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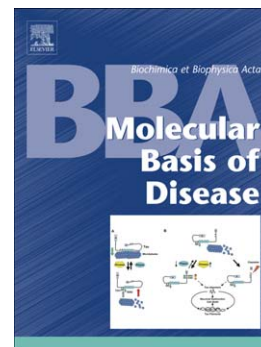
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**Hsp27 decreases inclusion body formation from mutated
GTP-cyclohydrolase I protein**

Yu-Wei Chiou ^{a,b}, Wuh-Liang Hwu ^c, and Yu-May Lee ^{a,b,*}

^aInstitute of Biological Chemistry, Academia Sinica, Taipei, Taiwan

^bInstitute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan

^cDepartment of Pediatrics and Medical Genetics, National Taiwan University Hospital
and National Taiwan University College of Medicine, Taipei, Taiwan

Running title: Hsp27 decreases mutated GCH inclusion

*Corresponding author. Institute of Biological Chemistry, Academia Sinica, Section 2,
Academy Road, Taipei 115, Taiwan. Tel: +886 2 2785 5696 ext 6120; Fax: +886 2 2788
9759; Email address: YML6120@gate.sinica.edu.tw

Key words: GTP-cyclohydrolase I, dopa-responsive dystonia, inclusion body formation, chaperone, heat shock protein 27, phosphorylation

Abbreviations used: Hsp, heat shock protein; GCH, GTP-cyclohydrolase I; BHK cells, baby hamster ovary cells; DRD, dopa-responsive dystonia

ACCEPTED MANUSCRIPT

Summary [185 words]

GTP cyclohydrolase I (GCH), an oligomeric protein composed of 10 identical subunits, is required for the synthesis of neurotransmitters; mutations in GCH are associated with dopa-responsive dystonia (DRD) and hyperphenylalaninemia. Mutated GCH proteins are unstable and prone to dominant-negative effect. We show herein that expression of the GCH mutant GCH-201E or the splicing variant GCH-II caused intracellular inclusion bodies. When Hsp27 was expressed together with the GCH mutants, Hsp27 expression decreased the formation of inclusion bodies by GCH (as assessed by immunofluorescence) and decreased the amount of insoluble GCH mutant proteins (as assessed by Western blot). Transfection of pcDNA-Hsp27-S3D, a phosphorylation-mimicry Hsp27 mutant, was more effective at the mutated GCH proteins than transfection with pcDNA-Hsp27, but okadaic acid, a phosphatase inhibitor, enhanced the effect of pcDNA-Hsp27. Hsp27-S3D also abolished the dominant-negative action of GCH-II. The mutated GCH proteins interacted with the wild-type GCH protein; the inclusion bodies were positive for lysosomal marker LAMP1, soluble in 2% SDS, and were not ubiquitinated. Phosphorylated Hsp27 also decreased the inclusion body formation by the huntingtin polyglutamines. Therefore, diseases involving mutated oligomeric proteins would be manageable by chaperone therapies.

INTRODUCTION

GTP cyclohydrolase I (GCH; EC 3.5.4.16) [1] is a homodecameric protein [2, 3], and mutations of GCH gene are associated with a wide range of clinical conditions, ranging from benign dopa-responsive dystonia (DRD) to malignant hyperphenylalaninemia [4-7]. The oligomeric quaternary structure of GCH seems to amplify its molecular defects and cause phenotype variabilities. Previously we have shown that mutated GCH proteins are unstable and prone to dominant-negative effect. In this study, we show that expression of GCH-201E (a mutation causing DRD) or splicing variant GCH-II (which encodes a shorter peptide) in baby hamster kidney (BHK) cells causes the formation of prominent punctate cytoplasmic inclusion bodies in immunofluorescence staining.

Protein aggregates are involved in neurodegenerative diseases including Alzheimer disease, Parkinson disease, Huntington disease, and prion diseases [8]. The aggregates consist of fragments of mutated proteins like the polyglutamines in Huntington disease, or the aberrantly cleaved amyloid beta protein in Alzheimer disease [8]. These polypeptides have a highly abnormal conformation, which prevents normal folding, and therefore form aggregates through polymerization. For example, polyglutamines form pleated sheets of beta-strands held together by hydrogen bonds

between their amides, and associate irreversibly into oligomers firmly interlocked by either strand- or domain-swapping [9] or covalent bonds [10]. The aggregates themselves, or early steps in the cascade, cause toxicity and subsequent death of the cells.

Molecular chaperones, or heat shock proteins, are initially known as cellular machineries against the accumulation of damaged proteins during stress. Hsp70 and other chaperones also protect partially synthesized peptides when they emerge from the ribosomes [11, 12]. Overexpression of chaperones has been evaluated as a therapeutic strategy to promote proper folding or degradation of misfolded proteins [13, 14]. It has been shown that chaperones HDJ-2/HSDJ [13], Hsp40 and Hsp70 [15], and MRJ [16] modulate polyglutamine pathogenesis. Chaperonin TRiC promotes the assembly of polyglutamine expansion proteins into nontoxic oligomers [17]. Actually, upregulation of Hsp70 may be an intrinsic cellular response against the neuronal degeneration mediated by the huntingtin mutant [18].

In this study, we tested the effect of small heat shock protein Hsp27 [19] on GCH inclusion body formation. We demonstrated that Hsp27, in its phosphorylated form, effectively prevented GCH-201E- and GCH-II-mediated formation of inclusion bodies, so chaperone therapy could be helpful in these conditions.

