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GTP Cyclohydrolase I 突變及 Splicing 之分子結構機轉(2/3)

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計畫主持人： 胡務亮

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GTP cyclohydrolase I 突變及 Splicing 之分子結構機轉(2/3)

(Regulation of GTP cyclohydrolase I protein, the spliced forms, and mutants by heat shock proteins)

Abstract

GTP cyclohydrolase I regulates the level of tetrahydrobiopterin and in turn the activities of nitric oxide synthase and aromatic amino acid hydroxylases. Mutations of the GCH gene induce human diseases including malignant hyperphenylalaninemia (HPA) and dopa-responsive dystonia (DRD). DRD is often caused by dominant GCH mutations with residual GCH activities up to 15% of normal. GCH protein is a multimeric protein composed of 10 identical subunits, and we have demonstrated that both some dominant GCH mutations and the type II cDNA exerted a dominant-negative effect toward the wild-type GCH protein. In this study, we demonstrated the expression of wild type GCH protein (by transfecting BHK cells) increased after heat shock. The mRNA levels were not changed, but we are not able to detect change in protein stability by pulse-chase and immunoprecipitation study. Cotransfection of hsp40 or hsp70 could not mimic the effect of heat shock. On the contrary, cotransfection of hsp27 greatly decreased the expression of GCH proteins. This study suggests regulation of GCH protein expression by a complex cellular reaction bringing up by heat shock and the heat shock proteins.

Key words: GTP cyclohydrolase I, Alternative splicing, Dominant-negative effect, Regulation

GTP 環狀水解酵素(GCH)突變造成二種疾病：肌肉張力不全(dopa-responsive dystonia, DRD)與苯酮尿症(malignant phenylketonuria)，後者伴隨嚴重的神經傷害。GCH 基因的突變，有些會以一種 dominant-negative 的方式，影響到細胞中正常的 GCH 蛋白。我們最近也發現 GCH 基因的第二型 splicing 也有 dominant-negative 的效果。當我們將細胞進行熱休克後，轉染到 BHK 細胞中之 GCH 基因會表現出比較大量的蛋白。GCH 基因的轉錄沒有改變，在 pulse-chase/immunoprecipitation 實驗中也看不到蛋白質穩定性的變化。轉染時如果同時加入 hsp40 及 hsp70 時，GCH 蛋白的表現並不會有變化。反而是轉染時如果同時加入 hsp27 時，GCH 蛋白的表現量會有明顯的減低。這個研究顯示 GCH 蛋白的表現會受到細胞內複雜，和熱休克有關之機制的控制。

關鍵詞：GTP 環狀水解酵素，基因調控，熱休克，熱休克蛋白

Introduction

Tetrahydrobiopterin (BH_4) is the essential cofactor of phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase, and nitric oxide synthase (NOS). The rate-limiting step of BH_4 biosynthesis is the cleavage of GTP to 7, 8-dihydroneopterin triphosphate by the enzyme GTP cyclohydrolase I (GCH, EC 3.5.4.16) (Nichol et al 1985). GCH is therefore necessary for the synthesis of the signaling molecules dopamine, norepinephrine, epinephrine, serotonin and nitric oxide as well as the detoxification of the amino acid L-phenylalanine (Kaufman et al 1959, Nagatsu et al 1964, Griffith and Dennis 1995). Mutations of the GCH gene induce human diseases including malignant hyperphenylalaninemia (HPA) and dopa-responsive dystonia (DRD) (Thony and Blau 1997). HPA is induced by recessive GCH mutations and is associated with hyperphenylalaninemia, dystonia, seizure and neurological degeneration, probably due to the lack of both catecholamines (dopamine, noradrenaline and adrenaline) and serotonin (Niederwieser et al 1984). Dystonia is the only symptom in DRD patients, and the response to low-dose L-dopa is excellent. DRD is often caused by dominant GCH mutations with residual GCH activities up to 15% of normal (Segawa et al 1976, Ichinose and Nagatsu 1997, Hwu et al 1999). GCH protein is a multimeric protein composed of 10 identical subunits (Nar et al 1995), and we have demonstrated that some dominant GCH mutations exerted a dominant-negative effect toward the wild-type GCH protein (Hwu et al 2000). Recently, we further demonstrated that the type II cDNA exerted a dominant-negative effect on the wild-type cDNA, similar to the effect of some GCH mutants. When we stimulate peripheral blood mononuclear cells by PHA, the transcription of full-length GCH mRNA increased, but that of type II mRNA decreased transiently. Therefore, type II mRNA may regulate GCH, and then contribute to the regulation of NO production by BH4-dependent iNOS in mononuclear cells. In this study, we demonstrated that the expression of heat shock proteins may affect the levels of GCH protein, or probably, alter the interaction between the wild type and mutant or spliced forms.

