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Spinocerebellar ataxia type 35 (SCA35)-associated transglutaminase 6 mutants sensitize cells to apoptosis

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ABSTRACT

Spinocerebellar ataxia type 35 (SCA35) is an autosomal dominant neurodegenerative disorder. In our previous study, using exome sequencing and linkage analysis, two missense mutations of the transglutaminase 6 (*TGM6*) gene were identified as causative for SCA35. *TGM6* encodes transglutaminase 6 (TG6), a member of the transglutaminase family of enzymes that catalyze the formation of a covalent bond between a free amine group and the γ-carboxamide group of protein- or peptide-bound glutamine. However, the precise role of TG6 in contributing to SCA35 remains unclear. In this study, we analyzed the subcellular distribution, expression and in vitro activity of two missense mutations of TG6 (D327G, L517W) and found that both mutants exhibited decreased transglutaminase activity and stability. Furthermore, overexpressing the TG6 mutants sensitized cells to staurosporine-induced apoptosis by increasing the activity of caspases. We propose that the pro-apoptotic role of these mutants might underlie the pathogenesis of SCA35.

1. Introduction

Spinocerebellar ataxias (SCAs) represent a large and complex group of neurodegenerative disorders characterized by progressive cerebellar ataxia associated with a variable combination of pyramidal and extrapyramidal signs, neuropathy, ophthalmoplegia, cognitive impairment and epilepsy [1]. The prevalence of SCAs is estimated to be 1–5 cases per 100,000 individuals, and disease onset typically occurs between 30 and 50 years of age [2]. SCAs are diagnosed genetically according to a specific mutation or mapped locus in addition to clinical findings.

Previously, we identified transglutaminase 6 (*TGM6*) as a novel SCA-causing gene (namely, SCA35) using exome sequencing. Two missense mutations of *TGM6* (L517W and D327G) were found in two Chinese families with ataxia [3]. Recently, another missense mutation in the *TGM6* gene (D510H) was identified in a Chinese SCA family in Hong Kong [4]. Transglutaminase 6 (TG6), the protein encoded by *TGM6*, belongs to the transglutaminase family of enzymes that are able to modify proteins through an acyl-transfer reaction between the γ-carboxamide group of peptide-bound glutamine and a primary amine. Transglutaminases have three functional domains: a catalytic core domain; an amino terminal β-sandwich domain, which forms a functional unit with the core domain; and two C-terminal β-barrel domains involved in the regulation of enzyme activity [5]. The SCA35-causing mutations are strictly conserved in *TGM6* among different species. Structural models suggest that the D327G mutation lies in the core domain and might interfere with the ability to bind Ca^{2+}, and the L517W mutation lies in the first transglutaminase C-terminal domain [3].

TG6 is expressed in the kidney, skin, eyes and neurons, but it is absent from skeletal muscle and joints [6]. Immunocytochemistry indicates that TG6 is expressed in neuronal cells, but not in astroglial or oligodendroglial cells [5]. Recently, TG6 deposits were reported to be present in the cerebellum of patients with gluten ataxia, a sporadic cerebellar ataxia with the presence of circulating anti-gliadin antibodies [7]. Therefore, antibody against TG6 could serve as a biomarker to identify a subgroup of gluten-sensitive patients who may be at risk of developing neurological diseases [8]. Animal studies have further shown that intraventricular injection of anti-TG2/3/6 cross-reactive antibody single-chain variable fragments provoked ataxia in mice [9]. Nevertheless, the underlying mechanisms whereby TG6 dysfunction causes ataxia are still unknown.

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In this study, we compared the enzymatic activity, stability, and subcellular distribution of SCA35-associated mutant TG6 with the wild type (WT) enzyme. In addition, we explored the relationship between TG6 and apoptosis, to understand the functional consequences of these mutations and gain insights into the pathogenic mechanisms involved in SCA35.

2. Materials and methods

2.1. Construction of TGM6 expression vectors

Full-length human *TGM6* complementary DNA (cDNA) cloned into the mammalian expression vector pcDNA3.1-myc-his(−)B (Invitrogen) was used as the template for mutagenesis. Mutations were introduced using the QuikChange site-directed mutagenesis protocol (Stratagene). All constructs were verified by Sanger direct sequencing.