MATERIALS AND METHODS

Vectors and chemicals

Expression vectors pCMV-A16-GCH-WT (GCH-WT), pCMV-GCH-II (GCH-II), and pCMV-GCH-201E (GCH-201E) have been described previously [4, 5]. N-terminal AGP/EBP A16 epitope was added to the GCH proteins for the convenience of detection, and the A16-tagged GCH proteins have molecular weights higher than the HA-tagged or untagged GCH proteins [20]. A rabbit antiserum (anti-N20) against the A16 epitope was developed in house. pcDNA3-Hsp27 (Hsp27) and pcDNA3-Hsp27-S3D (Hsp27-S3D) were gifts from Dr. Gaestel [21]. Replacing serine 15, 78, and 82 by aspartate, Hsp27-S3D mimics Hsp27 phosphorylation [21]. Okadaic acid was obtained from Sigma Chemical Company (St Louis, Mo, USA). A polyglutamine tract with alternating CAG/CAA repeats, encoding 71 glutamines (71Q-GFP), was inserted into the context of the huntingtin gene exon 1 and cloned into phrGFP (Clontech, Mountain View, Calif, USA). pcDNA3-HA-ubiquitin (HA-Ub) has been described by Ebisawa et al [22].

Cells and treatments

Baby hamster kidney (BHK) or NIH293 cells were cultured in DMEM supplemented

with 10% fetal calf serum. Transfection was performed by using the calcium phosphate method [23] or LipofectAmine 2000 (Invitrogen, Carlsbad, California USA). Forty hours after transfection, cells were disrupted by sonication in phosphate-buffered saline with 2.5 mM EDTA. The supernatant (Sup) and pellet (Pellet) fractions were separated by centrifugation 12,000 x g for 10 minutes at 4°C in a microcentrifuge. The 1X gel-loading buffer contained either 2% SDS and 5 M urea (urea gel-loading buffer, for most of the experiments unless specified), or 2% SDS and 10% glycerol (glycerol gel-loading buffer). The anti-GCH antibody was raised in rabbit previously [4]. Both anti-Hsp27 and anti-phospho-Hsp27 (Ser78) were obtained from Stressgen (Victoria, Canada). Western blot analysis was performed by using standard methods, and the signals were visualized by using the Western Lightning kit (NEN, Boston, Mass, USA). For immunoprecipitation experiments, cells were sonicated in 200 µl Tris-EDTA buffer containing 1% Triton X-100 and centrifugated at 14,000 rpm for 5 min, and after that 12 µl supernatant was loaded as input. Another 180 µl supernatant was incubated with HA beads for 2 hours at 4° C with continuous agitation. After wash for 3 to 4 times with the lysis buffer, samples were boiled in urea gel-loading buffer and subjected for Western blot analysis with anti-N20 or anti-HA to visualize GCH or ubiquitinated proteins.

For immunofluorescence staining, cells grown on cover slips were fixed by 4%

paraformaldehyde, and were permeabilized by 0.1% triton X-100. The secondary antibodies were either FITC-labeled anti-rabbit or Rodamin-labeled anti-mouse Ig. Slides were mounted in 50% glycerol and were visualized by fluorescence microscope or confocal microscope (Carl Zeiss, LSM510 Laser scanning confocal microscope). Okadaic acid was dissolved in 10% DMSO as a 10 μ M stock. The working concentrations of okadaic acid were from 20 nM to 100 nM. Okadaic acid was added to the medium 16 hours after transfection.

GCH cross-linking and filter retardation assay

Both wild-type GCH, GCH-II, and GCH-201E were cloned into pGEX-4 vector (GE Healthcare, Piscataway, NJ, USA) and expressed in *E. coli* as GST fusion proteins. The expressed GCH proteins were purified by GST isolation according to the manufacturers' instruction (GE Healthcare, Piscataway, NJ, USA). GCH cross-linking was performed in 10 μ L of 1X PBS, 2.5 mM EDTA buffer (pH 7.0) at a 7 mg/mL protein concentration. The protein mixture reacted with disuccinimidyl suberate (DSS, Pierce, Rockford, Ill, USA) at concentrations from 0.025 mM to 2 mM and was incubated at room temperature for 30 minutes. A 35-mM glycine buffer was used to terminate the reactions by incubation for 15 minutes at room temperature. The cross-linked protein mixture was resolved by electrophoresis on gradient

polyacrylamide gels in the presence of SDS. After electrophoresis, protein was transferred onto nitrocellulose membrane for Western blot analysis.

For filter retardation assay [24], the cell lysates were prepared in either PBS or 2% SDS, and were filtered through a cellulose acetate membrane with 0.2 μm pore size (Schleicher and Schuell, Keene, NH, USA) by the Bio-Dot apparatus (Bio-Rad, Hercules, CA, USA). Western blot analysis was then performed with anti-GCH.

Analysis of polyglutamine aggregation

BHK cells were transiently transfected with 71Q-GFP alone or in combination with pcDNA3-Hsp27 or pcDNA3-Hsp27-S3D. Expression of 71Q-GFP induced cytoplasmic inclusion bodies were visualized under fluorescence microscopy. The percentage of cells with inclusion bodies was determined by counting ten 200X fields for each sample. The values were expressed as mean \pm SD. Statistical analysis was performed using the unpaired *t* test, with $P < 0.05$ considered statistically significant.

RESULTS

Hsp27-S3D decreases inclusion body formation by mutated GCH proteins

We have previously demonstrated that both GCH-201E and GCH-II were unstable [4, 5, 25]. Here we showed that when GCH proteins were expressed in BHK cells, wild-type GCH (pCMV-A16-GCH-WT) gave a homogeneous cytoplasmic immunofluorescence stain, while both GCH-201E and GCH-II expression resulted in punctate cytoplasmic inclusion bodies (Fig. 1).

In order to determine if Hsp27 has an effect on the GCH inclusion body formation, we cotransfected expression vectors for both GCH and Hsp27 in BHK cells. We found that cotransfection of pcDNA-Hsp27 or pcDNA-Hsp27-S3D did not alter the staining pattern of wild-type GCH, although the intensity of the stain was stronger when pcDNA-Hsp27-S3D was cotransfected (Fig. 2, A and B). Cotransfection of pcDNA-Hsp27 did not alter inclusion body formation from GCH-II (Fig. 2C), but cotransfection of pcDNA-Hsp27-S3D changed the punctate GCH-II stain into a homogeneous pattern similar to that from the wild-type GCH (Fig. 2D).

