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Mutant γPKC that causes spinocerebellar ataxia type 14 upregulates Hsp70, which protects cells from the mutant's cytotoxicity

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ABSTRACT

Several missense mutations in the protein kinase Cγ (γPKC) gene have been found to cause spinocerebellar ataxia type 14 (SCA14), an autosomal dominant neurodegenerative disease. We previously demonstrated that the mutant γPKC found in SCA14 is misfolded, susceptible to aggregation and cytotoxic. Molecular chaperones assist the refolding and degradation of misfolded proteins and prevention of the proteins' aggregation. In the present study, we found that the expression of mutant γPKC-GFP increased the levels of heat-shock protein 70 (Hsp70) in SH-SY5Y cells. To elucidate the role of this elevation, we investigated the effect of siRNA-mediated knockdown of Hsp70 on the aggregation and cytotoxicity of mutant γPKC. Knockdown of Hsp70 exacerbated the aggregation and cytotoxicity of mutant γPKC-GFP by inhibiting this mutant's degradation. These findings suggest that mutant γPKC increases the level of Hsp70, which protects cells from the mutant's cytotoxicity by enhancing its degradation.

1. Introduction

Spinocerebellar ataxia type 14 (SCA14) is an autosomal dominant spinocerebellar ataxia that is clinically characterized by slowly progressive cerebellar ataxia and caused by missense or deletion mutations in the *PRKCG* gene, which encodes protein kinase Cγ (γPKC) [1,2]. γPKC is a neuron-specific isoform of PKC that is especially abundant in cerebellar Purkinje cells (PCs) [3]. We have previously demonstrated that missense mutations of γPKC make this protein prone to aggregation in cultured cell lines and primary cultured PCs [4–7]. The accumulation of misfolded proteins is a common feature of various neurodegenerative diseases, including Alzheimer's, Parkinson's and polyglutamine diseases [8,9]. Therefore, SCA14 is caused by a mechanism common to other neurodegenerative disorders.

Molecular chaperones are proteins that assist protein folding during synthesis and that stabilize misfolded proteins generated by various stresses, such as heat shock [10]. There are several reports demonstrating that molecular chaperones accumulate in inclusions of misfolded proteins in patient tissues and cellular models of neurodegenerative diseases [11,12]. In addition, the overexpression of molecular chaperones alleviates aggregate formation, neurodegeneration and neuropathological functions in model animals of neurodegenerative diseases [13,14]. In the present study, we focused on the effects of SCA14 mutant γPKC on the levels and distributions of molecular chaperones. Mutant γPKC significantly elevated the level of heat-shock protein 70 (Hsp70) in the presence or absence of aggregation. siRNA-mediated knockdown revealed that the induction of Hsp70 protected cells from the toxicity of mutant γPKC by enhancing the mutant's degradation. These findings suggest that Hsp70 could be a novel therapeutic target in SCA14.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), Alexa Fluor 546-conjugated anti-mouse IgG antibody and Alexa Fluor 350-conjugated anti-rabbit IgG antibody were obtained from Life Technologies (Carlsbad, CA, USA). The MISSION siRNA universal

Abbreviations: γPKC, protein kinase Cγ; SCA14, spinocerebellar ataxia type 14; Hsp70, heat-shock protein 70; Hsp40, heat-shock protein 40; Hsp90, heat-shock protein 90; GFP, green fluorescent protein; PCs, Purkinje cells; WT, wild-type; LDH, lactate dehydrogenase; CHIP, carboxyl terminus of Hsp70-interacting protein; JNK, c-Jun N-terminal kinase; Hsc70, heat-shock cognate protein 70; 17-AAG, 17-allylamino-17-demethoxygeldanamycin.

