Abnormal aggregates of transactive response DNA-binding protein-43 (TDP-43) and its hyperphosphorylated N-terminal truncated C-terminal fragments (CTFs) are deposited as major components of ubiquitinated inclusions in most cases of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U). The mechanism underlying the contribution of TDP-43 to the pathogenesis of these neurodegenerative diseases remains unknown. In this study, we found that a 2–5-fold increase in TDP-43 expression over the endogenous level induced death of NSC34 motor neuronal cells and primary cortical neurons. TDP-43-induced death is associated with up-regulation of Bim expression and down-regulation of Bcl-xL expression. siRNA-mediated reduction of Bim expression attenuates TDP-43-induced death. Accumulated evidence indicates that caspases are activated in neurons of ALS and FTLD-U patients, and activated caspase-mediated cleavage of TDP-43 generates CTFs of TDP-43. Here, we further found that the ER (endoplasmic reticulum) stress- or staurosporine-mediated activation of caspases leads to cleavage of TDP-43 at Asp89 and Asp169, generating CTF35 (TDP-43-(90–414)) and CTF27 (TDP-43-(170–414)) in cultured neuronal cells. In contrast to TDP-43, CTF27 is unable to induce death while it forms aggregates. CTF35 was weaker than full-length TDP-43 in inducing death. A cleavage-resistant mutant of TDP-43 (TDP-43-D89E/D169E) showed stronger death-inducing activity than wild-type TDP-43. These results suggest that disease-related activation of caspases may attenuate TDP-43-induced toxicity by promoting TDP-43 cleavage.

Transactive response DNA-binding protein-43 (TDP-43) has been identified as a major component of ubiquitinated inclusions in most cases of frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS). Multiple studies have suggested that these neurodegenerative diseases share a common pathogenesis linked to TDP-43 (see Refs. 3 and 4 for review).

FTLD-U is the most common variant of FTLD. FTLD, a heterogeneous syndrome characterized by progressive dementia, is caused by degeneration of the frontal and anterior temporal lobes, and is a leading cause of dementia in patients presenting before the age of 65 years. The pathogenesis of FTLD remains unknown. ALS is the most common motor neuron disease, characterized by progressive loss of motor neurons (6, 7). The pathogenesis of ALS remains undetermined although various hypotheses have been proposed: e.g., misfolded protein aggregates, mitochondrial dysfunction, glutamate toxicity, oxidative stress, disturbance of intracellular trafficking, and ER stress (8). About 10% of ALS cases occur in a genetically inherited manner.

Post-translational modifications of TDP-43 including truncation, hyperphosphorylation, and ubiquitination are assumed to be linked to abnormal aggregation (1, 2). In particular, C-terminal fragments (CTFs) of TDP-43 are prone to form cytoplasmic aggregates (9–11). Although it is apparent that cytoplasmic aggregates of TDP-43 are closely related to the pathogenesis of FTLD-U and ALS, it remains unknown how CTFs are generated and whether the aggregate formation of TDP-43 is a cause or a result of neuronal toxicity.

Caspase activation has been observed in motor neurons of ALS (12–15) and neurons in FTLD patients (16). One possible mechanism underlying the generation of CTFs is the cleavage of TDP-43 by activated caspases. In reality, multiple studies have shown that TDP-43 is cleaved in a caspase-dependent manner and the resulting CTFs of TDP-43 are constituents in the inclusion bodies (17–19).

Some clinical studies have suggested that the levels of TDP-43 are up-regulated in motor neurons of sporadic ALS patients, based on the finding that the levels of TDP-43 are up-regulated in cerebrospinal fluids (20) and skin cells (21). It has also been reported that TDP-43 expression is up-regulated in some FTLD-U patients (22). TDP-43 protein levels are elevated in wobbler mice, an animal model of ALS (23). Expression of TDP-43 is maintained at substantial levels in motor neurons throughout rodent lifetime, although it is decreased in other tissues (24). Transgenic rodents overexpressing TDP-43 exhibit ALS-like and/or FTLD-U-like phenotypes (25–28). These...
results suggest that neuronal up-regulation of TDP-43 expression may cause human ALS and FTLD-U.