Phosphorylation of Hsp27 is necessary for its function on GCH mutants

To further determine whether the phosphorylation of Hsp27 is necessary for its effect

on GCH mutant proteins, we utilized okadaic acid, an inhibitor of phosphatase PP2A which dephosphorylates phospho-Hsp27 [26], in the experiments. In immunofluorescence staining, we demonstrated that okadaic acid at concentrations 60 to 100 nM effectively decreased the number of GCH-II inclusion bodies in BHK cells (Fig. 3).

Hsp27 decreases insoluble GCH mutant proteins

We further used Western blot analysis to examine the effect of Hsp27. We first confirmed our observation from the immunofluorescence staining experiments, i.e., that Hsp27-S3D, but not Hsp27, increased the quantity of wild-type GCH protein (Fig. 4, A and B, $p=0.01$). In order to show that the effect of Hsp27 was not due to the induction of other chaperones, we measured the expressions of Hsp60, Hsp70, and Hsp90 in the experiments. Although these heat shock proteins were slightly induced by Hsp27 overexpression, the induction was lower with pcDNA-Hsp27-S3D which showed higher effects toward GCH (data not shown). Therefore, the effect of Hsp27-S3D on GCH mutant proteins should not be due to the induction of other heat shock proteins.

In BHK cells expressing GCH-II, cotransfection of pcDNA-Hsp27-S3D or pcDNA-Hsp27 plus okadaic acid decreased the amount of insoluble GCH (Fig. 5A,

Pellet). Okadaic acid treatment dramatically increased the quantity of phospho-Hsp27 in cells (Fig. 5A, phospho-Hsp27). pcDNA-Hsp27 plus okadaic acid also increased the quantity of soluble GCH-II, but pcDNA-Hsp27 itself or pcDNA-Hsp27-S3D was less effective (Fig. 5A, Sup). In BHK cells expressing GCH-201E, cotransfection with pcDNA-Hsp27, pcDNA-Hsp27-S3D, or pcDNA-Hsp27 plus okadaic acid decreased the amount of GCH in the pellet fraction, but again Hsp27 was less effective (Fig. 5B, Pellet). GCH-201E was not visible in the supernatant (data not shown).

Interaction between the wild-type and mutant GCH proteins and the dominant-negative effect

We first used the DSS cross-linking assay to evaluate the quaternary structures of GCH-201E and GCH-II. As shown in Figure 6, E. coli-produced recombinant wild-type GCH was monomeric or dimeric in urea gel-loading buffer (Fig. 6A, Lane 2). With increasing concentration of DSS, GCH oligomers appeared in the gel.

Recombinant GCH-II could be cross-linked in a similar way (Fig. 6B, left panel), but recombinant GCH-201E stayed as monomers throughout the experiments (Fig. 6B, right panel). This might explain why we were not able to bring GCH-201E into the soluble fraction.

We next approached the interaction between the wild-type and mutant GCH

proteins by coexpression studies in NIH293 cells. We demonstrated that in NIH293 cells when we expressed the HA-tagged wild-type GCH protein together with either A16-tagged GCH-201E or GCH-II, the mutated GCH proteins could be precipitated by anti-HA (Fig. 7A). This proves the interaction between the wild-type and mutated GCH proteins, even though GCH-201E itself might not be able to form stable oligomers. This result also supports our previous observation for the dominant-negative effect of the mutated GCH proteins where protein-protein interaction should be required.

We then tested whether Hsp27 would alter the dominant-negative effect of the mutated GCH proteins. Cotransfection of pCMV-GCH-II decreased the expression of wild-type GCH in BHK cells (Fig. 7B, lanes 1-4). The addition of Hsp27, but more effectively Hsp27-S3D, decreased the dominant-negative effect of GCH-II in a dose-dependent manner (Fig. 7B, lanes 5-10). Therefore both GCH-II and GCH-201E interact with their wild-type counter part, which could explain the dominant-negative effect, and the complex phenotypes and inheritance of the associated diseases.

The nature of the inclusion bodies

We first used the filter retardation assay to estimate the sizes of the GCH mutant proteins in the pellet fraction of the cells. Cell lysates were prepared in either PBS or 2% SDS, and were filtered through a 0.2 μm membrane (Schleicher and Schuell) by a

dot blot apparatus. After washing with PBS, Western blot analysis was performed with anti-GCH. The 71Q-GFP aggregation was used as a control, and the result showed that 71Q-GFP was not soluble in 2% SDS and was retained on the filter (Fig. 8A, A5 and B5) [24]. GCH mutant proteins, however, were completely solubilized by 2% SDS (Fig. 8A, B3 for GCH-II, and A4 and B4 for GCH-201E). GCH proteins dotted on nitrocellulose paper (NC) showed the presence of the proteins (Fig. 8A, A1 for GCH-II and B1 for GCH-Wt). It is interesting that, wild-type GCH, though stayed in the supernatant after sonication of the cell in PBS, could not pass the filter in PBS (A2). It may be due to the formation of multiple protein complexes.

We therefore changed the urea gel-loading buffer (2% SDS and 5 M urea) to glycerol gel-loading buffer (2% SDS) in Western blot analysis. The results showed that both GCH-II (Fig. 8B, lanes 2, 4, 6, and 8, white arrows) and GCH-WT (lanes 10 and 12, black arrows) contained oligomeric structures when glycerol gel-loading buffer was used. Either GCH-II in the cell pellets (lanes 6 and 8) or the GCH-II protein pushed to the supernatant by Hsp27-S3D revealed the same oligomerization as the wild-type GCH protein.

Since the mutated GCH proteins were soluble in SDS, which is different from the aggregated proteins formed by either polyglutamines or amyloid protein, we asked if the mutated GCH proteins would be within lysosomes. When BHK cells expressing

GCH-201E were stained for both the lysosomal marker LAMP1 and GCH, we could see that the two stains colocalize quite well (Fig. 9, upper panel, A-C). Because Hsp27 was not colocalized with GCH-II (Fig. 2C), Hsp27 didn't follow the GCH proteins to the lysosomes. The mutated GCH protein (GCH-201E) was also not ubiquitinated (Fig. 9, lower panel).