2.2. Establishment of stable cell lines overexpressing TG6

Human embryonic kidney HEK293 and mouse fibroblast NIH3T3 cells were grown at 37 °C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml of penicillin/streptomycin. The expression vectors for WT, D327G and L517W TG6 or the empty vector were transfected into cells with Lipofectamine 2000 (Invitrogen). HEK293 and NIH3T3 stable cell lines were established by selection with 1 mg/ml G418 (Calbiochem) and isolated by ring cloning. Positive clones were confirmed by Western Blot analysis and maintained in 600 μg/ml G418.

2.3. Transamidation activity assay

Twenty-four hours before transfection, HEK293 cells were seeded at approximately 75% confluency into 12-well plates and were then transfected with 1 μg of each plasmid. At 48 h after transfection, transamidation activity was detected by a transglutaminase assay kit (Sigma). The assay is based on the transglutaminase catalysis of a covalent bond formation between a free amine group of poly-L-lysine, which is covalently attached to the plate surface, and the γ-carboxamide group of a biotin-TVQQEL-OH substrate present in the assay buffer. The cells were washed with phosphate balanced solution (PBS) and lysed in 200 mM Tris–acetate buffer (pH 6.0) containing 1% Triton X-100. The total protein content of the lysate was measured with the BCA protein assay (Pierce). Equal amounts of total cell lysates were added into a 96-well substrate-coated plate. After incubation at room temperature for 30 min, the wells were washed 3 times with water and further incubated with a streptavidin-peroxidase solution for 20 min. After washing three times with PBS/0.05% Triton X-100, the tetramethylbenzidine (TMB) liquid substrate was added, and the plate was incubated for 10 min. The color development was stopped with addition of a stop solution, and the absorption at 450 nm was measured. Data were analyzed using Prism 5 software (GraphPad).

2.4. Immunofluorescence assays

HEK293 cells were grown on poly-D-lysine-coated coverslips and transfected with WT or mutant TG6 constructs. At 48 h after transfection, the cells were fixed in 4% paraformaldehyde. Anti-c-Myc primary antibody (Cell Signaling) was added, and the coverslips were incubated for 2 h at room temperature. At the same time, the cells were co-stained with antibodies against the endoplasmic reticulum marker calnexin (Sigma), the mitochondrial marker Tom20 (Santa Cruz) or the Golgi marker GM130 (BD Biosciences). Fluorescence-labeled secondary antibodies (Invitrogen) were then added, and the cells were incubated for 1 h in a dark room. After incubation with 4, 6-diamino-2-phenylindole (DAPI, Invitrogen) for 3 min, the cells were mounted in Fluoromount medium (Sigma) and examined with a laser scanning confocal system installed on a Carl Zeiss microscope. The images were analyzed using the Metaphor software package.

2.5. Western Blot assays

Proteins were separated by 10% SDS–PAGE and then transferred onto a polyvinylidene difluoride membrane (Millipore). The following primary antibodies were used: anti-c-Myc antibody (Cell Signaling), anti-caspase 3 antibody (Cell Signaling), or mouse anti-caspase 9 antibody (MBL). Sheep anti-mouse or anti-rabbit IgG-HRP antibodies (Sigma) were used as secondary antibodies. Detection was performed using the ECL plus kit (GE Healthcare). The bands were scanned and quantified with the NIH Image J software.

2.6. Analysis of TG6 stability

Twenty-four hours before treatment, the HEK293 stable cell lines were seeded into 12-well plates. Cycloheximide (CHX, Sigma) was added to a final concentration of 0.1 mg/ml to suppress new protein synthesis. The cells were lysed at 0, 4, 8 and 12 h after CHX treatment. Equal amounts of lysates were analyzed by Western Blot analysis.