Materials and Methods

Cells and cultures. BHK, HepG2, HeLa, and Huh7 cells were obtained from the American Type Culture Collection directly or through the National Health Research Institute Cell Bank. The cells were grown in Dulbecco's minimal essential medium (DMEM) with 10 % (v/v) fetal-calf serum (FCS) (Gibco, USA). For heat shock, the cells were moved to an CO2 incubator set as 45^{o}Cquantitative. Temperature and humidity control are set as usual.

Construction of plasmids. GCH cDNA was kindly provided by Dr. Nagatsu. This cDNA produced functional protein although it lacks a short 5' piece (Ichinose et al 1995). Type II cDNA was produced by PCR using GCH cDNA as a template, and was cloned into a CMV promoter expression vector (pCMV-GCH-II). pCMV-A16-GCH-wt was constructed by inserting full-length GCH cDNA (NarI/EcoRI fragment) into pCMV-AGP/EBP (replacing its BstBI/ EcoRI fragment) (Lee et al 1996). This generated an N-terminal AGP/EBP A16 epitope. The construction of pCMV-A16-GCH-249 and pCMV-A16-GCH-201 has been described (Hwu et al 1999, Hwu et al 2000). The expression vectors of the heat shock proteins were constructed by RT-PCR. mRNA was prepared and cDNA was synthesized and then subjected to PCR. PCR products were subcloned into pUC18 (Invitrogen)(Hsp70, *Bam*HI/*Eco*RI; Hsp40, *Bam*HI/*Pst*I). Primers sequences were (1) Hsp40: 5'-CGG GAT CCA GAT GGT GAA AGA AAC AAC TT-3' (sense) and 5'-CGG CTG CAG GCC ATT AAG AGG TCT GAC ACT-3' (antisence); (2) Hsp70: 5'-GAG AGT GAC TCC CGT TGT-3' (sense) and 5'-GGA ATT CAA ATG GCC TGA GTT AAG TGT A-3' (antisense).

Detection of GCH alternative splicing by reverse transcription-coupled PCR (RT-PCR). Cytoplasmic RNA was extracted from cells with a Total RNA Extraction Miniprep System (Viogene, Taiwan). For the measurement of GCH mRNA transcribed from transfected plasmids, mRNA was extracted by a QuickPrep Micro mRNA Purification kit (Amersham Pharmacia Biotech, USA). Upper strand PCR primers used were F2 (5'-GCCCCGCAGCGAGGAGGATAAC) and lower strand primers were R3 (5'-GACAGACAATGCTACTGGCAGT) for the full-length transcript and S1 (5'-GAAGCTATGGTTCTGCAGAC) for the spliced forms.

Transfection and western blot analysis. Calcium phosphate precipitation method was used for transfection (Graham and van der Erb 1973). BHK cells in 6 cm dishes were transfected with a combination of pCMV-A16-GCH-wt, pCMV-GCH-II and pCMV. Forty hours after transfection, cells were subjected to either mRNA extraction or western blot analysis. Western blot analysis was performed with a GCH antiserum (Hwu et al 1999) by standard methods. Blots were developed using the ECL detection system (Amersham Pharmacia Biotech, USA).