Hsp27 decreases inclusion body formation from polyglutamines

Although the mutated GCH proteins didn't have the same character as the polyglutamine aggregations, we still wanted to see the action of Hsp27 on the polyglutamines. The results showed that expression of an elongated huntingtin polyglutamine tract (71Q-GFP) in BHK cells produced prominent cytoplasmic inclusion bodies (Fig. 10A). Coexpression of pcDNA-Hsp27-S3D ($p=0.011$) and pcDNA-Hsp27 ($p=0.012$) decreased the percentage of cells showing polyglutamine inclusion bodies, but pcDNA-Hsp27-S3D was more effective ($p=0.018$) (Fig. 10B).

DISCUSSION

In this study, we demonstrated that mutated GCH proteins form intracellular inclusion bodies. Although precipitation of overexpressed proteins in cultured cells is occasionally seen, this study is particularly interesting because GCH itself is an oligomeric protein. A few cellular proteins oligomerize or polymerize by themselves, and there are plenty of examples of abnormal or precipitation of these proteins. In sickle cell disease, the mutated β -globin chains tend to form long chains or polymers [27]; in thalassemias, hemoglobin H (four β chains) is unstable and tends to precipitate in red blood cells and form characteristic inclusion bodies [28]. In Nemaline myopathy, mutations in the human skeletal muscle α -actin gene (*ACTA1*) causes the abnormal thread- or rod-like nemaline bodies [29]. Actin is also one of the components in inclusion bodies of uterine leiomyomas [30], and abnormal bundling and accumulation of F-actin mediates tau-induced neuronal degeneration *in vivo* [31]. Therefore, cellular proteins that naturally oligomerize may be prone to aggregation or precipitation upon unfavorable environments or mutations. Diseases involving this phenomenon may be treated by chaperone therapy, as in a recent paper that Hsp27-S3D prevents heat-induced aggregation of F-actin by forming soluble complex with denatured actin [32].

In the current study, we demonstrated that Hsp27 increases the expression of the wild-type GCH protein, causes the appearance of the soluble GCH-II protein, and decreases the quantities of insoluble mutated GCH protein. Therefore, it is likely that Hsp27 improves the folding of mutated GCH proteins, so they can stay in free cytosolic compartment. An enhancement in their degradation in the lysosomes is also possible, since we have previously demonstrated that the lysosome inhibitors NHCl4 and PMSF prevented the degradation of GCH-201E [4]. Although proteasome is the organelle responsible for protein degradation, and in polyglutamine-related diseases the inclusion bodies are immunopositive for ubiquitin and proteasome subunits [13, 33], a robust activation of the neuronal lysosomal system has been recently shown in Alzheimer and Huntington diseases [34, 35]. Since Hsp27 is not associated with GCH in the inclusion bodies, it should act on early stages of the maturation of the GCH protein. There have been concerns regarding aggregation-targeting therapy since it is likely that toxicity is due to the ongoing polymerization rather than the stable aggregates [36]. Nevertheless, using Hsp27 as a therapeutic molecule may not be a problem.

The role of phosphorylation in Hsp27 function has been a long debate. Hsp27 belongs to a family of small heat shock proteins [19] and protects cells from heat shock and oxidative stress [19, 37]. In unstressed cells, Hsp27 levels are generally low and Hsp27 exists predominantly as a large oligomeric unit of up to 800 kDa. During the

stress response, there is an increase in Hsp27 expression level which is preceded by a transient phosphorylation of Hsp27 by MAPKAP kinase 2/3 on Ser-15, Ser-78, and Ser-82, resulting in the redistribution of the large Hsp27 oligomer into smaller tetrameric units [19, 38]. The chaperonic activity of Hsp27 was traditionally tested via the suppression of turbidity formation from the thermal denaturation of citrate synthase, and it has been shown that phosphorylation of Hsp27 decreased this effect [21]. Some other studies employing this assay had similar findings [39, 40]. However, many recent studies have demonstrated that stress stimulates Hsp27 phosphorylation in the heart, and that phospho-Hsp27 protects the heart against oxidative stress [41], ischemia [42], atrial fibrillation [43], or inflammation [44]. The interpretation of these data may be difficult because Hsp27 possesses anti-apoptotic activity [45]. However, non-phosphorylated Hsp27 was also thought to have anti-apoptotic function [46, 47], although exceptions were documented recently [48]. It is possible that the mechanisms by which Hsp27 exerts its function are more complex than what we thought and can not be represented solely by the turbidity test. More studies are required to clarify those debates.

Although the mutated GCH proteins don't form tight SDS-resistant aggregation as the polyglutamines [24], the similar effect of phosphorylated Hsp27 on GCH and polyglutamines indicate that Hsp27 helps the folding, to prevent the untoward

consequences of these mutated proteins. Recently, it starts to be understood that the formation of protein aggregates is not a single-step process. Nucleation-dependent polymerization has been shown to be an essential component of amyloid-mediated neuronal cell death [36]. The initial aggregates could be soluble [49]. These initial aggregates or quasi-aggregates [50] may be more important in the pathogenesis of the diseases. Therefore, the application of Hsp27 in chaperone therapy could be promising, as shown recently that Hsp27 protects against α -synuclein-induced toxicity and aggregation [51]. The oligomeric protein-associated diseases, though not necessary associated with neurodegeneration, should also be good targets for future chaperone therapy.

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Figure legends**Figure 1. GCH-II and GCH-201E form cytoplasmic inclusion bodies.**

Immunofluorescence staining of BHK cells expressing pCMV-A16-GCH-WT (WT), pCMV-GCH-201E (G201E), or pCMV-GCH-II (GCH-II). The left panel shows GCH staining (green) and the right panel shows both GCH and 4',6-diamidino-2-phenylindole (DAPI) staining (blue). DAPI stains the nuclei. Both GCH-G201E and GCH-II form small punctate cytoplasmic inclusion bodies.

Figure 2. Hsp27-S3D reduces GCH-II inclusion body formation. BHK cells were transfected with pCMV-A16-GCH-WT (A16-GCH) or pCMV-GCH-II (GCH-II), with or without pcDNA3-Hsp27 (Hsp27) or pcDNA3-Hsp27-S3D (Hsp27-S3D). Cells were stained with both anti-GCH (left column) and anti-Hsp27 (middle column) antibodies. Images were captured by confocal microscope. The images for GCH and Hsp27 staining were merged on the right column.