2.7. Cell treatment and apoptosis assay

The NIH3T3 stable cell lines were seeded into a 6-well plate and treated with 0.5 μM staurosporine (STS, Sigma) for 24 h. The cells were lysed, and cleaved caspase-3 and caspase-9 were detected by Western Blot. For fluorescence-activated cell sorting (FACS) analysis, the NIH3T3 stable cell lines were treated with 0.5 μM STS for 24 h. The HEK293 stable cell lines were treated with 5 μM calcium ionophore A23187 (Sigma) or 4 μM STS for 30 h. The analysis of apoptotic cells was carried out with a FACSCalibur flow cytometer (BD Biosciences) using the Annexin V-FITC Apoptosis Detection Kit (Sigma). For each sample, 10,000 ungated events were acquired; AnnexinV(+)/PI(−) cells were considered to be the early apoptotic population, and Annexin V(+)/PI(+) cells were considered to be late apoptotic and necrotic cells.

3. Results

3.1. Mutations in TG6 affected its transamidation activity

Transglutaminases (TGs) are Ca^{2+}-dependent enzymes that modify proteins through an acyl-transfer reaction between the γ-carboxamide group of peptide-bound glutamine and a primary amine [10]. We first sought to study whether SCA35-associated mutations affect the transamidation activity of TG6. WT or mutant TG6 expression constructs were transfected into HEK293 cells, and the cell lysates were used to measure the transamidation activity. As shown in Fig. 1A, the lysates from cells transfected with the WT TG6 expression construct displayed significantly higher transamidation activity than the lysates from cells transfected with the empty vector. However, lysates from cells transfected with either of the mutant TG6 constructs exhibited significantly reduced enzymatic activity. To investigate whether the decreased activities of the lysates containing mutant TG6 were caused by the ectopic expression levels of mutant TG6, we performed Western Blot analysis on

