hybrid screening, we have identified the BAT3 as a RTA-binding protein. Through amino acid sequence analysis, we noticed a canonical caspase-3 cleavage site, DEQD^1001, in the C-terminal region of BAT3, which is absent in the corresponding region of Scythe. This observation prompts us to study the role of BAT3 in apoptotic regulation. In this report, we demonstrate the cleavage of BAT3 by ricin-induced caspase-3, suggesting that BAT3 is physiologically relevant to ricin-triggered apoptosis. Moreover, we identify a C-terminal caspase-3-generated fragment of BAT3 designated as CTF-131, which can execute apoptotic activities, such as cell rounding, shrinkage, nuclear condensation, and phosphatidylserine externalization. Silencing expression of endogenous BAT3 by antisense oligonucleotide transfection suppresses ricin-triggered apoptosis. Taken together, our results support a model that BAT3 is a novel cellular target of caspase-3 and its caspase-cleaved fragment, CTF-131, mediates ricin-induced apoptotic morphological changes.

EXPERIMENTAL PROCEDURES

Materials—The CaspACE® assay system was purchased from Promega (Madison, WI). The caspase-3 inhibitor, zDEVD-fmk, and fluorescent tetrapeptide protease substrates, Ac-LEHD-AFC and Ac-IETD-AFC, were purchased from Calbiochem (La Jolla, CA). All of the restriction enzymes used in this study were obtained from New England Biolabs (Beverly, MA). Anti-FLAG M2 monoclonal antibody was purchased from Eastman Kodak Co. Anti-active caspase-3 antibody (31A1067) was obtained from IMGENEX (San Diego, CA). Anti-human Bel-2 monoclonal antibody (4D7) was purchased from BD Pharmingen (San Diego, CA). High fluorescent FITC-labeled goat anti-mouse antibodies were from Chemicon International (Temecula, CA). Annexin-V-
Alexa 568 detection kit was purchased from Roche Applied Sciences (Ottawer, Germany). Anti-cytochrome c antibodies (6H2B4, and 7H8.2C12) were purchased from BD Pharmingen. Tandem and Cell Death Assays—HeLa and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 44 mM sodium bicarbonate, 0.1 mM non-essential amino acids, 10 units/ml penicillin G, and 10 μg/mL streptomycin in a humidified atmosphere containing 5% (v/v) CO2 at 37°C. The cells were seeded at 4–5 × 105 cells per 60-mm dish the day before transfection. Transfection reactions were performed by LipofectAMINE® and PLUS® reagents according to the manufacturer’s instructions (Invitrogen).

To examine caspase-3 activation, HeLa cells at 80% confluence were pretreated with 20 ng/ml of Z-DEVD-fmk for 30 min and then treated with ricin (100 ng/ml) for various periods of time. The cells were lysed with extraction buffer (10 mM HEPES-KOH, pH 7.4, 2 mM EDTA, 0.1% Triton X-100, 5 mM diethiothreitol, and 1 mM phenylmethylsulfonyl fluoride), and the lysates were subjected to SDS-PAGE and Western blotting analysis. The ability of BAT3 or CTF-131 to induce apoptotic morphological changes was assayed as follows. HeLa cells grown on coverslips were co-transfected with expression vectors encoding GFP (0.5 μg) and BAT3, CTF-131, or empty vector (2.0 μg each), and the apoptotic GFP-expressing cells were observed by conventional fluorescence or confocal microscopy. The percentage of apoptotic cells in each experiment was expressed as the number of round cells as a fraction of the total number of GFP-expressing cells. The data represent the mean ± S.E. of three independent experiments.

To assess the apoptotic morphological changes (Fig. 4C), HeLa cells were transfected with expression vector encoding BAT3 or CTF-131 for 24 h, and then cells were washed twice with incubation buffer (10 mM HEPES-KOH, pH 7.4, 140 mM NaCl, 5 mM CaCl2). The cells were incubated with annexin V-Alexa 568 labeling solution for 15 min in the dark. For analysis of ricin-triggered apoptosis (Fig. 1B), the cells were treated with ricin (100 ng/ml) for 4 h followed by annexin V staining as described above. The annexin V-labeled cells were fixed in 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, and then anti-FLAG anti-mouse IgG (Chemicon) for detection of overexpressed proteins. The FITC- or annexin V-Alexa 568-labeled cells were visualized under a confocal microscope (Leica TCS SP2).

To assess DNA ladder formation, HeLa cells were treated with ricin (100 ng/ml) for various periods of time at 37°C. The cells were lysed in lysis buffer (0.5 mg/ml proteinase K and 0.5% Sarcosyl in PBS), and the cell lysates were incubated at 37°C for 16 h and then treated with 10 μg/ml RNase A for 1 h followed by gentle extraction with phenol and chloroform. The extracted DNA was analyzed on a 1.5% agarose gel electrophoresis. The viability of transfected cells with various apoptotic stimuli was determined by measurement of their capacity to exclude the vital dye, ethidium bromide.