Figure 3. Okadaic acid enhances the effect of Hsp27 on decreasing GCH-II inclusion bodies. BHK cells were transfected with pCMV-GCH-II (GCH-II) alone (A), or together with pcDNA3-Hsp27 (Hsp27) (B, D, E, and F) or pcDNA3-Hsp27-S3D

(Hsp27-S3D) (C) followed by immunofluorescence staining with anti-GCH. Cells were treated with 40 to 100 nM of okadaic acid (OA) in panels D, E, and F.

Figure 4. Hsp27-S3D increases the expression of wild-type GCH. (A) BHK cells were transfected with pCMV-A16-GCH-WT (A16-GCH), with or without pcDNA3-Hsp27 (Hsp27) or pcDNA3-Hsp27-S3D (Hsp27-S3D). Western blot analyses were performed with anti-GCH (GCH) or anti-tubulin (Tubulin) on the supernatant fraction of the cell lysate. The quantities of plasmid used in the assays were either 0.6 μ g or 1.2 μ g. (B) Quantification of the results. The films were converted into digital images by a desk scanner. The signal intensities were quantified by using a densitometry software, and expressed by comparing to the GCH-only experiments. The lines above the bars represent one standard deviation calculated from 3 or more experiments.

Figure 5. Phospho-Hsp27 decreases insoluble GCH proteins and increases soluble GCH-II protein. Western blot analyses were performed on BHK cells transfected with pCMV-GCH-201E (GCH-201E) or pCMV-GCH-II (GCH-II), with or without pcDNA3-Hsp27 (Hsp27) or pcDNA3-Hsp27-S3D (Hsp27-S3D). For some experiments, cells were further treated with 100 nM okadaic acid (OA). (A) Effect of

Hsp27 on GCH-II. Western blots were performed with either anti-GCH (GCHII), anti-Hsp27 (Hsp27), anti-phospho-Hsp27 (Phospho-Hsp27), or anti-tubulin (Tubulin) on either the supernatant (Sup, or when not indicated) or pellet (Pellet) fraction. Lane 1 was a non-transfection control. (B) Effect of Hsp27 on GCH-201E. Western blot analyses were performed with anti-GCH (GCH-201E), anti-Hsp27 (Hsp27), or anti-tubulin (Tubulin).

Figure 6. Disuccinimidyl suberate (DSS) cross-linking assay. (A) Cross-linking of recombinant wild-type GCH protein with DSS at concentrations from 0.125 to 2 mM. Lane 1: untransfected control. Wild-type GCH was monomeric or dimeric in urea gel-loading buffer (Lane 2). With increasing concentration of DSS, GCH oligomers appeared (Lanes 3-7). (B) DSS cross-linking of GCH-201E and GCH-II. The input of GCH-II was less, but the formation of dimer and trimer was similar to those formed by the wild-type GCH protein. GCH-201E could not form any complex structures, and remained in monomeric form.

Figure 7. Interactions between the wild-type and mutant GCH proteins and the dominant-negative effect. (A) Both A16-GCH-WT, A16-GCH-201E, A16-GCH-II, and HA-GCH-WT were expressed in NIH293 cells. The crude cell lysates were

immunoprecipitated (IP) with anti-HA, and then Western blots (IB) were done with either anti-N20 or anti-HA. The figures show that both mutant GCH proteins can be brought down by wild-type GCH. (B) BHK cells were transfected with pCMV-A16-GCH-WT (A16-GCH) alone, or together with pCMV-GCH-II (GCH-II), pcDNA3-Hsp27 (Hsp27), or pcDNA3-Hsp27-S3D (Hsp27-S3D). Western blot analyses were performed with either anti-GCH (GCH) or anti-tubulin (Tubulin) on the supernatant fraction of the cell lysate. GCH-II was barely visible in the study and was omitted from the picture.

Figure 8. Structure and solubility of the GCH mutant proteins. Both GCH-WT, GCH-II and GCH-201E were expressed in BHK cells. Cell fractions used were: GCH-WT, Supernatant (Sup); GCH-II, Sup and Pellet; GCH-201E, Pellet. (A) Filter retardation assay. Cell lysates were prepared in either PBS or 2% SDS, and were filtered through a 0.2 μ m membrane (Lanes 2-5) or NC membrane (Lane 1) by a dot blot apparatus. Western blot analysis was performed with anti-GCH. A1-2: GCH-II in PBS; B1-2: GCH-WT in PBS; A3: GCH-II in SDS; B3: GCH-WT in SDS; A4 and B4: GCH-201E in SDS; A5 and B5: 71Q-GFP in SDS. (B) Western blot analysis of the GCH proteins. Samples were prepared in either urea gel-loading buffer (odd number lanes) or glycerol gel-loading buffer (even number lanes). The white arrows indicate

GCH-II monomer and oligomers; the black arrows indicate GCH-WT monomer and oligomers; the stars indicate cross-reacting materials.

Figure 9. Localization and ubiquitination of GCH-201E. Upper panel: BHK cells expressing pCMV-GCH-201E (GCH-201E) were stained by both anti-LAMP1 and anti-GCH. Phase contrast-confocal microscopy studies were shown for LAMP1 (A), GCH-201E (B), and the merge picture (C). The GCH stain colocalizes with the lysosome marker LAMP1. Lower panel: Ubiquitination assay for GCH-201E. A16-GCH-201E and HA-Ub were expressed in NIH293 cells. Immunoprecipitation (IP) was done by anti-HA, and Western blots (IB) were done with either anti-N20 or anti-HA. The results demonstrate that poly-ubiquitinated proteins (Poly-Ub) can be precipitated by anti-HA, but GCH-201E (arrow) is not ubiquitinated.