In this study, using an adenovirus expression system, we first show that overexpression of TDP-43 that is 2 to 5 times above the endogenous level induces death of NSC34 motor neuronal cells as well as primary cortical neurons, associated with up-regulation of Bim expression and down-regulation of Bcl-xL expression. We then showed that ER stress- or staurosporine-induced activation of caspases leads to the promotion of cleavage of TDP-43 at Asp⁸⁹ and Asp¹⁶⁹, generating CTF35 and CTF27, respectively. CTF35 and CTF27 have weaker or no death-inducing activity. In agreement, a cleavage-resistant mutant of TDP-43 showed stronger death-inducing activity than wild-type TDP-43. These results suggest that the disease-related activation of caspases may attenuate TDP-43-mediated neuronal toxicity by promoting TDP-43 cleavage.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Compounds**—The following antibodies were purchased from suppliers: TDP-43-N (10782-2-AP) and TDP-43-C (12892-1-AP), ProteinTech Group, Inc. (Chicago, IL); phospho-TDP-43 (pS409/410), Cosmo Bio Co., Ltd. (Tokyo, Japan); horseradish peroxidase (HRP)-conjugated FLAG M2, Sigma (St. Louis, MO); cleaved-caspase-3, GAPDH, Bim, Bcl-2, Mcl-1, and cleaved poly(ADP-ribose) polymerase, Cell Signaling Technology (Beverly, MA); HRP-conjugated HA antibody, Roche Diagnostics (Basel, Swiss); Bcl-xS/L, XBP-1, and GST, Japan); horseradish peroxidase (HRP)-conjugated FLAG M2, Sigma (St. Louis, MO); cleaved-caspase-3, GAPDH, Bim, Bcl-2, Mcl-1, and cleaved poly(ADP-ribose) polymerase, Cell Signaling Technology (Beverly, MA); HRP-conjugated HA antibody, Roche Diagnostics (Basel, Swiss); Bcl-xS/L, XBP-1, and GST, Santa Cruz (Santa Cruz, CA); CHOP, Affinity BioReagents (Rockford, IL); and HRP-conjugated goat anti-rabbit secondary antibody, HRP-conjugated Protein A, and HRP-conjugated goat anti-mouse secondary antibody, Bio-Rad. The TDP-43-N and -C antibodies were generated against N-terminal 260 amino acids and C-terminal 154 amino acids of human TDP-43, respectively (supplemental Fig. S1). Therefore, these antibodies recognized human TDP-43 (exogenous TDP-43) in a more sensitive fashion than mouse TDP-43 (endogenous TDP-43). Anti-TDP-43-N antibody recognized mouse TDP-43 only marginally. Thapsigargin was purchased from Sigma. Dithiothreitol and tunicamycin were purchased from Wako (Osaka, Japan); Boc-D-fmk, staurosporine, cycloheximide, MG132, and epoxomicin were purchased from Calbiochem (Darmstadt, Germany). Z-Asp-CH2-DCB and Z-ATAD-fmk were purchased from the Peptide Institute, Inc. (Osaka, Japan) and Biovision (Mountain View, CA), respectively.

**Plasmid Constructs**—A hemagglutinin (HA)-tagged human TDP-43 cDNA (pCMV-HA-TDP-43) was provided by Dr. Randal S. Tibbetts (University of Wisconsin). The TDP-43 cDNA was subcloned into the pEFl/Myc-His vector (Invitrogen) with a native stop codon for the construction of non-tagged TDP-43 expression vector (pEFl-TDP-43). A HA-TDP-43-encoding cDNA was subcloned into the pHAS-FLAG-CMV5 vector (Sigma) for the construction of an N-terminal HA- and C-terminal FLAG-tagged TDP-43 expression vector (pFLAG-CMV5-HA-TDP-43). TDP-43-D89E, -D169E, -D219E, and -M85A were obtained by site-directed mutagenesis. TDP-43 deletion mutants, such as TDP-(90–414), -(170–414), -(220–414), and -(1–89), were generated by the KOD-Plus Mutagenesis kit (Toyobo, Osaka, Japan). Methionine was N-terminal attached to the first amino acid of these deletion mutants (CTFs) of TDP-43 to generate an artificial initiation codon. The TDP-43-FLAG-encoding cDNA was subcloned into the pGEX-5X-1 vector (GE Healthcare) for the preparation of recombinant GST proteins in bacteria (supplemental Fig. S1).

**Cell Culture and Transfection**—NSC34 cell, a hybrid cell line established from a mouse neuroblastoma cell line and mouse embryo spinal cord cells, was a kind gift from Dr. Neil Cashman (University of Toronto). NSC34 cells and U2OS osteosarcoma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% of fetal bovine serum (Invitrogen). Transfection was performed using Lipofectamine (Invitrogen) and PLUS reagent (Invitrogen) under the manufacturer’s protocol.

**Primary Cortical Neurons**—Primary cortical neurons (PCNs), obtained from embryonic day 14 ICR mice, were seeded on poly-1-lysine-coated 96-well plates (Sumitomo Bakelite, Tokyo, Japan) at 5 × 10⁴ cells/well in Neuron medium (Sumitomo Bakelite) or neurobasal medium (Invitrogen) with B27 supplement (Invitrogen) and 0.5 mM 1-glutamine. Purity of neurons by this method was >98%. PCNs were infected by the indicated multiplicities of infection (m.o.i.) of adenoviruses.

**Western Blot Analysis**—Cells were homogenized with a cell lysis buffer (10 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1 mM EDTA, protease inhibitors) or 2 × sample buffer containing 4% SDS by a freeze-thaw cycle or sonication for 10 s. The samples in a SDS-containing sample buffer were boiled for 5 min at 95 °C, applied to SDS-PAGE, and blotted onto polyvinylidene fluoride membranes. Immunoreactive bands were detected with ECL Western blotting detection reagents (Amersham Biosciences). Intensities of immunodetected signals were densitometrically estimated with an ImageJ software. GAPDH was visualized as an internal control.

**Triton X-100 Solubilization Assay**—NSC34 cells were homogenized in a cell lysis buffer (10 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1 mM EDTA, protease inhibitors, phosphatase inhibitors) by pipetting and freeze-thaw cycles. The soluble fraction was defined as the supernatant of the cell lysates after centrifugation at 12,000 × g for 5 min. After complete removal of the supernatant, the cell pellets were resuspended in the buffer and sonicated for 10 s to homogenize the mixture. The solutions were then centrifuged for 5 min at 12,000 × g and the supernatant was completely removed. The resulting pellets were defined as the insoluble fractions.