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negative control and anti-β-tubulin 1 antibody were from Sigma Aldrich (St. Louis, MO, USA). Anti-Hsp40, anti-Hsp70 and anti-Hsp90 antibodies were from Enzo Life Sciences (Farmingdale, NY, USA). Anti-GFP antibody, Ham's F-12 medium and penicillin/streptomycin solution were from Nacalai Tesque (Kyoto, Japan). Anti-calbindin antibody was from Millipore (Billerica, MA). Hsp70 siRNA (sense: 5′-GAACCAGGUGGCGCUGAACdTdT-3′, antisense: 5′-GUUCAGCGCCACCUGGUUCdTdT-3′ [15]) was synthesized by Hayashi Kasei (Osaka, Japan). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and anti-rabbit IgG antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Glass-bottomed culture dishes (35-mm diameter) were from MatTek (Ashland, MA, USA). The Nerve-Cell Culture System (neuron culture medium and dissociation solutions) was from Sumitomo Bakelite (Tokyo, Japan).

2.2. Cell culture

SH-SY5Y cells were cultured in a DMEM/F-12 mixture (1:1) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 g/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. A mouse cerebellar primary culture was prepared as described previously [5]. Briefly, E14 embryos from pregnant ICR mice were dissociated using the dissociation solutions of the Sumitomo Nerve-Cell Culture System according to the manufacturer's protocol. Dissociated cerebellar cells were suspended in the neuron culture medium of the same system on a 35-mm diameter glass-bottomed culture dish. Cells were cultured for 28 days in vitro (DIV) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Transfection of γPKC-GFP and siRNA

γPKC-GFP (wild-type (WT) and S119P and G128D mutants) was expressed via adenoviral vectors using a tetracycline-regulated system, as described previously [16]. SH-SY5Y cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) during cell spreading. Two adenoviral vectors (Ad-CMV-tTA and Ad-TetOp-γPKC-GFP) were used for infection 1 day after cell spreading. For dissociated cerebellar culture, two adenoviral vectors (Ad-L7-tTA and Ad-TetOp-γPKC-GFP) were used for infection at DIV14 to selectively express γPKC-GFP in PCs.

2.4. Immunoblotting and immunostaining

The amounts of γPKC-GFP, Hsp40, Hsp70 and Hsp90 were assessed by immunoblotting cell lysates from SH-SY5Y cells expressing WT or mutant γPKC-GFP, as described previously [16]. Cells were harvested 2 days after adenoviral transfection. The degradation of γPKC-GFP was analyzed by a chase assay after the arrest of gene expression by tetracycline treatment [17] beginning 1 day after transfection. The cells were harvested before (0 h) or after 24 h treatment with tetracycline (1 μg/ml), followed by immunoblotting with anti-GFP antibody. All blots were probed with anti-β-tubulin antibody as an internal control.

SH-SY5Y cells on glass-bottomed dishes were fixed with 4% paraformaldehyde, followed by immunostaining with anti-Hsp70 mouse monoclonal antibody and Alexa Fluor 546-conjugated anti-mouse IgG antibody [18]. Primary cultured cerebellar cells were immunostained with anti-calbindin antibody and Alexa Fluor 350-conjugated anti-rabbit antibody to identify PCs, in addition to Hsp70 immunostaining. Images of GFP and Alexa Fluor 546 fluorescence were captured using a confocal microscope (LSM510META, Carl Zeiss, Oberkochen, Germany).

2.5. Cytotoxicity assay

One day after siRNA transfection, SH-SY5Y cells were infected with adenoviral vectors to induce γPKC-GFP expression. After further 2-day cultivation, the cytotoxicity of the γPKC-GFP-expressing