Figure 10. Phospho-Hsp27 decreases the formation of huntingtin polyglutamine inclusion bodies. BHK cells were transfected with 71Q-GFP (HT), with or without Hsp27 (27) or Hsp27-S3D (S3D). (A) Aggregation formed in the cytoplasm could be visualized under fluorescence microscopy (left panel, 1,000X), and inclusion body-positive cells were counted in ten 200X fields (right panel) for each experiments. (B) The results were expressed as percentages of aggregation-positive cells. The lines

above the bars indicate one standard deviation.

ACCEPTED MANUSCRIPT

Fig.1

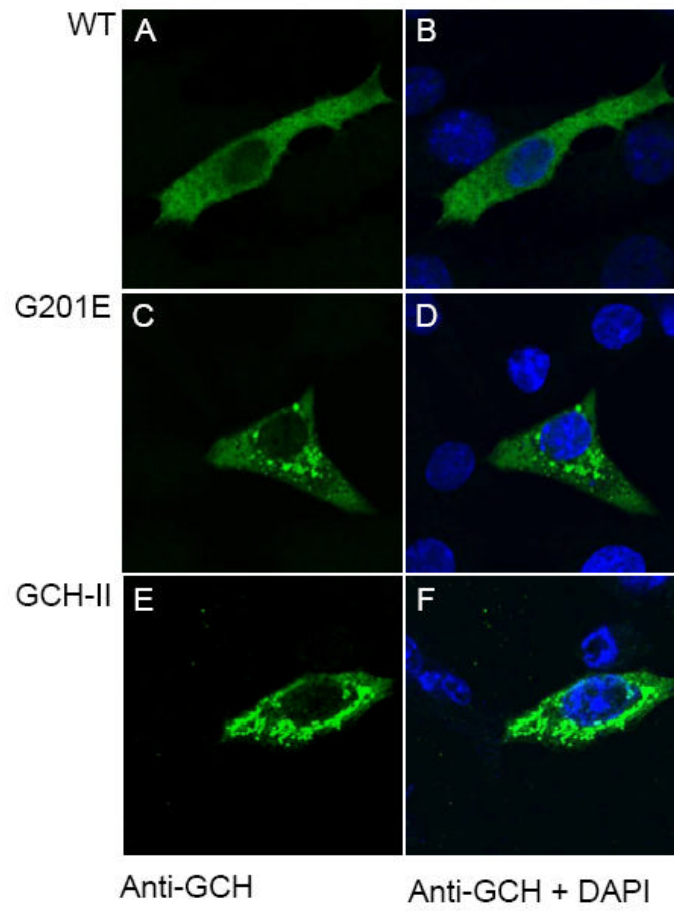


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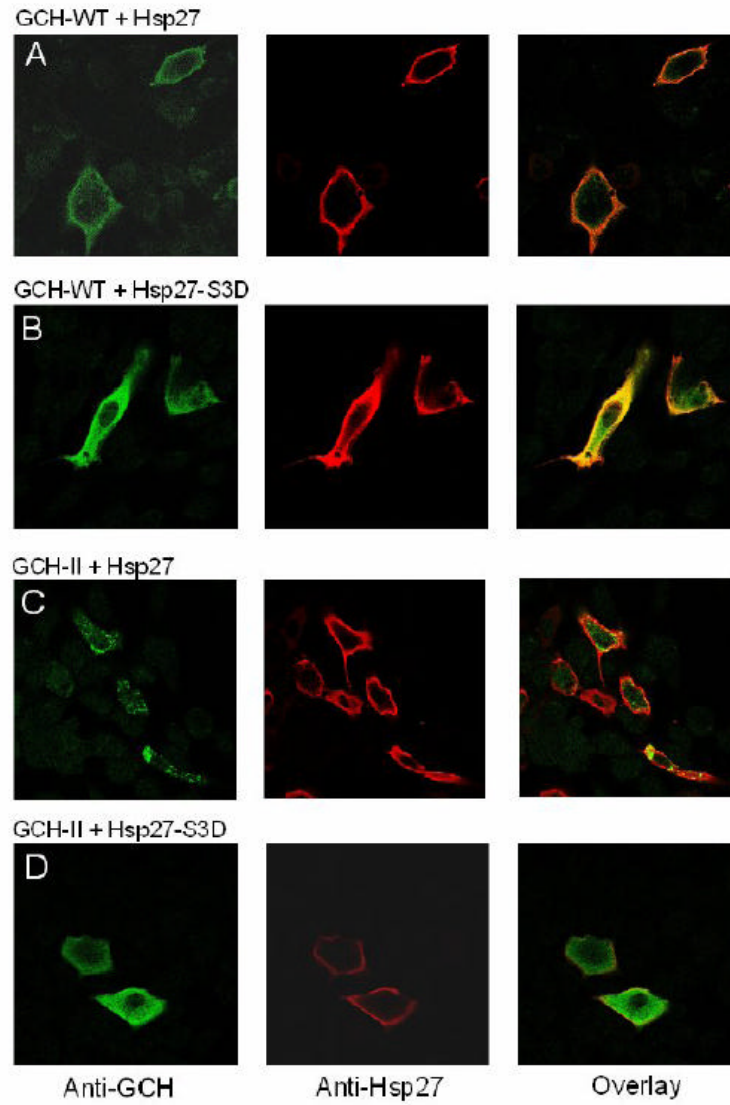


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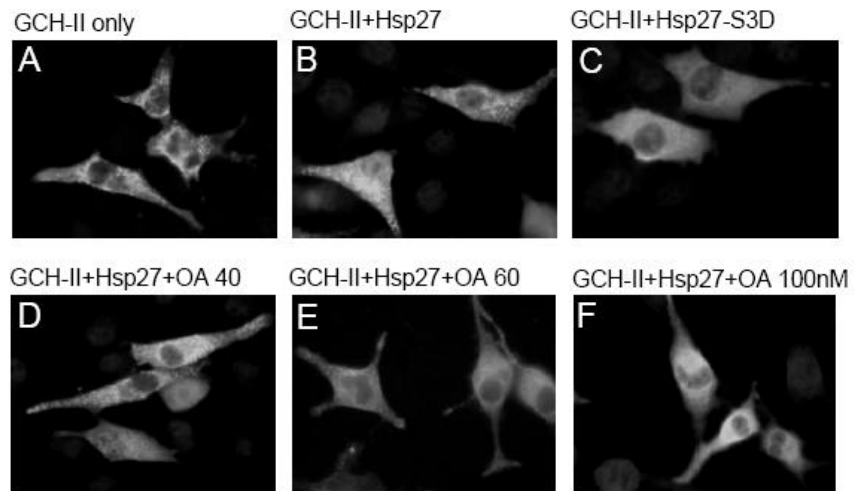