Yeast Two-hybrid Screening—Because wild type ricin A-chain (RTA) is detrimental to yeast, the RTA catalytic mutant (E177Q,R180L) was used as bait to screen a Jurkat cDNA library according to the instructions of the manufacturer (Clontech). Eighteen clones were isolated and 12 among these encoded the same fragment of BAT3 (BAT3-614–1044). For liquid culture assay, the yeast cells were grown overnight in the SD/Tp+Leu- medium, and the cells were diluted 5-fold inYPD media and grown to mid-log phase (A600, 0.4–0.8). The cells were collected by centrifugation and the cell pellets were resuspended in 100 μl of Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM MgCl2, and 50 mM β-mercaptoethanol) and 160 μl of o-nitrophenyl-β-D-galactopyranoside (ONPG) (1 mg/ml) was added. The β-galactosidase activity was measured in Miller units (47), and the results are expressed as the mean of triplicate measurements ± S.D.

In Vitro Binding Assay—GST-RTA fusion protein was expressed in Escherichia coli and purified by affinity chromatography on a glutathione Sepharose 4B column. Aliquots (1 μg) of recombinant GST fusion protein were incubated with GST-RNA binding domain and the GST pull down was performed with glutathione–Sepharose 4B (Amersham Life Science). Then, the immobilized proteins were eluted by incubation with 8 M urea. The recombinant proteins were separated by SDS-PAGE and visualized by staining with colloidal Coomassie Blue.

Measurement of Caspases Activities—The CaspaseC® assay system was used according to the instructions of the manufacturer. Briefly, cytosolic extracts (50 μg) were incubated with 240 μl of reaction buffer containing 80 μl of caspase buffer, 5 μl of 2-mercaptoethanol, and 10 μM dithiothreitol for 30 min at 30°C and then fluorescent tetra-peptide substrate was added at a final concentration of 50 μM. The reaction mixtures were incubated at 30°C for 1 h. Cleavage of fluorogenic substrate was quantitated by using a fluorescence spectrophotometer (F-4500, Hitachi) at 380/440 or 400/505 nm.

Preparation of Subcellular Fractions—HeLa cells were washed twice with ice-cold PBS and resuspended in 0.5 ml of buffer containing 50 mM HEPES-KOH, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, protease inhibitor mixture (Roche), and 250 mM sucrose, and then cells were homogenized by 30 strokes in a Dounce homogenizer. The homogenates were centrifuged at 500 × g for 10 min at 4°C to remove nuclei and broken cells, and the supernatant was added to 10 ml of 4% (w/v) Ficoll (dimehylpolyethylene glycol) to pellet the crude mitochondria fraction. The supernatant was further centrifuged at 20,000 × g for 30 min at 4°C to obtain the cytosolic fraction, and subjected to Western blotting with anti-cytochrome c antibody.

Western Blotting—For Western blotting analysis, cells were suspended in lysis buffer (50 mM HEPES-KOH, pH 7.4, 1% Triton X-100, 150 mM NaCl, and protease inhibitor mixture). Cell extracts were centrifuged at 20,000 × g for 30 min, and then the clear supernatant was separated in 10% SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane by a semidry blotting apparatus, and the blot was probed with anti-FLAG (1:1,000) monoclonal antibody for N-terminal FLAG-tagged proteins or with anti-CTF-131 polyclonal antibody (1:1,500) for detection of full length BAT3 and CTF-131 apopotic fragments. After the first antibody binding, the blot was washed in TBST (20 mM Tris-HCl, pH 7.6, 137.5 mM NaCl, 0.1% Tween 20) followed by horseradish peroxidase-conjugated secondary antibody (Chemicon, Temecula, CA) incubation for enhanced chemiluminescence detection (ECL) (Amersham Biosciences), and then developed on x-ray film (Kodak). For cytochrome c release and caspase-3 activation, cytosolic fractions were separated in 12.5% SDS-PAGE followed by immunoblotting with anti-cytochrome c and anti-active caspase-3 antibodies.