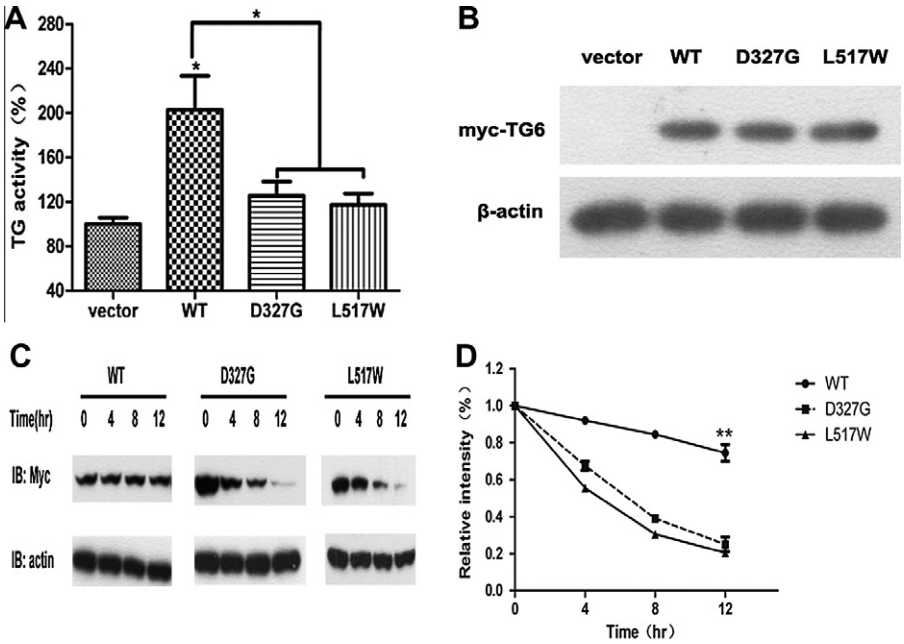

Fig. 1. The transamidation activity, exogenous expression and stablity of TG6. (A) HEK293 cells were transfected with empty vector, WT or mutant TG6 expression plasmids. The cells were lysed, and the transamidation activity was detected with a transglutaminase assay kit. The data are reported as the percentages of TG activity, with 100% corresponding to the activity for cells transfected with empty vector. The data are presented as the mean ± SEM from three independent experiments. ($^{*}P < 0.05$ by one-way ANOVA with Dunnett's multiple comparison tests). (B) HEK293 cells were transfected with WT or mutant TG6 plasmids. The expression of TG6 was visualized using a c-Myc antibody. Actin was used as a loading control. (C) HEK293 stably transfected cells were harvested at 0, 4, 8 and 12 h after CHX (0.1 mg/ml) treatment. The expression of TG6 was visualized by Western Blot using a c-Myc antibody. (D) The graph illustrates the quantitation of WT and mutant TG6 by densitometry of triplicate experiments (mean ± SEM, $^{**}P < 0.01$ by two-way ANOVA with Bonferroni tests). The relative intensity of an untreated sample was set as one.

the proteins extracted from the HEK293 cells transfected with WT or mutant TG6. As shown in Fig. 1B, WT and mutant TG6 proteins were detected at the expected size. There was no apparent difference in expression levels between WT and mutant TG6.

3.2. Mutations in TG6 affected the stability of the TG6 protein

We further compared the stability of mutant TG6 with the WT enzyme. The HEK293 stable cell lines were treated with CHX (0.1 mg/ml) to suppress protein synthesis. The cells were harvested at 0, 4, 8 and 12 h after the addition of CHX, and the protein levels of TG6 were measured by Western Blot analysis. As shown in Fig. 1C and D, WT TG6 was quite stable under this condition, with a half-life longer than 12 h. However, both the D327G and the L517W mutant of TG6 exhibited a significantly shorter half-life, suggesting that SCA35-causing mutations resulted in destabilization of the TG6 protein.

3.3. Subcellular distribution of TG6 proteins

Exogenously expressed WT and mutant TG6 in HEK293 cells were visualized by immunofluorescence with confocal laser scanning microscopy. As shown in Fig. 2, WT TG6 was localized in the cytoplasm. TG6 did not co-localize with any of the three organelle (ER, Golgi, and lysosome) markers [11]. Notably, neither of the mutations changed the subcellular localization of TG6. The same expression pattern was also observed in NIH3T3 and mouse neuroblastoma Neuro2a cell lines (data not shown).

3.4. Mutant TG6 sensitized the cells to apoptosis

Activation of the apoptotic pathway may be responsible for the neuronal death observed in SCAs [12]. Notably, TG2 has been implicated in the regulation of apoptosis [13]. We thus speculate that TG6 may be involved in apoptosis regulation and that mutant TG6 may disturb this process. To determine the role of TG6 in apoptosis, we established HEK293 and NIH3T3 cell lines stably expressing WT or mutant TG6. There was no difference in cell viability under normal culture conditions. STS, a protein kinase inhibitor, is one of the most potent and frequently used pro-apoptotic stimuli that trigger the classical mitochondrial apoptosis pathway [14]. As shown in Fig. 3A–C, STS induced significant apoptosis in both NIH3T3 and HEK293 cell lines. The cell lines stably expressing WT TG6 exhibited significantly less apoptosis than the controls. However, the cell lines expressing mutant TG6 had significantly increased levels of apoptosis compared with the empty vector controls. This phenomenon was recapitulated with A23187 (Fig. 3D), a calcium ionophore that increased the cytosolic free calcium concentration and induced apoptosis in HEK293 cells.

3.5. Mutant TG6 sensitized cells to STS-induced apoptosis by increasing the activities of caspases

Caspases exist as inactive proenzymes in cells and are activated through processing into two subunits in response to apoptotic stimulation. Caspase-9, a 45 kDa protein, is triggered by cytochrome c released from mitochondria, and activated caspase-9 activates the downstream caspase-3, a critical executioner of apoptosis [15]. Western Blot assays were performed to detect the cleavage of caspase-3 and caspase-9 in stably transfected NIH3T3 cells after STS treatment (Fig. 4A). The activated form of caspase-3/9 appeared after 16 h in STS-treated NIH3T3 cells, whereas treatment of the NIH3T3 cells stably expressing WT TG6 with STS resulted in the decreased appearance of caspase-3/9 fragments compared with the controls. However, cells stably expressing mutant TG6 were more prone to activate caspase-3/9 than the controls (Fig. 4B). These results indicate that mutant TG6 might sensitize cells to STS-induced apoptosis by increasing caspase-3/9 activities.