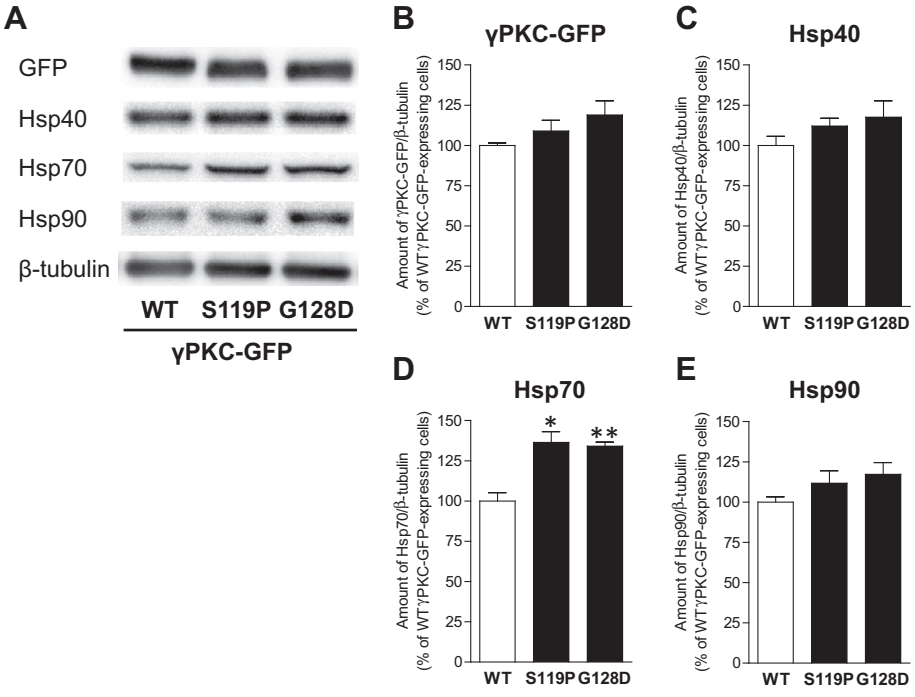

Fig. 1. SCA14 mutant γPKC-GFP upregulated Hsp70, but not Hsp40 and Hsp90. (A) Amount of γPKC-GFP, Hsp40, Hsp70 and Hsp90 in SH-SY5Y cells expressing WT or mutant (S119P or G128D) γPKC-GFP, as detected by immunoblotting. (B–D) Quantitative analyses of immunoblotting results for γPKC-GFP (B), Hsp40 (C), Hsp70 (D) and Hsp90 (E). The amount of each protein was normalized to the amount of β-tubulin in the same blot. Mean values from five independent experiments are shown. Error bars, SE. $^{*}p < 0.01$, $^{**}p < 0.001$ vs cells expressing WT γPKC-GFP (unpaired *t*-test).

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cells was assessed by a lactate dehydrogenase (LDH) assay using the CytoTox 96 NonRadioactive Cytotoxicity Assay (Promega, Fitchburg, WI, USA) according to the manufacturer's protocol.

3. Results

3.1. SCA14 mutant γPKC upregulates Hsp70

To examine whether SCA14 mutant γPKC affects the levels of molecular chaperones, the amounts of Hsp40, Hsp70 and Hsp90 were assessed by immunoblotting in SH-SY5Y cells expressing wild-type (WT) or missense mutant γPKC-GFP (Fig. 1A). There was no significant difference between the amounts of expressed WT and mutant γPKC-GFP (Fig. 1B). The amount of Hsp70 was significantly elevated in cells expressing mutant γPKC-GFP (Fig. 1D), whereas Hsp40 and Hsp90 levels were slightly increased, but these increases were not significant (Fig. 1C and E). We have previously demonstrated that mutant γPKC-GFP tends to form aggregates [4]. In various neurodegenerative diseases, molecular chaperones are reported to accumulate in neural inclusions [11,12]. Therefore, the distribution of Hsp70 was examined by immunostaining in cells expressing WT or mutant γPKC-GFP. As we reported previously, mutant γPKC-GFP tended to form aggregates, whereas WT γPKC-GFP was diffusely expressed in the cytoplasm (Fig. 2A). Consistent with immunoblotting results, the immunoreactivity of Hsp70 was elevated in cells expressing mutant γPKC-GFP (Fig. 2A). Unexpectedly, this elevation was observed in many cells without aggregates of mutant γPKC-GFP, in addition to strong colocalization of Hsp70 immunoreactivity to the mutant's aggregates (Fig. 2A). This elevation of Hsp70 expression was also observed in primary cultured PCs expressing mutant γPKC-GFP (Fig. 2B). These results indicate that Hsp70 is upregulated in cells expressing mutant γPKC independently of the mutant's aggregation.