Production of CTF-131 by Baculovirus and Its Polyclonal Antibody—The CTF-131 cDNA was PCR amplified and cloned in-frame into the baculovirus transfer vector, pAc5SG-His (BD Pharmingen) with 5′-NotI and 3′-KpnI flanking sites and five copies of the FLAG-coding sequence (DYKDDDDK) in the position of the N-terminal FLAG-tag. The CTF-131 expression plasmid, pAc5SG-His-CTF-131 and BaeVector-3000 (Novagen, Madison, WI) were used. Four hours later, cells were washed with 500 mM NaCl and proteinase inhibitors mixture. The cell extracts were obtained by centrifugation, and the cytosolic fraction was identified by Southern blotting analysis. For production of recombinant proteins, 1 × 106 S9 cells were seeded in a T75 flask for 15 min, and the seeded cells were then infected with a high-titer stock solution of recombinant baculoviruses at 27°C for 3 days. The transfected cells were centrifuged at 1,000 × g for 10 min and the cell pellets were resuspended in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitors mixture) at 4°C for 45 min. The clear cell extracts were obtained by centrifugation, and the N-terminal His-tagged CTF-131 was purified from the nickel-nitrioltriacetic acid-agarose beads (Novagen).

To obtain anti-CTF-131 serum, the purified N-terminal His6-tagged CTF-131 was subjected to a 12.5% SDS-PAGE, and the CTF-131 band was excised from the gel and used to immunize New Zealand White rabbits. After three courses of immunization, the serum was isolated and tested for its specificity against endogenous BAT3 and the CTF-131 apoptotic fragment.

Immunofluorescence and Confocal Microscopy—HeLa cells grown on coverslips were transfected as described above. Immunostaining was performed on cells fixed with 4% paraformaldehyde in PBS on coverslips. For detection of FLAG-tagged proteins, cells were permeabilized in 0.2% Triton X-100 for 2 min and blocked in blocking buffer (10% fetal bovine serum in PBS) for 30 min at room temperature. The cells were then probed with anti-FLAG antibody (1:1,000) for 1 h at room temperature followed by extensive washing in
antisense oligonucleotide (AS) consisting of the sequence 5'-phosphorothioate-substituted DNA oligonucleotides (Invitrogen). An nuous BAT3 as described above. immunostaining for observation of the endocytosed ricin and endoge-
to remove free and membrane-bound ricin. The cells were subjected to
mounting in mounting solution (80% glycerol in PBS), and were sealed

Experimental Procedures.
ricin (100 ng/ml) for 4 h followed by annexin V and Hoechst dye staining
in a 1.5% agarose gel followed by ethidium bromide staining and UV

Fig. 1. Characteristics of apoptosis triggered by ricin. A, DNA
fragmentation in ricin-treated cells. HeLa cells were treated with ricin
(100 ng/ml) for various periods of time or mock treated (−) for 24 h as
indicated on the top of this figure. DNA samples were electrophoresed
in a 1.5% agarose gel followed by ethidium bromide staining and UV
detection. The DNA laddering pattern appeared after exposure of cells
to ricin for 24 h. Molecular weight markers (M) in kb are shown on the
left. B, ricin induces the exposure of PS. HeLa cells were treated with
ricin (100 ng/ml) for 4 h followed by annexin V and Hoechst dye staining
for cell surface-exposed PS and the nucleus, respectively. The staining
and confocal microscopy analyses were performed as detailed under
“Experimental Procedures.” Arrowheads indicate that the cells display
PS exposure (left panel), nuclear condensation (central panel), and cell
rounding (right panel) after treatment with ricin in the same field. Scale bar = 20 μm.

blocking buffer. The washed cells were stained with FITC-conjugated
goat anti-mouse IgG (1:1000) for 45 min at room temperature. During
the last 15 min of secondary antibody staining, Hoechst 33258 (5 μg/ml)
was applied for visualization of the nucleus. After washing, slides were
mounted in mounting solution (80% glycerol in PBS), and were sealed
with nail polish. For analysis of the cytochrome c release, mouse mono-
oclonal antibody against cytochrome c (6H2 B4) was used to probe the
cells at a dilution of 1:1000. For visualization of endogenous BAT3, the
first and second antibodies were replaced with anti-CTF-131 (1:500)
and Alexa 568-conjugated IgG (1:1000), respectively.

To determine co-localization of BAT3 with ricin in vivo, HeLa cells
grown on coverslips were washed with ice-cold Dulbecco’s modified
Eagle’s medium and incubated with FITC-labeled ricin (10 ng/ml) at
4 °C for 30 min for ricin attachment on the cell membrane. To allow
endocytosis of ricin, the cells were transferred to an incubator at 37 °C
in the dark for 1 h, and then washed with 0.1% galactose in PBS twice
to remove free and membrane-bound ricin. The cells were subjected to
immunos staining for observation of the endocytosed ricin and endoge-
nous BAT3 as described above.