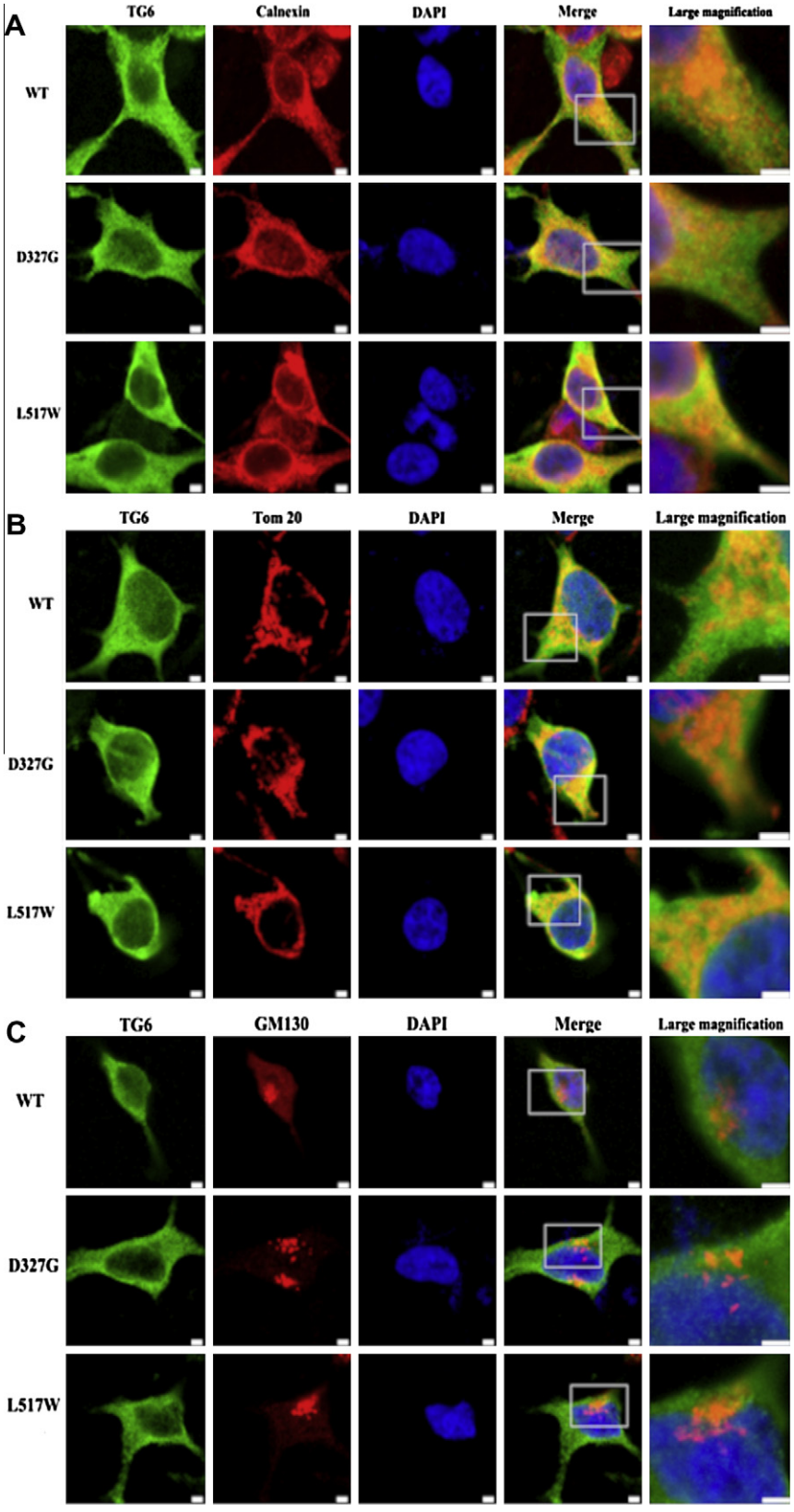

Fig. 2. Subcellular distribution of TG6. HEK293 cells were transfected with WT or mutant TG6 plasmids. An anti-c-Myc antibody was used for detecting exogenous TG6. Calnexin, Tom20 and GM130 were used as markers for endoplasmic reticulum, mitochondria and Golgi, respectively. Co-localization was analyzed by confocal laser scanning microscopy. TG6 is shown in green, DAPI-stained nuclei are blue, the three organelles are shown in red, merged images are shown in the 4th row, and magnified merged images of boxed regions are shown in the 5th row. Scale bar = 2.5 μm