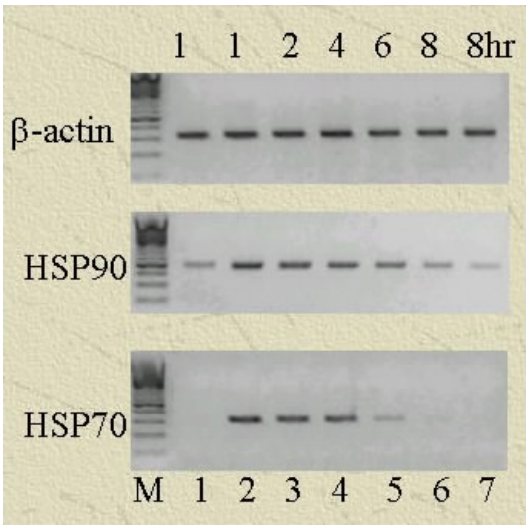
Disuccinimidyl suberate (DSS) cross-linking assay. GCH cross-linking reactions were performed in 10ì l of 1X PBS-EDTA buffer (pH 7.0) at a 7 mg/ml protein concentration. The protein mixture reacted with disuccinimidyl suberate (DSS, PTERCE) at concentrations varying from 0.025 mM to 2 mM and was incubated at room temperature for 30 min. 35mM glycine buffer was used to terminate the reactions for 15 min at room temperature. Followed by adding 10ì l of 2x SDS sample buffer into the mixture, the cross-linked protein mixture was resolved by

electrophoresis on gradient polyacrylamide gels in the presence of SDS. After electrophoresis, protein was transfer onto nitrocellulose membrane and proceeded as Western blot analysis Method.

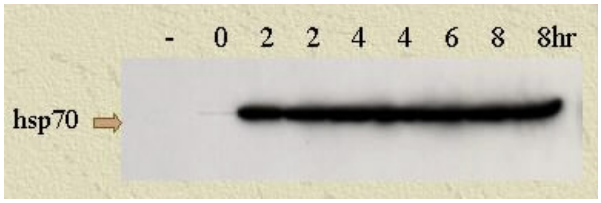
Pulse-chase study and immunoprecipitation. Ten μg pCMV-A16-GCH was transfected into BHK cells. Forty hours after transfection, cells were incubated in medium without methionine and cysteine for 1 hour, pulsed with Pro-mix (Amersham, USA) for 5 or 30 minutes, and then chased for another 1, 2 or 4 hours. Immunoprecipitation was done by anti-GCH antibody and protein A-sepharose. Proteosome inhibitor N-acetyl-leu-leu-norleucinal (ALLN, 100 nM, Sigma, USA), protease inhibitors PMSF (1 mM) and leupeptine (Leu, 1 mM), and lysosomotropic agent NH_4Cl (50 mM) were added 30 minutes prior to the pulse-chase experiment. Autoradiography and quantification were done by BAS 1500 Image Analyzer (FUJI, Japan)

Results and Discussions

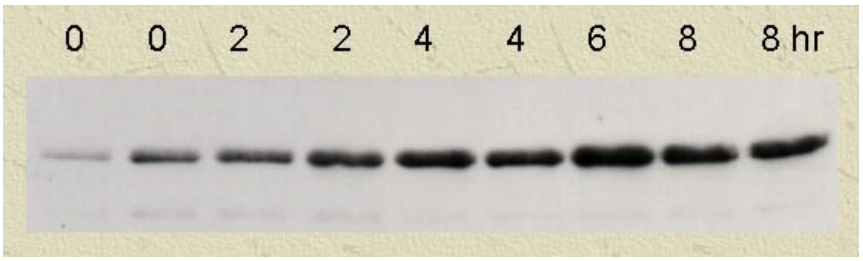
Verification of the heat shock response could be shown by measurement of the transcription of the heat shock protein genes.



As the figure shows, the mRNA of both hsp90 and hsp70 increase transient in BHK cells after heat shock. In the figure below, the hsp70 protein increased rapidly sine the second hour and persisted beyond the 8th hour (a western blot analysis).