3.2. Hsp70 is elevated to degrade mutant γPKC, protecting cells from the mutant's toxicity

To explore the role of Hsp70 upregulation, we attempted to knock down Hsp70 using siRNA in cells expressing mutant γPKC-GFP. siRNA against Hsp70 successfully decreased the level of Hsp70 compared with control siRNA-transfected cells (Fig. 3A and B). siRNA-mediated knockdown of Hsp70 significantly increased mutant γPKC-GFP levels (Fig. 3A and C). To elucidate the

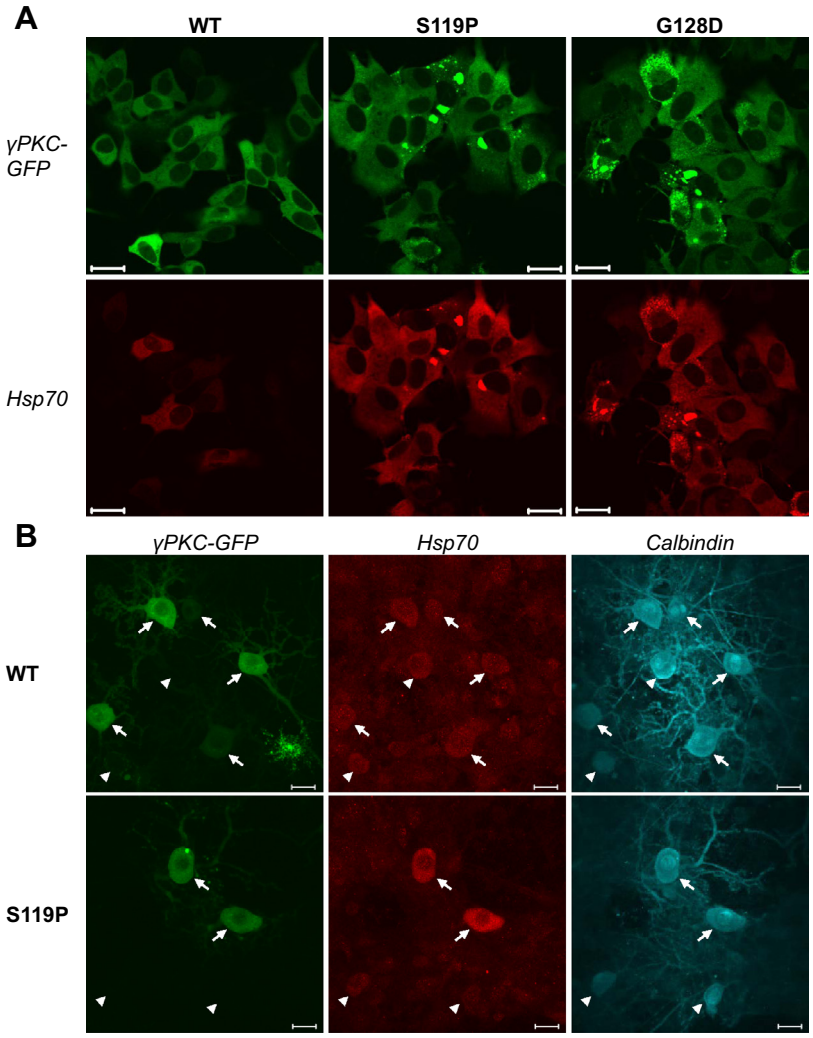

Fig. 2. Mutant γPKC-GFP upregulated Hsp70 in the presence or absence of the mutant's aggregation. (A) Hsp70 immunostaining of SH-SY5Y cells expressing WT or mutant (S119P or G128D) γPKC-GFP. Hsp70 strongly colocalized to aggregates of mutant γPKC-GFP, although Hsp70 levels also increased in cells without aggregation of mutant γPKC-GFP. Scale bar, 20 μm. (B) Hsp70 and calbindin immunostaining of primary cultured cerebellar PCs expressing WT or mutant (S119P) γPKC-GFP. The Hsp70 levels of PCs expressing WT γPKC-GFP (arrows) were similar to levels in non-expressing PCs (arrowhead). In contrast, Hsp70 immunoreactivity was obviously increased in PCs expressing mutant γPKC-GFP (arrows). Scale bar, 20 μm.