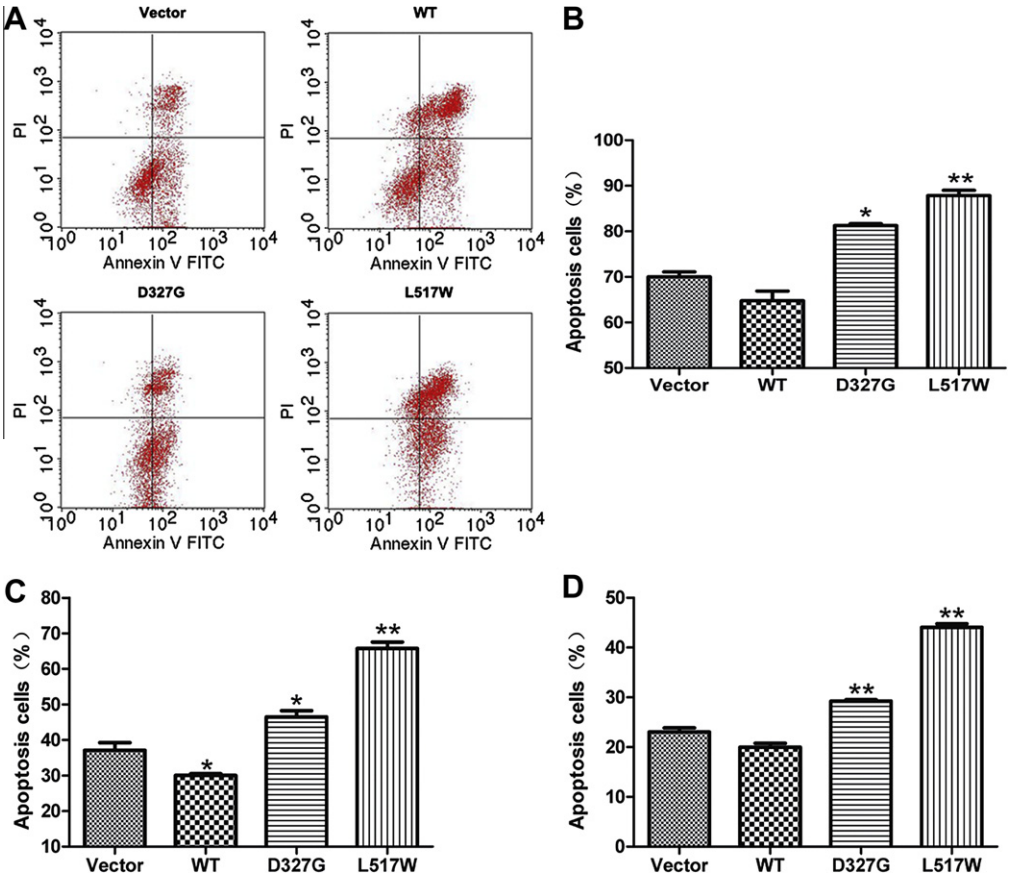

Fig. 3. Mutations in TG6 sensitized cells to apoptosis. (A) Representative images for FACS analysis of apoptosis induced by 0.5 μM STS treatment for 24 h in NIH3T3 cells. The upper right quadrant [Annexin V (+)/PI(+)] contains the early apoptotic population, the upper left quadrant [Annexin V (+)/PI(−)] contains the late apoptotic and necrotic population. (B) Statistical analysis of the FACS analysis of apoptosis induced by 0.5 μM STS treatment for 24 h in NIH3T3 cells. (C) Statistical analysis of the FACS analysis of apoptosis in HEK293 cells induced by 4 μM STS for 30 h. (D) Statistical analysis of the FACS analysis of apoptosis in HEK293 cells induced by 5 μM A23187 for 30 h. Asterisks indicate statistically significant differences compared with empty vector controls ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by one-way ANOVA with Dunnett's multiple comparison tests)