**Immunocytochemistry**—NSC34 cells were transfected using Lipofectamine 2000 (Invitrogen) under the manufacturer’s protocol. At 48 h after transfection, the cells were fixed with 4% paraformaldehyde-PBS and immunostained with TDP-43-C antibody and the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit polyclonal antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Nuclei were stained with Hoechst 33258 (Sigma). The cells were observed with a confocal microscope LSM510 (Carl Zeiss).

**Adenoviral Vector-mediated Expression**—The systems of adenovirus expression vectors were purchased from Takara Co. (Shiga, Japan). The TDP-43-wt, -D89E/D169E, deletion mutants (CTFs), and human Bcl-xL cDNA (kindly provided by...
Dr. Stanley J. Korsmeyer, Dana Farber Cancer Institute) were inserted into the Swal site of a cosmids adenoviral vector, pAXCaLNLw. In this vector, a stuffer DNA fragment, sandwiched by two loxP sequences, is located just upstream of cDNA and interferes with gene expression. The TDP-43 and HA-TDP-43 cDNAs were also inserted into the pAXCaWt vector to generate adenoviruses that can express proteins without co-infection of the cre-recombinase-encoding virus. pAXCa-LacZ was obtained from TaKaRa to express LacZ. All viruses were grown in HEK293 cells and purified by CsCl2 gradient ultracentrifugation.

**Cell Death Assay and Cell Viability Assays**—Cells, seeded on six-well plates, were incubated with virus-containing medium at the indicated multiplicities of infection at 37 °C for 60 min with constant agitation. After 24 h infection, cell medium was replaced with the DMEM/N2 supplement (Invitrogen) for LDH release assay. 24 h after the medium replacement, LDH release from cells was measured with an LDH assay kit (Wako). Absorbance (Abs) of the mixtures at 560 nm wavelength was measured by a multilabel reader 2030 ARVOTM X5 (PerkinElmer Life Sciences). For the measurement of sub-G1 fractions, cell nuclei were stained with propidium iodide and analyzed by FACS analysis with FACS CaliburTM from BD Biosciences. The numbers of cells belonging to the sub-G1 fractions were determined with the MODFIT program.

Cell viability was measured by WST-8 assay and calcein staining assays. The WST-8 assay, performed using Cell Counting kit-8 (Dojindo, Osaka, Japan), was based on the ability of cells to convert a water-soluble 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt into a water-soluble formazan. Cells were treated with WST-8 reagent for 5 h at 37 °C, and absorbance was measured at 450 nm. For calcein staining, 6 μM calcein AM (3′,6′-di-O-acetyl)-2′,7′-bis(N,N-bis(carboxymethyl)aminomethyl) fluorescein, tetraacetoxymethyl ester; Dojindo) was added to cells, and more than 30 min after calcein AM treatment, calcein-specific fluorescence (excitation, 485 nm; emission, 535 nm) was measured using a spectrofluorometer (Wallac1420 ARVOx Multi Label Counter).

**siRNA-mediated Knockdown**—Bim siRNA and non-targeting control siRNA were purchased from RNAi Co., Ltd. (Tokyo, Japan). The siRNA sequence for Bim#1 and Bim#2 are 5’-CGGATCGGAGACGAGTTCAAC-3’ and 5’-CAGCTTCGTAGATTTGCTTT-3’, respectively. NSC34 cells were transfected with Lipofectamine2000 according to the manufacturer’s reverse transfection protocol. Briefly, 7 × 104 cells per well on a 6-well plate were combined with 5 nm siRNA and Lipofectamine2000 reagent complexes in Opti-MEM I Reduced Serum Medium (Invitrogen). After a 24-h incubation, cells were infected with adenovirus vectors as described above.

**Quantitative Real Time PCR Analysis**—Total RNA was extracted from NSC34 cells using the RNAiso (TaKaRa). Reverse transcription and PCR were performed on an Applied Biosystems StepOnePlus™ Real Time PCR System (Applied Biosystems, Carlsbad, CA) using the TaqMan RNA to CT 1-Step Kit (Applied Biosystems). The primers pairs and TaqMan probes for the target mRNAs were designed based on the mouse mRNA sequence using TaqMan Gene Expression Assays (Applied Biosystems, Assay ID: Bim, Mm01333921_m1; Bcl-xL, Mm00437783_m1; Bcl-2, Mm00477631_m1; Mcl-1, Mm01257352_g1; GAPDH, Mm99999915_g1). Data analysis was performed using StepOne Software version 2.0.2 (Applied Biosystems). Relative mRNA expression was analyzed by the relative standard curve method. Data were normalized to the mRNA expression of GAPDH.

**Luciferase Assay**—A Bim luciferase reporter plasmid (Bim-luc) (provided by Dr. Markus Schwaninger, University of Heidelberg) is a reporter plasmid containing the luciferase gene under control of the 5′-flanking sequences of the mouse Bim (−3600/+96). NSC34 cells, seeded onto 48-well plates at 2.5 × 104 cells/well, were transfected with the Bim-luc together with a TDP-43-encoding vector. At 48 h after transfection, luciferase assays were performed with the Dual Luciferase Reporter Assay (Promega, Madison, WI). The pRL-TK Renilla luciferase vector (0.025 μg/well) (Promega) was co-transfected to monitor transfection efficiency. Calculated luciferase activities were normalized for transfection efficiency.