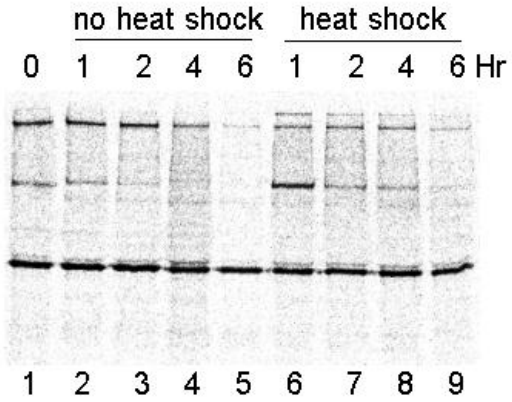


When we transfected BHK cells with the wild type GCH plasmid, and heat shock was performed in the morning of the second day after a medium change, and harvested 2, 4, 6 and 8 hours thereafter.



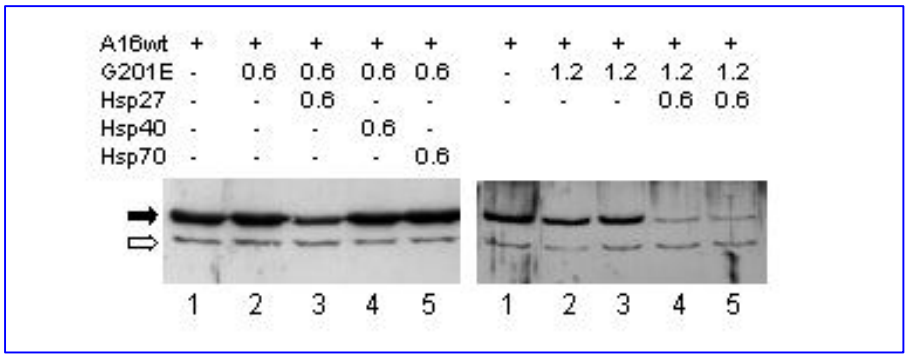
From the above figure we can see that the amount of GCH protein expression increased after heat shock, peaked at 4th to 6th hours, and persisted for more than 8 hours.

We tried to see the reason that the expression of GCH increased. The mRNA expression level was not changed. We performed a pulse-chase study, followed by immunoprecipitation.



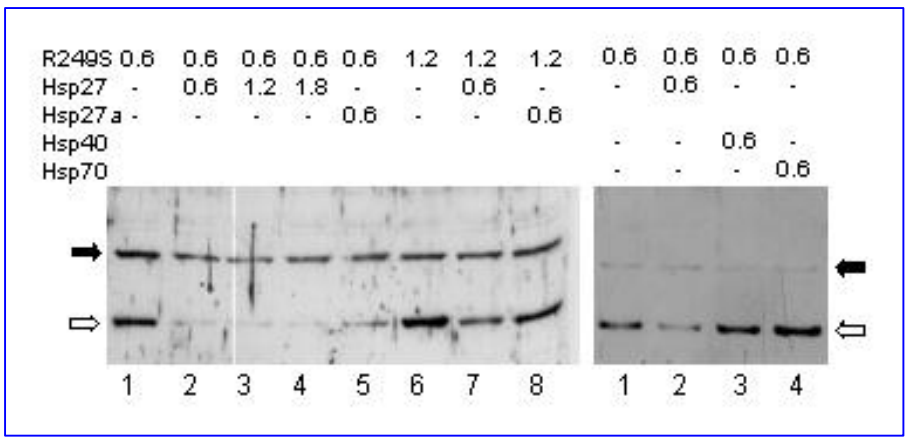
From the data above, the amount of labeled GCH either after pulse (time 0) or after a chase of 1 to 6 hours, with or without heat shock, the amount of GCH protein remained the same. Therefore the protein is stable and the half lives can not be estimated from the current experimental design.