Oligonucleotides Used to Inhibit Endogenous BAT3 Expression—
Translation of BAT3 was suppressed by treatment of cultures with
phosphorothioate-substituted DNA oligonucleotides (Invitrogen). An
antisense oligonucleotide (AS) consisting of the sequence 5′-ACTAT-
specific substrates for caspase-1, caspase-3, caspase-8, and caspase-9, respectively. As shown in Fig. 3A, the caspase-9 activity was gradually increased during 4–6 h of incubation with ricin (panel III). The caspase-3 activity was dramatically increased to 4-fold after treatment of cells with ricin for 5 h as compared with that of the control group (Fig. 3A, panel IV). These results indicate that caspase-9 and caspase-3 were activated at the early onset of ricin-triggered apoptosis. In addition, no significant increases of caspase-1 and caspase-8 activities were detected in ricin-treated cells (Fig. 3A, panels I and II). It was reported that caspase-8 is the key apoptotic initiator in the death-receptor pathway (extrinsic) (22, 23). It was further demonstrated that caspase-8 mediates cytochrome c release in the Fas-receptor pathway through cleavage of Bid (24, 25). Ricin activates caspase-9 and -3, but not caspase-8 suggesting that it triggers apoptosis through the cellular stress-induced mitochondria pathway (intrinscic) (26, 27). We showed that cytochrome c was released from mitochondria within 4 h of treatment with ricin (Fig. 3B, upper panel). Besides, caspase-3 activation determined by the appearance of active caspase-3 (Fig. 3B, lower panel) and cleavage of fluorescent tetrapeptide substrates (Fig. 3A, fourth panel) followed a similar time course as compared with the cytochrome c release. These results suggest that ricin induces apoptosis through the mitochondria pathway by activating caspase-9 and a downstream executor, caspase-3.

Amino acid sequence analysis of BAT3 revealed that there is a consensus caspase-3 cleavage site at DEQD1001 (28, 29), whereas Scythe does not have this cleavage site at the corresponding site (TQED1001(G). Because BAT3 is a protein with 1132 amino acid residues, cleavage at the C-terminal end of the Asp1001 residue would release a C-terminal fragment with 131 amino acid residues designated as CTF-131. To examine whether the endogenous BAT3 was cleaved by caspase-3, HeLa cells were treated with ricin (100 ng/ml) for various periods of time (1–6 h) to induce apoptosis. As shown in Fig. 3C, the CTF-131 appeared with approximately the same kinetics of pro-caspase-3 activation as revealed by immunoblotting using anti-CTF-131 and anti-active caspase-3 antibodies (central and lower panels). Pretreatment with the caspase-3-specific inhibitor, zDEVD-fmk (50 μM) completely blocked the generation of the CTF-131, indicating that it was a caspase-3-mediated event (Fig. 3C, central panel). To determine whether caspase-3 was necessary for the apoptotic proteolysis of endogenous BAT3 in vivo, we made use of the human breast carcinoma cell line MCF-7 that is devoid of caspase-3 because of a deletion in the caspase-3 gene (30). In MCF-7 cells, no CTF-131 was detected during ricin treatment (Fig. 3D), demonstrating that the cleavage of BAT3 was mediated by caspase-3. To map the caspase-3 cleavage site on BAT3, a cleavage mutant of BAT3, BAT3-D1001A, with a N-terminal FLAG tag was generated. HeLa cells expressing N-terminal FLAG-tagged wild type BAT3 or BAT3-D1001A were treated with ricin for 6 h, and the results indicated that BAT3-D1001A was resistant to caspase-3 cleavage, whereas wild type BAT3 was sensitive (Fig. 3E). These data indicate that the cleavage of BAT3 occurred at the caspase-3 reactive site. To further examine whether other apoptotic inducers can also induce the cleavage of BAT3, cytosolic extracts from HeLa cells treated with protein synthesis inhibitors, ricin or cycloheximide, endoplasmic reticulum stress inducers, thapsigargin or tunicamycin, and topoisomerase inhibitors, camptothecin or etoposide, for 5 h were analyzed by Western blotting with anti-CTF-131 antibody. Strikingly, the CTF-131 was generated only after treatment with ricin (Fig. 3F, central panel). The other potent apoptotic inducers used in this experiment could not induce cleavage of BAT3 even though they could activate caspase-3 activity from 3–5-fold as compared with the control level (Fig. 3F, lower panel). Note that the molecular size of the N-terminal FLAG-tagged CTF-131 is larger than that of the endogenous CTF-131 fragment because of cloning requirements (Fig. 3F, center panel). These results suggest that BAT3 is specifically cleaved by caspase-3 during ricin-triggered apoptosis.