**Statistical Analysis**—All values in the figures are shown as mean ± S.D. All experiments that were statistically analyzed were performed with n = 3. Statistical analysis was performed using one-way analysis of variance, followed by Turkey-Kramer post hoc analysis unless specifically indicated in the figure legends. All data were analyzed using StatView (version 5.0.1) software from SAS Institute (Cary, NC) (**, p < 0.001; ***, p < 0.01; *, p < 0.05; N.S., not significant).

**RESULTS**

**Low Level Overexpression of TDP-43 Induces Death of Motor Neurons and Primary Cortical Neurons**—To examine the mechanism underlying TDP-43-induced neuronal toxicity, we employed an adenovirus expression system to introduce TDP-43 protein into cells with high efficiency. We generated an adenovirus encoding non-tagged TDP-43, in which the cDNA encoding TDP-43 is located downstream of a stuffer DNA, and is sandwiched by two loxP sequences interfering with gene expression. If an adenovirus expressing cre-recombinase is co-introduced into the cells, the stuffer is removed and TDP-43 begins to be expressed. Although all earlier studies using mammalian cells employed tagged TDP-43 constructs, we used non-tagged TDP-43.

Using this expression system, we overexpressed human TDP-43 by 2–3 times over the endogenous level in NSC34 motor neuronal cells (Fig. 1A). The TDP-43 antibody (anti-TDP-43-C), used in this immunoblot analysis, was raised against a C-terminal peptide of human TDP-43 and recognized human TDP-43 (exogenous TDP-43) slightly more preferentially than mouse TDP-43 (endogenous TDP-43). Such low level enforced expression of TDP-43 reduced the number of viable NSC34 motor neuronal cells attached to cell dishes (Fig. 1B) and increased the percentage of cells belonging to the sub-G1 fraction, as assessed by FACS analysis (Fig. 1C). Employing another TDP-43-encoding adenovirus that expresses TDP-43 without co-infection of cre-recombinase virus for TDP-43 expression and an LDH release assay as a cell death assay, we confirmed that an increase of TDP-43 expression by 2–3 times over the endogenous level induced death (supplemental Fig. S2, A and B).
We performed similar experiments using mouse PCNs under multiple assay conditions (Fig. 1, D and E). WST-8 or calcein assays were used in addition to the LDH assay to determine cell viability in these experiments. TDP-43 virus was infected at a m.o.i. of 100 or 400, at which overexpression levels were within twice the endogenous level. Harvest was done at 5 or 7 days after adenoviral infection. Because results were basically similar under all assay conditions, we showed representative results in Fig. 1, D and E. Overexpression of TDP-43 by less than twice the endogenous level of TDP-43 (Fig. 1E) caused death of PCNs (Fig. 1D) and an increase in cleaved caspase-3 (Fig. 1E, middle panel). Based on these results, the following experiments were performed using NSC34 cells as indicator cells and LDH release assays as death assays.

**Caspases Attenuate TDP-43-induced Death**

**FIGURE 1. TDP-43 induces death of motor neuronal NSC34 cells and primary cortical neurons.** A–C, NSC34 cells, seeded on 6-well plates at 1 × 10^5/well, were co-infected with LacZ or TDP-43 virus at a m.o.i. of 400 in association with cre-recombinase or LacZ virus at a m.o.i. of 40. Cell mortality was assessed by microscopic views of cells attached to the dishes (B) and FACS analysis (C) at 48 h after infection. TDP-43 was detected with immunoblot (IB) analysis with TDP43-C antibody (A). TDP43-C antibody was raised against the C-terminal fragment of “human” TDP-43. D and E, primary cortical neurons, seeded on 96-well plates at 5 × 10^4 cells/well, were infected with LacZ or TDP-43 virus at a m.o.i. of 100 (WST-8 assay) or 400 (LDH assay and Calcein assay). These viruses are able to express proteins without co-expression of cre-recombinase. Cell mortality (LDH assay) and cell viability (WST-8 assay and Calcein assay) were assessed at 5 and 7 days after infection, respectively (D). Data were analyzed with Student’s t test. Immunoblot (IB) analyses were performed with antibody to TDP43-C or cleaved caspase-3 (E).

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**TDP-43-induced Death Is Associated with Increase of Bim Expression and Decrease of Bcl-xL Expression**—TDP-43-induced death of NSC34 cells was completely blocked by Z-Asp-CH₂-DCB, a general caspase inhibitor (Fig. 2A). TDP-43-induced death was associated with increased cleavage of poly(ADP-ribose) polymerase, a caspase substrate (Fig. 2B), and cleavage of caspase-3 (Fig. 2C and supplemental Fig. S2B). These results indicate the involvement of the caspase cascades in TDP-43-induced death.

