Molecular Chaperones Affect GTP Cyclohydrolase I Mutations in Dopa-Responsive Dystonia

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Unstable GTP cyclohydrolase I (GCH) mutations in dopa-responsive dystonia (DRD) can exert a dominant-negative effect in the HeLa cell model, but in a batch of cells this effect could not be shown. Through differential display, we found a higher Hsc70 expression in the non-dominant-negative cells. We further demonstrated that ectopic expression of Hsp40/Hsp70 stabilized the GCH mutant G201E. Moreover, Hsp90 inhibitor geldanamycin destroyed the wild-type GCH level, and heat shock increased the synthesis of GCH protein. Therefore, the dominant-negative effect produced by unstable proteins would be susceptible to the status of molecular chaperones, which could be the modifying genes and therapeutic targets for DRD and other genetic diseases.


Dopa-responsive dystonia (DRD), characterized by a prominent diurnal change of dystonia, is induced by mutations in the GTP cyclohydrolase I (GCH; EC 3.5.4.16) gene, which leads to tetrahydrobiopterin (BH4) deficiency.1–4 Homozygous recessive GCH mutations cause low GCH activity, neurotransmitter deficiencies, and hyperphenylalaninemia.4 However, heterozygous GCH mutations in DRD also result in GCH activities less than 20% of normal.2 Because DRD is characterized by its low penetrance and female predominance, modifying genes may exist but have not been defined.1–4

We have established a HeLa cell model to show the dominant-negative effect of the GCH G201E mutation,5 but recently we found a batch of HeLa cells that did not demonstrate the effect (the non–dominant-negative cells). In this study, by differential display, we found higher Hsc70 expression in these cells. We further demonstrated the roles of Hsp40/Hsp70, Hsp90, and heat shock on GCH synthesis. We propose that chaperones could be the modifying genes for DRD.

Materials and Methods

Polymerase Chain Reaction–Select cDNA Subtraction

Polymerase chain reaction (PCR)–select cDNA subtraction was conducted according to the manufacturer’s instruction (Clontech, Palo Alto, CA).6 Sixteen forward clones (enriched in the dominant-negative cells) and 16 reverse clones (enriched in the non–dominant-negative cells) were identified after sequencing and data bank searching.

Plasmids and Cells

GCH cDNA was kindly provided by Dr Nagatsu. pCMV-GCH (wild type), pCMV-FLAG-GCH (FLAG epitope), pCMV-A16-GCH (A16 epitope), pCMV-GCH-G201E, and pCMV-FLAG-GCH-G201E have been described.5 Hsp70 and Hsp40 cDNA were cloned in pUC18 (Invitrogen, La Jolla, CA). Human embryonic kidney 293 cells (HEK293), HeLa cells, and baby hamster kidney cells (BHKs) were cultured in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum. Calcium phosphate precipitation method was used for transfection with 1μg pRSV-B-galactosidase as efficiency control and pCMV as DNA carrier. Forty hours after transfection, cells were harvested for assays. BHK-A16 was a stable line derived from BHK cells transfected with pCMV-A16-GCH and pCMV-neo. Geldanamycin was obtained from Sigma-Aldrich (Milwaukee, WI).

Western Blot, GTP Cyclohydrolase I Assay, Reverse Transcription Polymerase Chain Reaction, and Cross-linking

Western blot analysis was performed with monoclonal anti-A16 antibody,7 anti–GCH antibody,5 anti–FLAG antibody (M2 antibody; Kodak, Rochester, NY), anti-tubulin, anti-Hsc70 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-Hsp70 (Stressgen, Vancouver, BC, Canada) and was detected with the ECL system (Amersham, Arlington Heights, IL). GCH activity was determined by an high-performance liquid chromatography method. GCH mRNA expressed in
BHK-A16 cells was estimated by reverse transcription–PCR with primers F2 (5′-GCCCGGCAGCGAGGAGGATAAC) and R3 (5′-GACAGAACATGCTACTGGCCAGT) after adjusting cycle numbers for linear amplification. GCH cross-linking reactions were performed in 10 μl of 1 × phosphate-buffered saline 2.5mM EDTA buffer (pH 7.0) at a protein concentration of 7mg/ml by disuccinimidyl suberate (DSS; Pierce, Rockford, IL).

Pulse-Chase Experiment

BHK-A16 cells were incubated in medium without methionine and cysteine for 1 hour, pulsed with Pro-mix (Amerham) for 30 minutes, heat-shocked (44°C) for 1 hour, and then chased for a variety of time. Immunoprecipitation was done by anti–GCH antibody and protein A–Sepharose.