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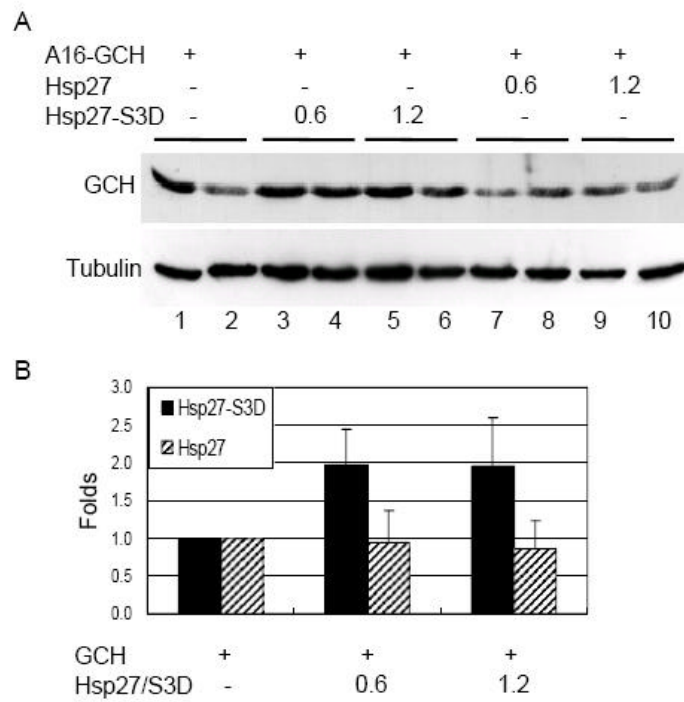


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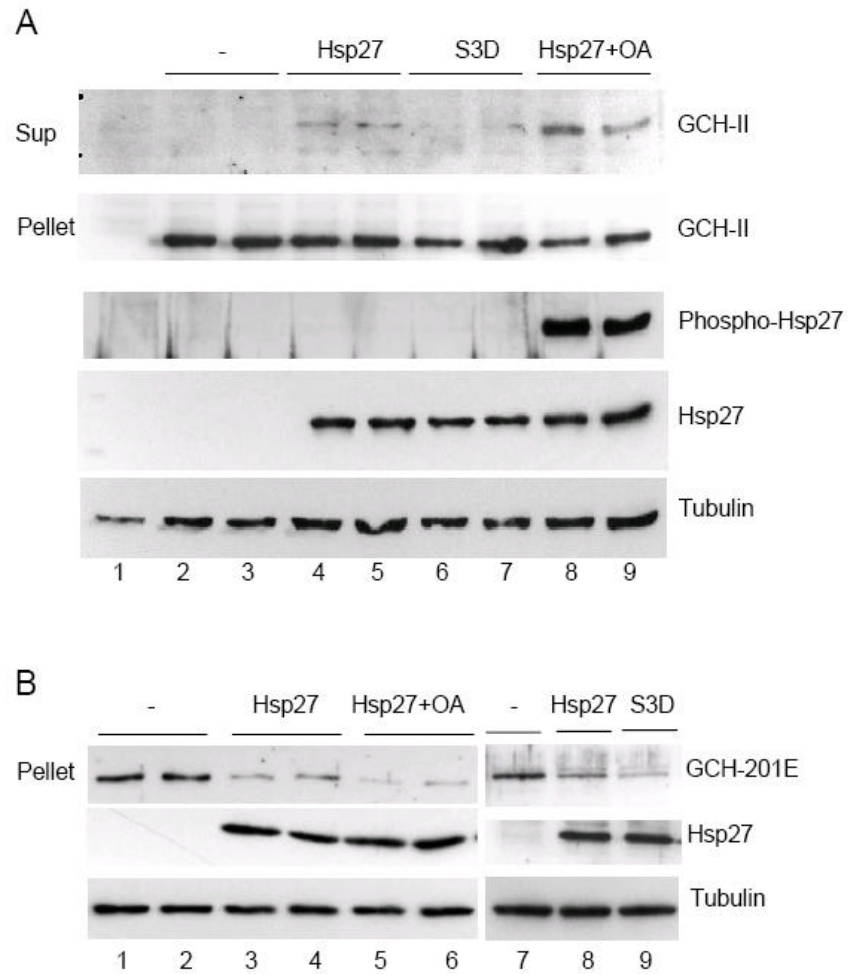


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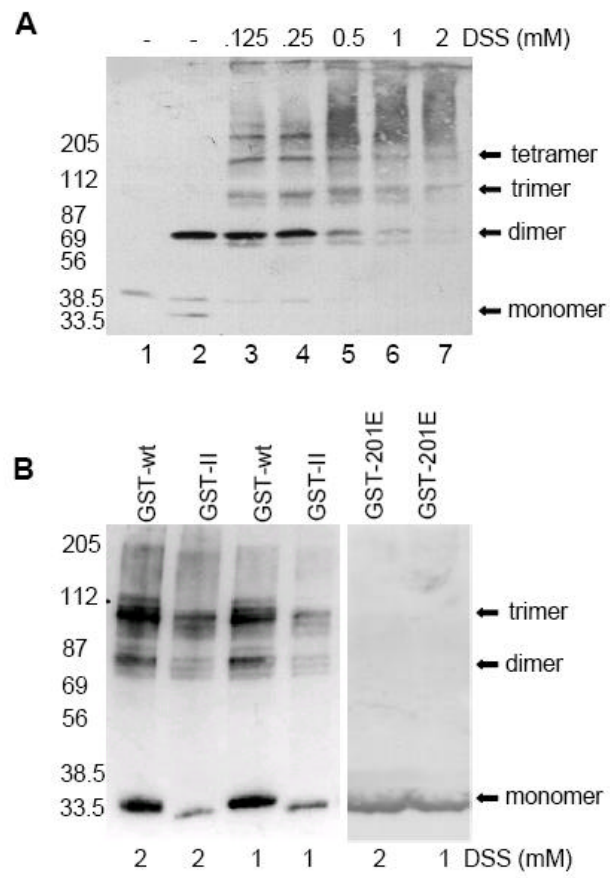