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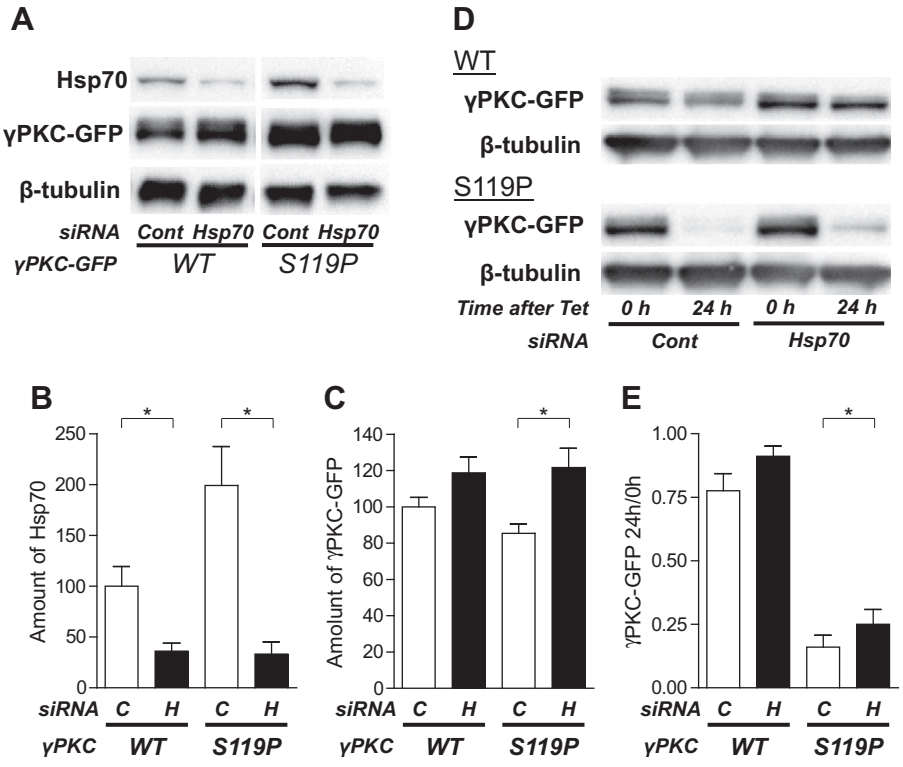

Fig. 3. Knockdown of Hsp70 increased mutant γPKC-GFP levels by inhibiting the mutant's degradation. (A) Amount of γPKC-GFP and Hsp70 in γPKC-GFP-expressing SH-SY5Y cells transfected with negative control (Cont or C) or Hsp70 (H) siRNA, as detected by immunoblotting. (B and C) Quantitative analyses of immunoblotting results for γPKC-GFP (B) and Hsp70 (C). The amount of each protein was normalized to the amount of β-tubulin in the same blot. Mean values from three independent experiments are shown. Error bars, SE. $^{*}p < 0.05$ (unpaired *t*-test). (D) The degradation of WT and mutant (S119P) γPKC-GFP was determined by a chase assay in cells transfected with control or Hsp70 siRNA. The expression of γPKC-GFP was arrested by treatment with 1 μg/ml tetracycline (Tet). Cells were harvested before (0 h) or 24 h after Tet treatment, followed by immunoblotting with anti-GFP and anti-β-tubulin antibodies. (E) The degradation of γPKC-GFP was quantitatively evaluated based on the amount of γPKC-GFP at 24 h divided by the amount of γPKC-GFP at 0 h. Mean values from four independent experiments are shown. Error bars, SE. $^{*}p < 0.05$ (paired *t*-test).