4. Discussion

Thirty-one different types of SCA have been identified to date. Most of the SCAs are caused by an abnormal expansion of a CAG repeat sequence encoding an expanded tract of polyglutamine (polyQ) residues within the mutated protein [16]. One possible way that might induce neuronal death is by activating caspases and the associated cell death pathways directly [17]. Neuronal apoptosis can be induced both in vivo and in vitro by different stimuli, including oxidative stress, perturbed calcium homeostasis, mitochondrial dysfunction and the deficiency of survival factors [18].

Several lines of evidence suggest that activation of the apoptotic pathway is responsible for the neuronal death observed in SCAs. (i) Expression of a portion of the MJD/SCA3 locus, including the expanded CAG repeat, has been shown to cause apoptosis in cultured cells [19]. (ii) Activated caspase-3 is recruited into the inclusions in both SCA7 cell models and human SCA7 brain biopsies, and its expression is up-regulated in cortical neurons [20]. (iii) Sanchez et al. demonstrated that expression of expanded polyQ tracts in primary rat neurons leads to the recruitment of caspase-8 to the polyQ aggregates, resulting in its activation [21]. (iv) The CAG expansion in SCA12 is located in a 5′-untranslated region of the gene *PPP2R2B*, encoding the Bβ1 and Bβ2 regulatory subunits of protein phosphatase 2 (PP2). The CAG repeat expansion upregulates Bβ2 expression and increases PP2A activity at the outer mitochondrial membrane, leading to mitochondrial fragmentation and ultimately neuronal death [22]. (v)SCA5 is caused by mutations of the *SPTBN2* gene, which is highly expressed in cerebellar Purkinje neurons. Studies suggest the mutant *SPTBN2* may affect the localization of the glutamate transporters EAAT4 and GluRδ2, thus resulting in a glutamate signaling abnormality and Purkinje cell death [23]. (vi) Mutant *γPKC*, the causative protein of SCA14, is susceptible to aggregation and induces apoptosis in cultured cell lines [24].

As one of the proteins selectively expressed during apoptosis, TG2 has been widely related to programmed cell death, and its cross-linking activity is implicated in the formation of apoptotic bodies [13]. However, the exact role of TG2 in apoptosis is still controversial. Several studies have suggested that TG2 can exert a protective role in apoptosis. For example, TG2 suppresses thapsigargin-induced apoptosis in HCT116 cancer cells by cross-linking caspase-3 into a nonfunctional oligomer [25]. Cao et al. found that TG2 prevents apoptosis induced by cisplatin by activating the NF-kB survival pathway in ovarian cancer cells [26]. TG2 can also protect HEK293 cells from Ca^{2+} ionophore-induced cell death through the down-regulation of Bax expression and suppressing caspase-3 and caspase-9 activities and mitochondrial membrane depolarization [27]. Knocking out endogenous TG2 resulted in a significant exacerbation of caspase-3 activity and PARP cleavage in mouse embryonic fibroblast (MEF) cells subjected to apoptotic stimuli [28]. However, some studies have suggested that TG2 might facilitate the apoptotic process. By using U937 cells and neuroblastoma cell lines, Oliverio et al. showed that overexpression of