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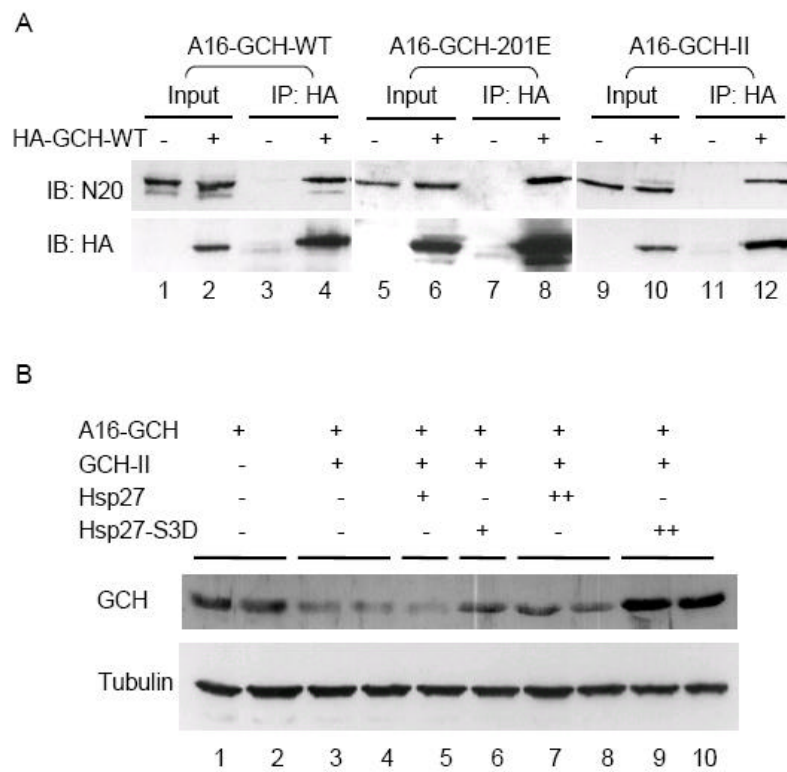


Fig.8

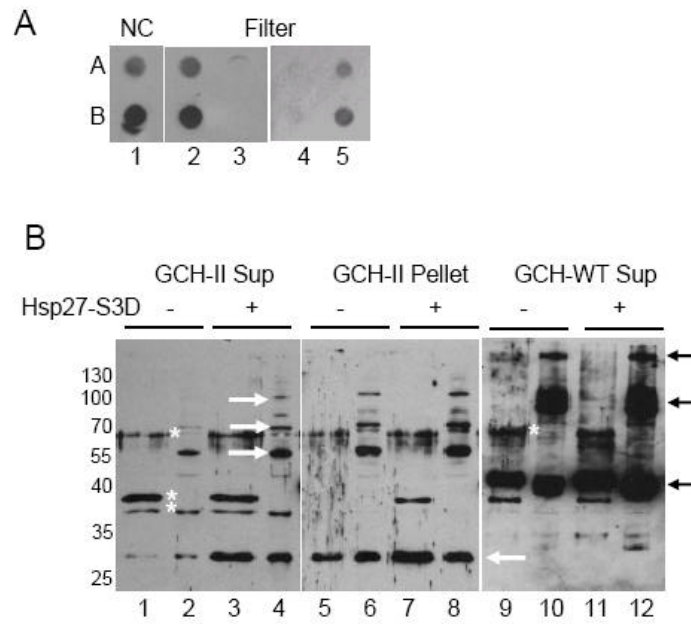
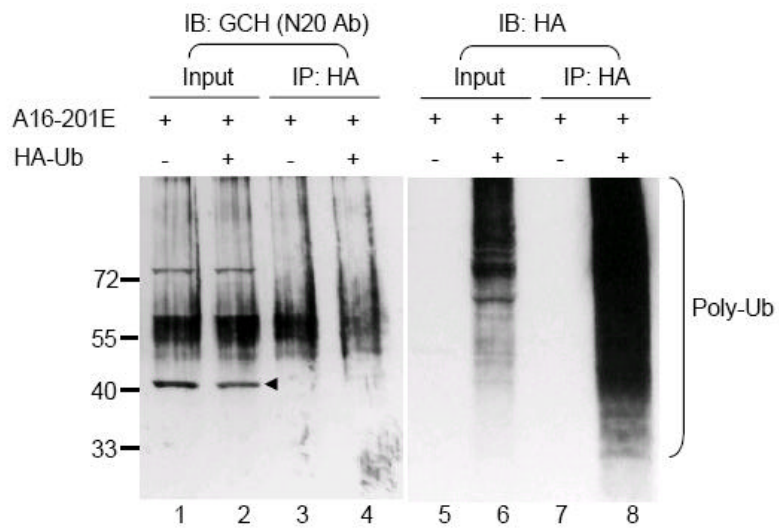
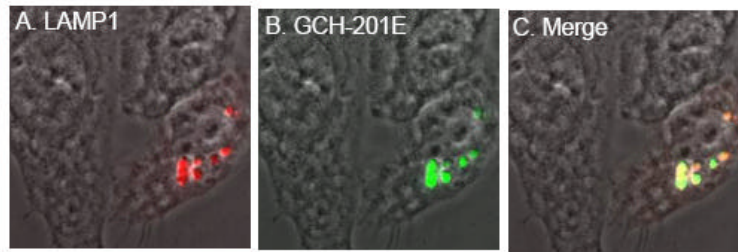


Fig.9



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Fig. 10