Induction of Apoptotic Morphologies by CTF-131—As shown above, the CTF-131 was released from BAT3 by caspase-3. To determine the effects of this fragment on apoptosis regulation, CTF-131 was expressed in HeLa cells and morphological changes were quantitatively evaluated. Cells were co-transfected with expression vectors encoding GFP and N-terminal FLAG-tagged BAT3 or CTF-131 at a ratio of 1:4, and the transfected cells were identified by confocal microscopy. In GFP and BAT3 or mock co-expressing cells, the cellular morphology was normal (Fig. 4A, a–c and d–f), whereas the CTF-131 and GFP co-expressing cells showed typical apoptotic features, including cell rounding, shrinkage, and chromatin condensation (Fig. 4A, CTF-131 panel). Expression of the CTF-131 caused 46% cell death in the GFP-positive cells (Fig. 4B). These results suggest that the caspase-3-generated CTF-131 acquires apoptotic activities and mediates ricin-induced cell morphologic changes.

An event in apoptosis is the loss of plasma membrane asymmetry, resulting in the exposure of PS residues on the outer plasma membrane leaflet. Annexin V has been shown to specifically bind to the externalized PS in the presence of calcium (31). To determine the involvement of BAT3 and CTF-131 in apoptosis, HeLa cells expressing BAT3 or CTF-131 were assayed by confocal microscopy for the externalization of PS. The BAT3 expressing cells showed normal nuclear morphology, whereas the annexin V signals were negative (Fig. 4C, BAT3 panel). The CTF-131-positive cells displayed cytoplasm shrinkage, cell rounding, and nuclear condensation and caused externalization of the PS (Fig. 4C, CTF-131 panel). After transfection for 24 h, 25% of CTF-131-positive cells showed PS externalization (data not shown). Expression levels of BAT3 and CTF-131 were examined by Western blot-
FIG. 3. **BAT3 is a novel caspase-3 substrate.** A, time courses for activation of caspase-1, caspase-8, caspase-9, and caspase-3, respectively. After treatment with ricin (100 ng/ml) for various periods of time (1–6 h), or mock treated (−) for 6 h, cytosolic extracts were isolated and incubated with caspase fluorescent substrates and assayed as described under “Experimental Procedures.” *First panel*, ricin could not induce caspase-1 activation. *Second panel*, no significant caspase-8 activation occurred during ricin treatment. *Third panel*, caspase-9 was gradually activated between 4 and 6 h of treatment with ricin. *Fourth panel*, ricin dramatically induced caspase-3 activation after treatment for 4 h. The data are presented as the mean ± S.D. of three independent experiments. **B**, analyses of cytochrome c (cyt. c) release and pro-caspase-3 (pro-casp-3) activation. HeLa cells were treated as described above. Equal amounts (50 μg) of cytosolic fraction were loaded in each well of a 12.5% SDS-PAGE followed by Western blotting analysis using anti-cytochrome c antibody. The arrow indicates cytochrome c, which is present in the cytosol (upper panel). For pro-casp-3 activation, total cell extracts (100 μg) in each well were analyzed by Western blotting using anti-active caspase-3 antibody. The arrowhead and arrows indicate pro-casp-3 and the active fragment of caspase-3, respectively (lower panel). **C**, endogenous BAT3 is cleaved by
ting with anti-FLAG antibody (Fig. 4D). Together, these results suggest that caspase-3-cleaved CTF-131 induces apoptotic phenotypes.

Overexpression of Full-length BAT3 Inhibits Ricin-induced Cytochrome c Release and Cell Death—Because CTF-131 causes apoptotic morphologies, we reasoned that full-length BAT3 might strengthen ricin-triggered apoptosis. To test this hypothesis, the ricin-induced cytochrome c release was monitored in BAT3- or CTF-131-transfected cells. Surprisingly, overexpression of BAT3 did not reinforce apoptosis, but suppressed ricin-induced cytochrome c release. Expression of CTF-131 was unable to prevent cytochrome c release (Fig. 5A).

caspase-3 in cells undergoing ricin-induced apoptosis. HeLa cells were treated with ricin (100 ng/ml) for various periods of time (1–6 h). Pretreatment with zDEVD-fmk (50 μM), a caspase-3 specific inhibitor, for 2 h was used to evaluate the involvement of caspase-3. Cell extracts (100 μg) were analyzed by Western blotting using anti-CTF-131 and anti-active caspase-3 antibodies. The CTF-131 was generated by ricin-induced caspase-3 at the early onset of apoptosis (central panel). The zDEVD-fmk completely blocked the cleavage of BAT3 and generation of CTF-131 (upper and central panels). CTF-131 appeared with similar kinetics of pro-caspase-3 (pro-casp-3) activation (central and lower panels). D, caspase-3 is necessary for the cleavage of BAT3. HeLa and MCF-7 cells were treated with ricin (100 ng/ml) for various periods of time as indicated in the figure. In caspase-3-deficient MCF-7 cells, there is no detectable CTF-131 (lanes 6 and 7), and the BAT3 protein level has remained constant (upper panel). In HeLa cells, the CTF-131 was released from BAT3 during ricin treatment (lanes 2 and 3). The production of the CTF-131 was thoroughly abolished by zDEVD-fmk (lane 4). Equal amounts (100 μg) of extracts loaded in each lane were determined by immunoblotting using anti-tubulin antibody (lower panel).