Results

The Dominant-Negative and Non–Dominant-Negative Cells

The expressions of wild-type GCH (pCMV-GCH) were equal in both kinds of HeLa cells (Fig 1A, lanes 1 and 3). However, when the mutant plasmid (pCMV-GCH-G201E) was transfected together, GCH protein was abolished in the dominant-negative cells (see Fig 1A, lane 2) but not in the non–dominant-negative cells (see Fig 1A, lane 4). This established the difference between these two cells.

Enrichment of Hsc70 in the Non–Dominant-Negative Cells

Sixteen forward clones (enriched in the dominant-negative cells) and 16 reverse clones (enriched in the non–dominant-negative cells) were obtained from differential display study. Ferritin represented 5 of the 16 forward clones. The reverse clones included Hsc70 (NM_006597) and RNF4 (a ring-finger transcriptional coactivator, GenBank HSU95140). The differential expression of these genes was proved by Northern blot analysis (see Fig 1B).

Hsp70/Hsp40 Stabilize GCH G201E Mutant

To prove the role of chaperones in GCH expression, we transfected pcDNA-Hsp40 and pcDNA-Hsp70 with pCMV-FLAG-GCH-G201E in HEK293 cells, a cell line with high transfection efficiency. GCH-G201E mutant was scanty after transient expression of pCMV-FLAG-GCH-G201E (Fig 2A, lane 2). Hsp40 or Hsp70 alone did not significantly alter the steady state of GCH-G201E (see Fig 2A, lanes 3 and 4). However, cotransfection of Hsp40 and Hsp70 allowed for a higher expression of GCH-G201E (contained no GCH activity, data not shown). To eliminate protein aggregates, we performed ultracentrifugation for the cell lysate, and a small amount of G201E protein remained in the supernatant (see Fig 2B). The wild-type GCH could be cross-linked by DSS, suggesting a multimeric structure (see Fig 2C), but GCH-G201E remained monomeric. Therefore, chaperones stabilized GCH-G201E, even though the mutant protein demonstrated no activity nor self-assembly.

Geldanamycin Abolishes GTP Cyclohydrolase I Expression

Hsp70/Hsp40 primarily acted on the unfolded GCH mutant, and it did not alter the protein level of the wild-type GCH (data not shown). Hsp90 is specialized in guiding the maturation of conformationally labile proteins, such as steroid receptors and cell cycle kinases, and may play a regulatory role. To explore the dependence of GCH protein on chaperones, we used Hsp90 inhibitor geldanamycin. When geldanamycin was added to HEK293 cells, GCH expression was completely abolished (Fig 3A, lanes 1 and 2). This shows that the steady state level of wild-type GCH depends on the presence of Hsp90.

Heat Shock Increases the Synthesis of Wild-type GCH

To ask if the chaperones could enhance the expression of GCH, we established a GCH stable line (BHK-A16) and tested the effect of heat shock. Two hours after heat shock, the GCH protein level started to in-
crease (see Fig 3B), but the level of GCH mRNA did not change. To know if GCH protein was stabilized by heat shock, we performed a pulse-chase study. The result showed that the half-life of GCH was not changed by heat shock (see Fig 3C), and therefore protein synthesis increased.

Discussion
BH₄ is widely involved in neurotransmission, signal transduction, vasodilation, inflammation, and free radical production. The regulation of BH₄ mainly depends on the rate-limiting step catalyzed by GCH, whose expression can be controlled at transcriptional level or by alternative splicing in tissues. Our results suggest that GCH also could be regulated at protein level by molecular chaperones. The multiple disciplinary controls of GCH expression increase the...
flexibility of GCH levels upon different physiological conditions.

Overexpression of Hsc70 in the non–dominant-negative cells, from spontaneous mutation or environment selection, gives a clue to the role of chaperones on GCH expression. It then was proved by the heterologous expression of Hsp70/Hsp40. Hsp70 chaperones assist folding of both newly translated proteins and misfolded proteins.\textsuperscript{12} Stabilization of GCH-G201E mutant by Hsc70 may explain the loss of dominant-negative effect in the cells. The dependence on Hsp90 for GCH expression indicates Hsp90 may assist GCH conformation. In the maturation of GCH, including folding and assembly, the chaperone systems are required and could be a good therapeutic target for DRD, like the trial of Hsp40 for Huntington disease.\textsuperscript{13}

DRD is characterized by its low penetrance. Data from this study suggest that subtle changes in chaperones can alter the manifestation of the disease. Therefore, we propose that chaperones could be the modifying genes in DRD and other genetic diseases involving labile proteins and multimeric protein complexes.

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References