Immunoblot analysis showed that expression of Bim, a BH3-only pro-apoptotic Bcl-2 family protein, was increased whereas expression of Bcl-xL, an anti-apoptotic Bcl-2 family protein, was reduced by overexpression of TDP-43 (Fig. 2B). In agreement, quantitative real time PCR analysis indicated that mRNA expression of Bim was increased, whereas mRNA expression of Bcl-xL was decreased by overexpression of TDP-43 (Fig. 2D). Protein levels of Bcl-2 and Mcl-1, two Bcl-2 anti-apoptotic family proteins, were unaffected by overexpression of TDP-43 although mRNA expression of Bcl-2 was reduced (Fig. 2D). Based on these results, it is assumed that a recovering system post-translationally increasing the protein level of Bcl-2 may be activated and keep the Bcl-2 protein level...
constant in TDP-43-expressing cells in which the mRNA level of Bcl-2 was reduced by overexpression of TDP-43. Luciferase assay confirmed that Bim promoter activity was enhanced by expression of TDP-43 (Fig. 2E).

Knocking Down Bim Expression Inhibited TDP-43-induced Death —To investigate whether Bim is involved in TDP-43-induced death, we examined the effect of siRNA-mediated silencing of Bim expression on TDP-43-induced death of NSC34 cells (Fig. 3). We constructed two Bim siRNAs. As shown in Fig. 3A, the siRNA-mediated reduction of Bim expression inhibited TDP-43-induced LDH release (Fig. 3A) and potentiation of caspase-3 cleavage (Fig. 3B).

Because significant amounts of cleaved caspase-3 were still present in cells overexpressing TDP-43 when endogenous Bim was knocked down by siRNA, it is highly likely that TDP-43-induced activation of caspases is partly independ-
Caspases Attenuate TDP-43-induced Death

FIGURE 3. TDP-43 induces cell death by up-regulating Bim. A and B, NSC34 cells, seeded on 6-well plates at 7 × 10^5 cells/well, were transfected with 5 nM si-control (Cont.), siBim1, or siBim2 using Lipofectamine 2000 reagent. 24 h after transfection, cells were infected with LacZ or TDP-43 virus at a m.o.i. of 800. All samples were co-infected with cre-recombinase virus at a m.o.i. of 40. 24 h after infection, medium was replaced with DMEM/N2 supplement. 24 h after medium replacement, LDH release was measured (A). Immunoblot (IB) analyses were performed with antibodies to TDP43-C, Bim, and cleaved caspase-3 (B).

Caspases Attenuate TDP-43-induced Death

Overexpression of TDP-43 Down-regulated Bcl-xL Expression—Earlier studies have shown that Bcl-xL expression is reduced in motor neurons of mouse models of ALS and in human ALS cases (29, 30). In accordance, Bcl-xL expression was reduced by overexpression of TDP-43 (Fig. 2, A and B).

We also found by chance that TDP-43-induced death and cleavage of caspase-3 were inhibited by blockers of proteasomal cleavage sites (D-G/S/A) (36) at Asp23, Asp65, Asp89, Asp169, and Asp406. Based on expected molecular weights, weak protein bands, migrating around CTF27, also became apparent in the TDP-43-(D169E) lane (Fig. 6B). These proteins may represent other CTFs, some of which have been already reported (9, 10, 17, 37). The D219E mutation did not affect the generation of CTF35 and CTF27, respectively (Fig. 6B). A very weak protein band, migrating slightly slower than CTF35 in SDS-PAGE analysis, became apparent in the TDP-43-(D89E) lane. Multiple weak protein bands, migrating around CTF27, also became apparent in the TDP-43-(D169E) lane (Fig. 6B). Notably, staurosporine treatment induced TDP-43 cleavage at the same sites as those induced by thapsigargin (Fig. 6C). This result indicates that activated caspases, including caspases 3, 7, and 12, cleave TDP-43 at Asp89 and Asp169.

A recent study revealed an alternative translational product of TDP-43, resembling CTFT5, whose initiation methionine is Met85 (37). To address whether CTFT5 is identical to this product, NSC34 cells transiently expressing the mutant TDP-43-(M85A), which lacked the alternative initiation site, were treated with thapsigargin. The M85A mutation did not restore the generation of CTFT5 (supplemental Fig. S4C).

In contrast to CTFs, N-terminal fragments (NTFs) of TDP-43 were not detected in NSC34 cells by immunoblot analysis. This suggests that activated caspases, including caspases 3, 7, and 12, cleave TDP-43 at Asp89 and Asp169.

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In contrast to CTFs, N-terminal fragments (NTFs) of TDP-43 were not detected in NSC34 cells by immunoblot analysis (supplemental Fig. S5, A–C). However, an in vitro reconstituted cleavage assay using purified recombinant GST–TDP-43-

Caspases Attenuate TDP-43-induced Death

ER Stress Induces TDP-43 Cleavage—Multiple TDP-43 fragments are deposited in neurons of ALS and FTLD-U patients (1, 2, 17, 18). Caspase activation has been observed in ALS and FTLD patients (12–16). TDP-43 is cleaved in a caspase-dependent manner and the resulting CTFs of TDP-43 are constituents of inclusion bodies (9–11, 17, 18). Recent studies of autopsied ALS patients and ALS models have suggested that ER stress markers are up-regulated in ALS motor neurons (32, 33). Because ER stress induces caspase activation (34), it is likely that ER stress may affect ALS pathogenesis (35). Therefore, we first examined whether proteolytic cleavage of TDP-43 is induced by various ER stress inducers in NSC34 cells. Treatment with thapsigargin reduced endogenous TDP-43 expression and increased the levels of 35- (CTF35) and 27-kDa (CTF27) fragments (Fig. 5A). TDP-43 and HA–TDP-43–FLAG were also cleaved by treatment with other ER stress inducers (Fig. 5, B and C). These results suggest that the ER stress-induced activation of caspases may lead to TDP-43 cleavage and the generation of CTFs.