E, mapping the caspase-3 cleavage site at BAT3. HeLa cells expressing N-terminal FLAG-tagged wild type (WT) BAT3 or caspase-3 cleavage mutant (D1001A) were treated with ricin or mock treated (−) for 6 h. Cell extracts were analyzed by immunoblotting with anti-FLAG antibody. A prominent N-terminal cleavage product (arrow) was released from BAT3 after treatment with ricin. The BAT3-D1001A (D1001A) cleavage mutant was resistant to ricin-activated caspase-3 cleavage. F, effects of various apoptotic inducers on the cleavage of BAT3. HeLa cells were treated with ricin (100 ng/ml), cycloheximide (CHX, 30 μg/ml), thapsigargin (TSG, 1.3 μg/ml), tunicamycin (TUN, 10 μg/ml), camptothecin (CMT, 20 μg/ml), etoposide (ETS, 20 μg/ml), or mock treated (PBS) for 5 h. Cell extracts (100 μg) were analyzed by immunoblotting using anti-CTF-131 antibody. The FLAG-tagged CTF-131 is also recognized by anti-CTF-131 antibody (right lane). Ricin specifically induced the generation of CTF-131 (central panel). The caspase-3 activities induced by various apoptotic inducers were determined by cleavage of fluorogenic tetrapeptide substrates (lower panel).
FIG. 4. CTF-131 induces apoptotic events. A, CTF-131 induces apoptotic morphological changes. HeLa cells were co-transfected with vectors encoding GFP (0.5 µg) and BAT3, CTF-131, or empty vector (2.0 µg each). The GFP-positive cells were visualized by confocal microscopy. Co-expression of GFP with mock (a–c) or BAT3 (d–f) was not detrimental to cells. The CTF-131 and GFP co-expressed cells, however, show characteristics of apoptosis including cell rounding and chromatin condensation (g–i and arrowheads). Scale bars = 40 µm. B, quantitative analysis
Overexpression of BAT3 or CTF-131 alone did not induce the release of cytochrome c (data not shown). To further confirm the inhibitory activity of BAT3 overexpression on the release of cytochrome c, HeLa cells expressing C-terminal Myc-tagged BAT3 or CTF-131 were subjected to ricin treatment followed by immunofluorescence staining and confocal microscopic analysis. Ricin induced the accumulation of untransfected cells with a diffuse cytochrome c staining pattern (Fig. 5B, a and b, arrows). Intriguingly, in the BAT3 overexpressing cell, a normal cytochrome c punctate staining pattern was observed (Fig. 5B, a and b, arrowheads). Nevertheless, a large number of CTF-131-positive cells displayed the diffuse cytochrome c staining pattern (Fig. 5B, e and f, arrowheads). By quantitative analysis, we noticed that the cytochrome c was retained in the mitochondria in each BAT3 expressing cell (Fig. 5C). The cell morphology of these BAT3-positive cells was normal. However, 45% of CTF-131-positive cells were susceptible to ricin and displayed a cytochrome c release pattern and cell rounding (Fig. 5C). HeLa cells transfected with various amounts of plasmids encoding BAT3 were treated with ricin and the release of cytochrome c was decreased in a dose response manner (Fig. 5D). To examine whether overexpression of BAT3 suppresses ricin-triggered cell death, cells transfected with vector alone or plasmids encoding BAT3 were treated with ricin (100 ng/ml), and cell death was quantitated by trypan blue exclusion. A 42.7% reduction in cell death was observed in BAT3-transfected cells (Fig. 5E). The transfection efficiency for BAT3 was 42.7% reduction in cell death was observed in BAT3-transfected cells (Fig. 5E). The transfection efficiency for BAT3 was indicated by Western blotting analysis (Fig. 5F). These data suggest that overexpression of BAT3 suppresses ∼92.8% ricin-induced cell death. It has been shown that overexpression of Bel-2 inhibits programmed cell death induced by various stimuli (32–34). Moreover, the Bel-2 has been suggested to block apoptosis by inhibiting the release of cytochrome c from mitochondria (35, 36). Surprisingly, an elevated protein level of endogenous Bel-2 was found in BAT3 transfectants, but not in the control as revealed by Western blotting analysis (Fig. 5F). These findings suggest that overexpression of full-length BAT3 inhibits ricin-induced cytochrome c release partly through the Bel-2.