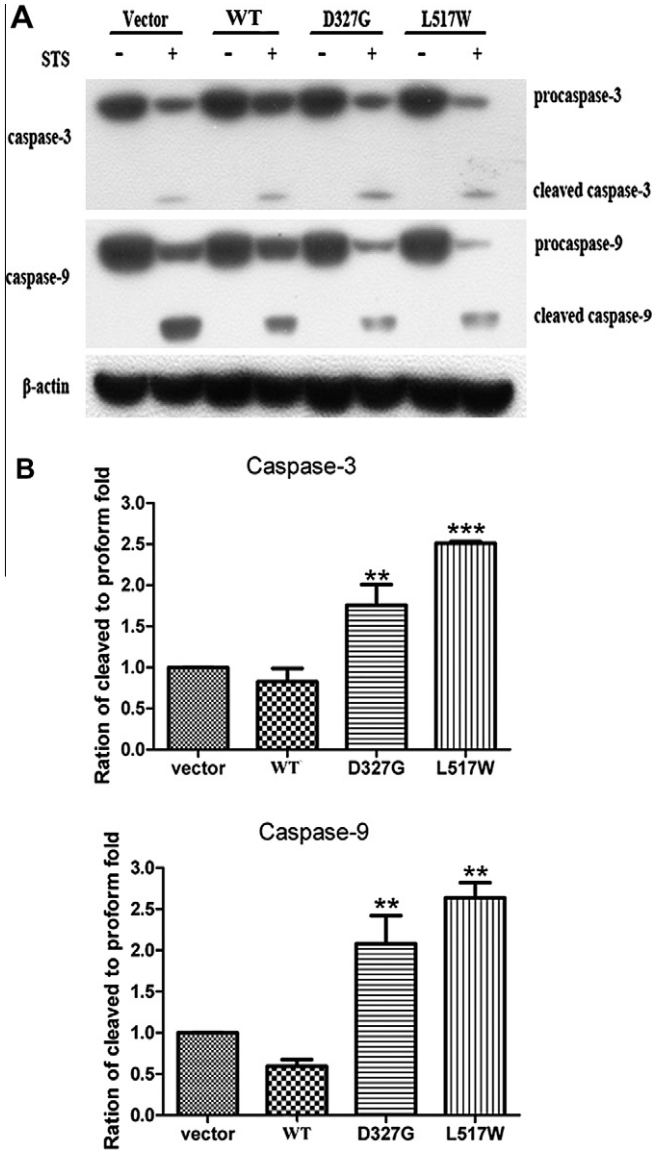

Fig. 4. Mutant TG6 sensitized cells to apoptosis by increasing activities of caspases. (A) Western Blot was performed to detect caspase-3 and caspase-9 in NIH3T3 stably transfected cells after 0.5 μM STS treatment for 16 h. (B) Quantitation of the Western Blot shows the ratio of cleaved caspase-3/9 compared with procaspase-3/9. Each value shown is the mean ± SEM of three replicates from a single assay. Asterisks indicate statistically significant differences compared with empty vector controls. ($^{**}P < 0.01$; $^{***}P < 0.001$ by one-way ANOVA with Dunnett's multiple comparison tests).

TG2 resulted in increased cell death [29]. Overexpression of TG2 sensitizes SK-N-BE cell lines to apoptosis by increasing mitochondrial membrane potential and cellular oxidative stress [30]. Furthermore, TG2 promotes the apoptosis of cardiomyocytes by reducing phospholipase C-δ1 and active PKC levels during oxidative stress [31]. In this study, we demonstrated that TG6 protects cells from apoptosis induced by A23187 and STS. Notably, SCA35-associated mutant TG6 could sensitize cells to apoptosis.

Caspases, a family of cysteine proteases, are the central regulators of apoptosis. Caspase-9, as an initiator caspase, can cleave and activate the downstream effector caspase-3, thereby executing the caspase-cascade and cell-death program [32]. Previous studies have found that TG2 can suppress thapsigargin-induced apoptosis in HCT116 cancer cells by cross-linking caspase-3 into a nonfunctional oligomer [25]. It is unknown whether the transglutaminase function of TG6 is required for its role in apoptosis. Nevertheless, the mutant TG6 enzymes were deficient in transglutaminase activity, and the overexpression of mutant TG6 resulted in increased caspase activation after STS stimulation. In conclusion, we found that the SCA35-associated TG mutations resulted in disturbed transglutaminase activity and stability. Furthermore, we showed that SCA35-associated TG6 mutants sensitize cells to apoptosis by increasing the activities of caspases, which may underlie the pathogenesis of SCA35.

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