mechanism of how Hsp70 knockdown elevates the γPKC-GFP level, we examined the degradation rate of γPKC-GFP by gene arrest by tetracycline treatment [17]. Hsp70 knockdown slightly but significantly inhibited the degradation of mutant γPKC-GFP (Fig. 3D and E). Next, we examined whether the aggregation and cytotoxicity of mutant γPKC is affected by Hsp70 knockdown. siRNA against Hsp70 significantly exacerbated the aggregation of mutant γPKC-GFP (Fig. 4A and B). An LDH assay revealed that this siRNA did not affect cytotoxicity to cells expressing WT γPKC-GFP (Fig. 4C). As we previously reported, mutant γPKC-GFP significantly increased the cytotoxicity, and this toxicity was significantly exacerbated by siRNA-mediated knockdown of Hsp70 (Fig. 4C). These findings suggest that Hsp70 is upregulated to enhance the degradation of mutant γPKC, protecting cells from the mutant's toxicity.

4. Discussion

In the present study, we found that the SCA14 missense mutant γPKC induced the upregulation of Hsp70 in cultured cell lines and primary cultured PCs (Figs. 1 and 2). Consistent with its behavior in other neurodegenerative diseases [11,12], Hsp70 strongly colocalized to aggregates of mutant γPKC. Because the overexpression of molecular chaperones reduces the aggregation of mutant proteins [13,14], chaperones would stabilize the misfolded proteins to prevent their aggregation. In the case of mutant γPKC, the Hsp70 level was also increased in cells without any aggregates (Fig. 2). We have previously demonstrated that mutant γPKC forms oligomers in cells [5]. Accumulating evidence suggests that oligomers of mutant protein have higher toxicity than do aggregates [19,20]. Hsp70 might prevent the oligomerization of mutant γPKC in cells without aggregation.

siRNA-mediated knockdown of Hsp70 strengthened the notion that the Hsp70 level increased to protect cells from the toxicity of mutant γPKC. Because Hsp70 knockdown significantly reduced the rate of mutant γPKC degradation, Hsp70 may have a role in degrading mutant γPKC (Fig. 3). We have previously demonstrated that mutant γPKC is degraded more rapidly than the wild-type protein via both proteasomal and lysosomal pathways [17]. Although we did not determine which pathways are involved in the Hsp70-mediated degradation of mutant γPKC, Hsp70 is known to participate in both of these protein degradation pathways. Regarding the proteasomal pathway, it is known that carboxyl terminus of Hsp70-interacting protein (CHIP), an E3 ubiquitin ligase, interacts with Hsp70 and ubiquitinates Hsp70 client proteins, leading to their degradation via the proteasome [21]. CHIP is reported to be involved in the degradation of many causal proteins of neurodegenerative diseases [22]. Therefore, it is possible that mutant γPKC is also regulated by CHIP and that knockdown of Hsp70 prevents CHIP from detecting and ubiquitinating mutant γPKC. Regarding the lysosomal proteolytic pathways, Hsp70 enhances macroautophagy, one type of lysosomal protein degradation, via c-Jun N-terminal kinase (JNK) pathways [23]. In addition, Hsp70 is known to interact with heat shock cognate protein 70 (Hsc70) [24], which is mainly involved in chaperone-mediated autophagy, another type of lysosomal protein degradation [25]. Therefore, Hsp70 might also affect chaperone-mediated autophagy. Furthermore, a small-molecule inhibitor of Hsp70 inhibits both proteasome-mediated and lysosome-mediated protein degradation [26]. Thus, Hsp70 may render the rapid degradation of mutant γPKC