**BAT3 Is Crucial for Ricin-induced Apoptosis**—Ricin-induced caspase-3 cleavage of BAT3 and generation of CTF-131, moreover, CTF-131 caused apoptotic morphologies (Fig. 4). Therefore, it appears that CTF-131 mediates ricin-induced apoptotic morphological changes. To test this, we used antisense oligonucleotides to inhibit expression of endogenous BAT3 and examined ricin-induced apoptosis. An AS oligonucleotide used in this study was the inverse complement of the coding sequence −2 to +19 of human BAT3 transcript. As shown in Fig. 6A, AS treatment significantly lowered the protein level of BAT3. The SC transfection did not alter the BAT3 protein level as compared with oligo-free control (Fig. 6A). The actin immunoblot served as an internal control. In addition, silencing expression of BAT3 accompanied down-regulation of Bel-2, supporting the notion that BAT3 might function as an intrinsic regulator for Bel-2 protein level (Fig. 5E). To analyze effects of antisense treatment on ricin-induced apoptosis, the oligo-free, SC- or AS-transfected cells were treated with ricin and cell morphology was examined. As shown in Fig. 6B, the oligo-free or SC-treated cells were more susceptible to ricin-induced apoptosis than the AS-treated cells. The oligo-free and SC-treated cells had displayed apoptotic cell rounding, 33 and 35%, respectively. However, only 15% of AS-treated cells showed apoptotic morphology under treatment with ricin (Fig. 6C). To further assess the inhibitory effects of AS treatment on apoptosis induced by ricin and to study whether or not cells with reduced levels of BAT3 become more resistant only to ricin-induced apoptosis, the transfected cells were treated with various apoptotic stimuli and cell death was quantitated by trypan blue exclusion. As shown in Fig. 6D, cells with reduced levels of BAT3 (AS treatment) become more resistant to ricin but not to other apoptotic stimuli-induced cell death. These results suggest that BAT3 (CTF-131) plays a crucial role in ricin-induced apoptosis.

**Intracellular Localization of BAT3 and CTF-131**—To detect the intracellular localization of BAT3 and CTF-131, we stained BAT3 or CTF-131-overexpressed cells with anti-FLAG antibody, and then investigated by confocal microscopic analysis. Most of the overexpressed BAT3 was localized in the nucleus of HeLa cells (Fig. 7A, BAT3 panel), whereas the CTF-131 was localized in the cytoplasm (Fig. 7A, CTF-131 panel).

Recently, the BAG (Bcl-2-associated athanogene) domain was found in the C-terminal region of BAT3 (residues 1055–1111) (37, 38). To examine whether this functional domain would play roles in regulating the intracellular translocation of CTF-131, we generated the BAG domain-deleted CTF-131 fragment, designated as CTF-ΔBAG. HeLa cells expressing N-terminal FLAG-tagged CTF-131 or CTF-ΔBAG were visualized by confocal microscopy. The CTF-131 was localized in the shrunken cytoplasm (Fig. 7B, CTF-131 panel). Interestingly, the morphology of CTF-ΔBAG expressing cells was normal, and the CTF-ΔBAG was prominently localized in the nucleus (Fig. 7B, CTF-ΔBAG panel). These results suggest that the BAG domain of CTF-131 plays an important role in cytoplasmic targeting after caspase-3 cleavage.

**DISCUSSION**

Ricin inhibits cell growth by inhibiting protein biosynthesis and inducing apoptosis. We have identified BAT3 as a novel caspase-3 substrate during ricin-triggered apoptosis. BAT3 is cleaved by caspase-3 at a consensus sequence, DEQD1001→G, and the caspase-cleaved C-terminal fragment of BAT3, CTF-131, exerts its apoptotic activities, such as cell rounding, nuclear condensation, and phosphatidylserine exposure. Our results not only demonstrate the active role of CTF-131 in apoptosis but also identifies a previously unknown apoptotic mechanism (Fig. 8).

Several studies have shown that in addition to inhibition of translational machinery, ricin induces apoptosis by activating caspases and causing apoptotic morphology changes (7, 9, 39). Human BAT3 is the first molecule shown to be involved in ricin-induced cellular changes associated with apoptotic cell death. Among cell stress inducing reagents such as ricin, cycloheximide, thapsigargin, tunicamycin, camptothecin, and etoposide, only ricin causes the cleavage of BAT3 and the release of CTF-131, suggesting that the interaction between BAT3 and ricin might promote this proteolytic process. Furthermore, we found that ricin A-chain (RTA) specifically interacts with BAT3 as revealed by GST pull-down, co-localization, and yeast two-hybrid analyses. With regard to the cleavage of BAT3, the activated caspase-3 has to be transported from the cytoplasm to the nucleus where it can act on BAT3. It has been suggested that caspase-9, which is activated earlier than
Overexpression of BAT3 inhibits ricin-induced cytochrome c (cyt. c) release and cell death. A, BAT3 attenuates release of cytochrome c during treatment with ricin. Cytosolic extracts (50 μg) of BAT3- or CTF-131-expressed cells with ricin (100 ng/ml) treatment for 4 h were collected and processed for immunoblotting with anti-cytochrome c antibody. Expression of BAT3 significantly suppresses the release of cytochrome c as compared with mock or CTF-131-transfected cells. B, BAT3 is able to inhibit cytochrome c release from the mitochondria to the cytosol, whereas CTF-131 is not. Immunostaining with anti-Myc and anti-cytochrome c antibodies and confocal microscopy were applied to visualize the cytochrome c staining pattern in BAT3- or CTF-131-expressing cells with ricin treatment. The BAT3-positive cells maintained...
caspase-3 increases the diffusion limit of the nuclear pores, allowing caspase-3 to pass through the nuclear pores during apoptosis by diffusion (40). It also showed that caspase-3 activation was initiated first in the cytosol and then in the nucleus (41). These findings indicate that ricin could specifically promote caspase-3 cleavage of BAT3 in the nucleus.