Cleavage of TDP-43 at Asp89 and Asp169 is Mediated by Caspase Activation—Treatment with Boc-D-FMK, a pan-caspase inhibitor, or Z-ATAD-fmk, an inhibitor of ER stress-related caspase-12, reduced cleavage of TDP-43, induced by thapsigargin (Fig. 6A, supplemental Fig. S3). These results suggest that ER stress-induced cleavage of TDP-43 is mediated by activation of caspases including caspase-12.

TDP-43 contains three caspase-3/7 cleavage consensus sites (DXXD) at Asp13, Asp89, and Asp169, and at least 5 potential caspase cleavage sites (D-G/S/A) (36) at Asp23, Asp45, Asp89, Asp169, and Asp406. Based on expected molecular weights, Asp89, Asp169, and Asp219 were artificially replaced by glutamate (supplemental Fig. S1) and these TDP-43 mutants were transiently expressed in NSC34 cells, followed by treatment with thapsigargin. Immunoblot analysis indicated that the D89E and D169E replacements disrupted the generation of CTFT5 and CTFT27, respectively (Fig. 6B). A very weak protein band, migrating slightly slower than CTFT35 in SDS-PAGE analysis, became apparent in the TDP-43-(D89E) lane. Multiple weak protein bands, migrating around CTFT27, also became apparent in the TDP-43-(D169E) lane (Fig. 6B). These proteins may represent other CTFs, some of which have been already reported (9, 10, 17, 37). The D219E mutation did not affect the generation of CTFs (supplemental Fig. S4A). We confirmed that the molecular sizes of CTFT5 and CTFT27 were almost identical to those of transiently expressed TDP-43-(90–414) and TDP-43-(170–414), in which methionine was N-terminal attached to the first amino acid to generate ATG initiation codons (supplemental Fig. S4B).

Notably, staurosporine treatment induced TDP-43 cleavage at the same sites as those induced by thapsigargin (Fig. 6C). This result indicates that activated caspases, including caspases 3, 7, and 12, cleave TDP-43 at Asp89 and Asp169.

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FLAG and active caspase-7 showed two GST-NTFs (supplemental Fig. S5D). The protein sizes suggest that the 37-kDa GST-NTF corresponds to N-terminal fragment (1–89) of TDP-43, from which CTF35 was removed, whereas the NTFs, generated by removal of CTF27 from TDP-43, were undetected. Based on these results, it is likely that NTFs of TDP-43 are highly prone to degradation in the cells.

CTF27, a Triton X-100-insoluble, Hyperphosphorylated, and Aggregated Protein, Is Prone to Degradation by the Proteasomal System—To characterize the CTFs of TDP-43 biochemically, we performed a Triton X-100 solubilization assay. As shown in the upper panel of Fig. 7A, the majority of TDP-43 and CTF35 was contained in Triton X-100-soluble fractions. In contrast, relatively larger amounts of CTF27 were contained in the Triton X-100-insoluble fraction. Immunoblot analysis using an antibody recognizing phosphorylated Ser409/Ser410 of TDP-43 revealed that CTFs in the Triton X-100-insoluble fraction was more prone to phosphorylation, as indicated by a supershifted band, according to the previous studies (Fig. 7A, lower panel) (9–11, 19, 38).

Immunocytochemical analysis showed that the major portions of TDP-43 and CTF35 were diffusely localized to the nucleus and cytoplasm, respectively (Fig. 7B). Minor portions of TDP-43 and CTF35 appeared to be localized in the cyto-
plasm and nucleus, respectively (Fig. 7B). CTF27 was localized in the cytoplasm and tended to form aggregates (Fig. 7B).

The TDP-43 cleavage assay using NSC34 cells treated with ER stress inducers (Fig. 5A) showed that the amount of CTF27 generated was smaller than that of CTF35. In contrast, the in vitro reconstituted cleavage assay indicated that the amount of CTF27 generated was nearly equal to that of CTF35 (supplemental Fig. S5D). These observations prompted us to examine whether CTF27 was more prone to degradation than CTF35. NSC34 cells, transfected with CTF35 or CTF27, were treated with cycloheximide for 6–12 h. As shown in Fig. 7C, CTF27 was more rapidly degraded than CTF35, which was inhibited by the proteasomal inhibitors (Fig. 7D).

TDP-43 Cleavage Leads to the Attenuation of TDP-43-induced Death—Earlier studies showed that overexpression of GFP-tagged CTFs as well as that of GFP-tagged TDP-43 was toxic to mammalian cells (11). Adenovirus-mediated overexpression of CTF35 induces death of NSC34 cells but its induction of death was weaker than full-length TDP-43 (Fig. 8A). CTF35 as well as full-length TDP-43-induced death was associated with increased Bim expression and decreased Bcl-xL expression (Fig. 8B). In contrast to CTF35, CTF27 appeared to lose the ability to induce cell death (Fig. 8A). The low levels of CTF27 expression may be caused by its high degradation rate (Figs. 7C and 8B). By co-incubating cells expressing CTF27 with MG132, we increased the expression levels of CTF27 (Fig. 8B).
but did not observe CTF27-induced death (Fig. 8A). This result was anticipated because TDP-43-induced death was inhibited by MG132 treatment (Fig. 4, A and B).