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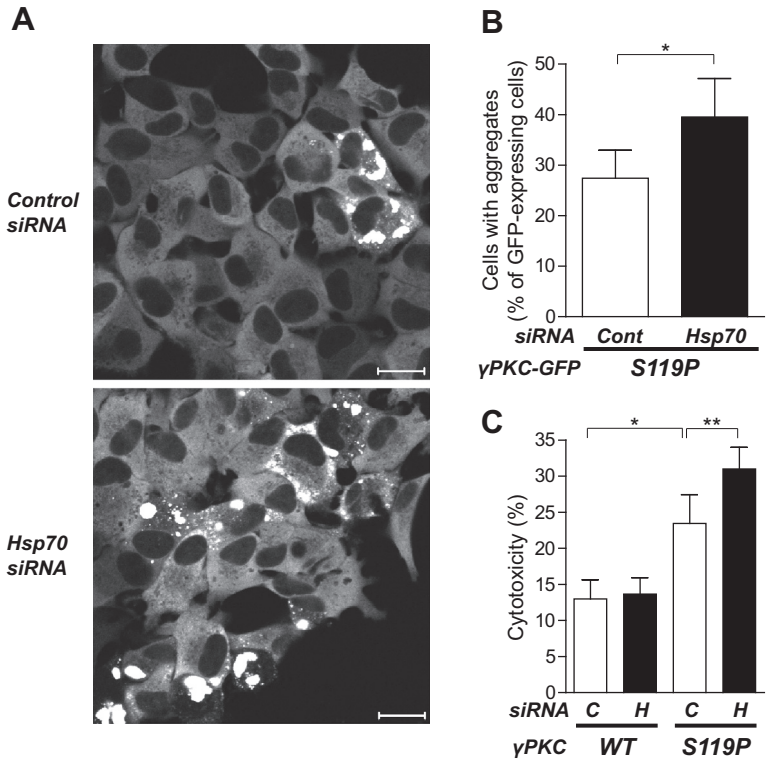

Fig. 4. Knockdown of Hsp70 exacerbated the aggregation and cytotoxicity of mutant γPKC-GFP. (A) Aggregate formation in cells expressing mutant (S119P) γPKC-GFP with control (upper) or Hsp70 (lower) siRNA. Scale bar, 20 μm. (B) Quantification analyses of the aggregation. Mean values from three independent experiments are show. Error bars, SE. $^{*}p < 0.05$ (paired *t*-test). (C) An LDH assay was conducted to evaluate cytotoxicity to WT or mutant (S119P) γPKC-GFP-expressing cells transfected with control (C) or Hsp70 siRNA. Mean values from four independent experiments are shown. Error bars, SE. $^{*}p < 0.05$, $^{**}p < 0.01$ (paired *t*-test).

via these two degradation pathways. Moreover, Hsp70 can stabilize lysosomal membranes, resulting in the protection of cells from apoptosis by lysosomal rupture [27]. This property of Hsp70 might contribute to its protective effect against the cytotoxicity of mutant γPKC.

Our present findings indicate novel roles for Hsp70, which is upregulated by SCA14 mutant γPKC, in therapeutic strategies for treating neurodegenerative disease. Inhibitors of Hsp90 (17-allylamino-17-demethoxygeldanamycin (17-AAG) and celastrol) are reported to increase the levels of molecular chaperones, including Hsp70 [28,29]. These chemicals have been demonstrated to have the potential to inhibit aggregation and prevent neurodegeneration in several neurodegenerative disease models [30–33]. Our present findings strongly suggest that these chemicals have potential as novel therapeutics for SCA14.

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