FIG. 6. BAT3 is crucial for ricin-induced apoptosis. A, immunoblotting analysis for the protein level of endogenous BAT3 after treatment with oligo-free, SC or AS oligonucleotides. Cells were transfected with AS or SC, each 250 nM for 24 h. Cell extracts (100 µg) were analyzed by immunoblotting. The protein level of BAT3 is significantly reduced in the presence of AS treatment (right lane). Actin immunoblot is indicated as an internal control. B, phase-contrast and immunofluorescence images of oligo-free (left panel), SC (central panel), or AS (right panel) transfected HeLa cells with ricin (100 ng/ml) treatment for 4 h. AS transfection not only silences expression of endogenous BAT3 (BAT3 panel) but also suppresses ricin-induced apoptotic cell rounding. Scale bar = 20 μm. C, quantification of apoptosis. Cells show apoptotic morphological changes as evaluated as described in B. Four hundred cells were counted in each of three independent experiments. The fraction of cells with apoptotic morphology as a percentage of all cells in the same field was calculated. The percentage is expressed as the mean ± S.D. D, overexpression of BAT3 causes elevation of the endogenous Bcl-2 protein level. Cell lysates (100 µg) were analyzed by immunoblotting with anti-FLAG, anti-human Bcl-2, or anti-actin antibody. The actin immunoblot was indicated as an internal control.
ptosis. Inconsistence with these findings, CTF-131 dramatically induced cell morphological changes, which were in association with apoptosis. However, it could not induce DNA fragmentation (data not shown). Thus the CTF-131 is responsible for extranuclear apoptotic events and nuclear condensation in ricin-triggered programmed cell death.

We noticed that cytochrome c was retained in the mitochondria in each BAT3-transfected cell after treatment with ricin. Nevertheless, the cytochrome c release was found in a large number of untransfected cells with a normal level of BAT3. Moreover, the endogenous Bcl-2 protein level was up-regulated in BAT3 transfectants. These findings suggest that overexpression of BAT3 induces the anti-apoptotic effect. Further studies are required to elucidate the mechanism(s) by which BAT3 overexpression regulates the protein level of Bcl-2.

Silencing the expression of endogenous BAT3 prominently reduced the number of cells with apoptotic morphologies, suggesting that BAT3 (CTF-131) plays a crucial role in ricin-induced apoptosis. Impaired ability to undergo programmed cell death in response to tumor suppressor or cellular pro-apoptotic factors acquires in tumor cells a selective advantage for progression as well as their notorious resistance to therapy (42). It has been reported that the level of rat BAT3 mRNA was down-regulated in some transformed cells rather than in the parental cells, suggesting that BAT3 is a pro-apoptotic factor (43).

BAT3 contains nuclear localization signals between amino acid residues 1030–1053 (18). Interestingly, we observed that CTF-131 was localized in the cytoplasm even though it contains the nuclear localization signals. Deletion of the BAG domain of CTF-131 (ΔH9004–1111) markedly induces nuclear targeting of this fragment, suggesting that the BAG domain might contain cytoplasmic targeting signals that function after the caspase-3 cleavage.

The major dose-limiting toxicity of immunotoxin-based tumor therapy is vascular leak syndrome (VLS). Some evidence shows that RTA rapidly damages endothelial cells (human umbilical endothelial cells) by inducing early changes in morphology including cell rounding and disruption of cell monolayer (44–46). The damaged cells display an increase in permeability, which resembles a typical characteristic of the VLS. To reduce side effects and optimize the therapeutic index of immunotoxins, elimination of VLS is desirable. Our studies imply that caspase-3-cleaved CTF-131 of BAT3 is responsible for morphological changes in ricin-treated cells. Because the link between caspase-3 and VLS is still unclear at present, it would be intriguing to study the relationship between CTF-131 and VLS for facilitating the clinical development of immunotoxins.

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