To obtain higher expression of CTF27, we increased the m.o.i. of CTF27-encoding adenovirus to 800 for infection of U2OS cells. The expression levels were higher than those of TDP-43 expressed by infection of TDP-43-encoding virus at a m.o.i. of 100 (Fig. 8C). However, cell death assays indicated that overexpressed CTF27 did not induce death, whereas full-length TDP-43 did (Fig. 8D). We were unable to increase expression of CTF27 in NSC34 cells (data not shown).

To confirm that caspase-induced cleavage of TDP-43 reduces TDP-43-induced death, we constructed an adenovirus encoding TDP-43-(D89E/D169E) that is resistant to cleavage by caspases. Immunoblot analysis showed that thapsigargin- and staurosporine-induced generation of CTF35 and CTF27 was disrupted and expression of the full-length TDP-43 was increased by the D89E/D169E mutations (supplemental Fig. S6). We compared the extent of death induced by TDP-43-(D89E/D169E) with that by TDP-43-wt (Fig. 8E, F). Immunoblot analysis, simultaneously performed, indicated that the TDP-43 level was slightly increased by the mutations (Fig. 8E, short exposure). As a consequence, TDP-43-(D89E/D169E) showed stronger death-inducing activity than TDP-43-wt when expressed by infection of adenovirus at the same m.o.i. Note that an uncharacterized protein, migrating slightly slower than CTF35, again became apparent in the TDP-43-(D89E/D169E) lane instead of CTF35 (Fig. 8E), as already shown in Fig. 6B. TDP-43-(D89E/D169E) showed subcellular localization similar to that of TDP-43-wt (supplemental Fig. S7).

It is possible that CTFs show toxicity only in the presence of appropriate amounts of full-length TDP-43. To examine this possibility, we adenovirally co-expressed CTF35 or CTF27 in association with full-length TDP-43. Co-expression of TDP-43 did not appear to increase CTF35- or CTF27-induced toxicity (Fig. 8G and H). Notably, co-expression of CTF27 appeared to attenuate TDP-43-induced death and cleavage of caspase-3 (Fig. 8G and H).

**DISCUSSION**

TDP-43 may exhibit neuronal toxicity in gain-of-function and loss-of-function manners (see Refs. 3 and 4 for review). In many earlier studies, the gain-of-function phenotypes of TDP-43 were examined by overexpression of TDP-43. Overex-
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FIGURE 7. CTF27, a Triton X-100-insoluble, hyperphosphorylated, and aggregated protein is prone to degradation by the proteasomal system. A, NSC34 cells transiently overexpressing TDP-43 or CTFs of TDP-43 were harvested at 48 h after transfection for fractionation into Triton X-100-soluble or -insoluble fractions. Immunoblot (IB) analyses were performed with TDP43-C and phospho-TDP-43 (pS409/p410) antibodies. *, phosphorylated TDP-43. B, NSC34 cells transiently overexpressing TDP-43 or CTFs of TDP-43 were fixed and immunostained with anti-TDP43-C antibody (green). Nuclei were stained with Hoechst 33258 (blue). C, NSC34 cells transiently overexpressing CTFs of TDP-43 were treated with 50 μg/ml of cycloheximide (CHX) for 6–12 h and harvested for subsequent immunoblot analysis with TDP43-C antibody. <, TDP-43-(90–414); ≤, TDP-43-(170–414). D, NSC34 cells transiently overexpressing TDP-43-(170–414) were treated with 50 μg/ml of cycloheximide for 6 h in the presence or absence of 2 μM MG132 or 2.5 μM epoxomicin and harvested for subsequent immunoblot analysis with TDP43-N antibody.

expression of TDP-43 resulted in toxicity in yeast cells and mammalian cells in vitro (11, 39). Transgenic flies overexpressing human TDP-43 recapitulate the neuropathological and clinical features of human TDP-43 proteinopathy (40, 41). Transgenic rodents overexpressing TDP-43 exhibit ALS-like and/or FTLD-U-like phenotypes (25–28). Recent clinical studies have suggested that TDP-43 expression is up-regulated in human ALS and FTLD-U cases (20–22). In the present study, we showed that adenovirus-mediated very low-grade overexpression of TDP-43 causes death of NSC34 motor neuronal cells and PCNs.

The mechanism underlying TDP-43-induced neuronal death has not been clarified. In this study, we have shown that TDP-43-induced toxicity is at least partly mediated by up-regulation of Bim expression and associated with down-regulation of Bcl-xL expression. Up-regulation of Bim expression has been noted in motor neurons of the G93A-SOD1-transgenic ALS model mice (42, 43). Disruption of the Bim gene has been shown to prevent motor neurons death (42). Puma, another BH3-only Bcl-2 family protein, has been shown to be involved in motor neuron death (43). Bcl-xL and Bcl-2 were reduced in brain areas where motor neuron death occurred in ALS model mice (29). Bcl-xL expression was found to be reduced in motor neurons of human ALS cases (30). These findings together suggest that the abnormal regulation of Bcl-2-related proteins may be responsible for the progression of motor neuron death in ALS. However, it remains unknown whether expression of Bcl-2 family proteins other than Bim, Bcl-xL, Bcl-2, and Mcl-1 is altered in association with TDP-43-induced cell death.

Our results have shown that CTF35 and CTF27 are generated by caspase activation including that induced by ER stress or staurosporine. We have preliminarily found that overexpression of TDP-43 by itself appears to induce ER stress (supplemental Fig. S8) due to an uncharacterized mechanism. Although multiple CTFs have been already identified (9, 10, 17, 37), CTF27, identical to TDP43-(170–414), is newly identified in this study. In the cells treated with ER stress inducers or staurosporine, we did not identify CTF-(220–414), which had been speculated to be produced, based on the fact that Asp219 is a consensus site (DXXD) of caspase 3/7–mediated cleavage (17).

Low-grade TDP-43 cleavage was also observed in normal cells and in ER-stressed cells treated with a caspase inhibitor (Figs. 5A and 6A). One possible reason accounting for this observation is that other unknown molecules than caspases may be involved in the TDP-43 cleavage.

We confirmed that CTFs are more prone than full-length TDP-43 to form cytoplasmic aggregates (Fig. 7B), as shown in earlier studies (9–11). We also reproduced the finding, shown in an earlier report, that CTF is toxic to yeast cells (supplemental Fig. S9) (39). On the other hand, our in vitro death assays using NSC34 cells have shown that CTFs elicit no or weaker toxicity, compared with full-length TDP-43 (Fig. 8). Furthermore, it is likely that CTF27 may possess preventive activity against TDP-43-induced cell death (Fig. 8, G and H). Based on these observations, it is likely that caspases, activated by many neuronal stressors, may attenuate TDP-43 toxicity to neurons by inducing TDP-43 cleavage. However, the results of this study do not rule out the possibility that CTFs cause additional toxicity to neurons via surrounding non-neuronal cells in vivo (44).

Inconsistently with our results, an earlier report showed that GFP-tagged CTF25 (TDP-43-(220–414)) was more toxic than full-length TDP-43 to M17 neuroblastoma cells (11). The rea-
son for the discrepancy between our observation and that of the earlier study remains speculative. The most important difference between the earlier study and the present study is the presence of tags attached to TDP-43. In the present study, non-tagged full-length TDP-43 was compared with non-tagged CTFs of TDP-43. The function of TDP-43 may be affected by

FIGURE 8. Cleavage of TDP-43 leads to attenuation of TDP-43-induced death. A, NSC34 cells, seeded on 6-well plates at 1 × 10^5 cells/well, were co-infected with the indicated virus at a m.o.i. of 400 in association with cre-recombinase or LacZ virus at a m.o.i. of 40. 24 h after infection, cell medium was replaced with DMEM/N2 supplement. B, immunoblot (IB) analyses were performed with antibodies to cleaved caspase-3, Bim, Bcl-xL, and TDP43-C. #, GAPDH band. C and D, U2OS cells, seeded on 6-well plates at 5 × 10^4/well, were co-infected with TDP-43-(1–414) or TDP-43-(170–414) virus at a m.o.i. of 100 for TDP-43-(1–414) or 800 for TDP-43-(170–414) in association with cre-recombinase or LacZ virus at a m.o.i. of 40. 24 h after infection, cell medium was replaced with DMEM/N2 supplement. E and F, NSC34 cells, seeded on 6-well plates at 1 × 10^5/well, were co-infected with an indicated adenovirus at a m.o.i. of 400. All samples were co-infected with cre-recombinase virus at a m.o.i. of 40. 24 h after infection, cell medium was replaced with DMEM/N2 supplement. 24 h after medium replacement, LDH release from cells was measured (G). Immunoblot analysis was performed with antibodies to cleaved caspase-3, Bim, Bcl-xL, and TDP43-C (H).
the presence of tags, as shown for the VAPB-induced unfolded response reaction and solubility of VAPs (45). In this study, we have found that an N-terminal HA tag and a C-terminal FLAG tag, both of which are relatively short tags, markedly affected TDP-43-induced death of NSC34 motor neuronal cells and the TDP-43-induced activation of the Bim promoter (supplemental Fig. S10, A and B). It is notable that HA-tagged TDP-43-induced death of NSC34 cells was not associated with increased Bim expression, whereas non-tagged TDP-43 induced death is associated with increased Bim expression (supplemental Fig. S10A). Immunocytochemical analysis showed that the HA tag did not alter nuclear localization (supplemental Fig. S10C). This result suggests that a tag may alter not only the extent of TDP-43-induced death but also the nature of TDP-43-induced death.

Another important difference is the degree of overexpression. In the present study, the level of TDP-43 overexpression is regulated by the adenovirus expression systems only at 2–5 times over the endogenous level, whereas in earlier studies the overexpression levels were not regulated. Conflicting results may also originate from the difference in the cells and the expression system used.

Multiple studies have reported the caspase-mediated fragmentation/degradation and inactivation of apoptotic inducers as negative feedback mechanisms (46–48) that act similarly to the activated caspase-mediated attenuation of TDP-43-induced death, shown in this study. Bim may be degraded by caspase-3 as a negative feedback mechanism in osteoclasts (46).

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