The next step of this study is to over-express heat shock proteins together with GCH. We transfected 293 cells with the wild type GCH plasmid (A16wt) with a variety of plasmids including the dominant negative mutant G201E, hsp27, hsp40 and hsp70.



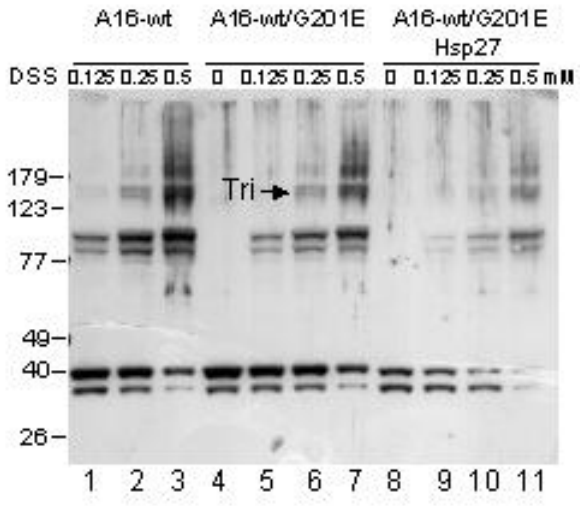
The results revealed that the dominant negative mutant (G201E) did not affect the expression of the wild type protein (open arrow). Either hsp40 or hsp70 did not change the expression. However, hsp27 significantly decreased the expression of wild type GCH.

The next we want to test the action of chaperones on the unstable mutant R249S.

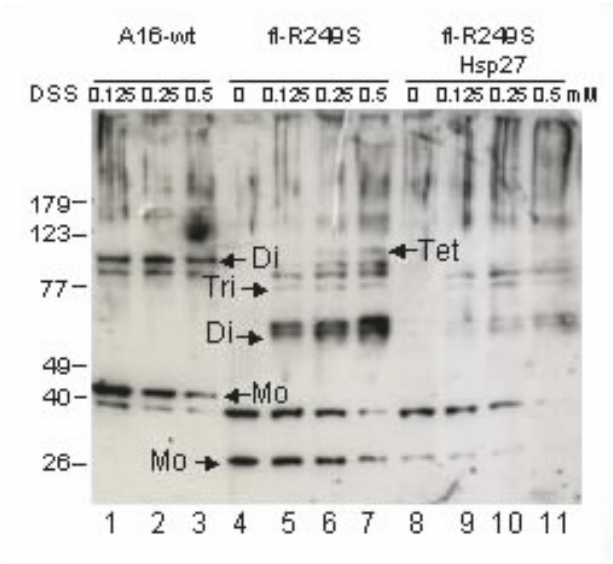


We observed that hsp27 also significantly decreased the amount of the R249S GCH protein (open arrow) that we can detect. The inhibition was dose-dependent. Therefore studies suggested that although heat shock could increase the amount of GCH protein expression, coexpression of some of the individual heat shock protein might on the contrary decrease the levels of GCH protein expression.

Since we observed that hsp27 could decrease the expression of GCH protein, we want to explore if hsp27 exert its function either on subunit protein stability, or on subunit assembly. We used the DSS cross-linking test to assess the assembly of GCH protein.



From the results, we can see GCH monomer, dimmer, and trimer (Tri) clearly on the gel. The total protein decreased in the presence of hsp27. However, the pattern of oligomerization did not change. Therefore, there is no evidence that hsp27 affected the assembly of GCH subunit. Also, G201E did not show dominant-negative effect on the wild type protein. In the following, we did the same test on the unstable GCH mutant R249S. The results showed similar decrease of protein levels in the presence of hsp27, but no change in the assembly pattern.



In conclusion, the expression of wild type GCH protein, but we are not able to detect change in protein stability by pulse-chase and immunoprecipitation study. Cotransfection of hsp40 or hsp70 could not mimic the effect of heat shock. On the contrary, cotransfection of hsp27 greatly decreased the expression of GCH proteins. Therefore, regulation of GCH protein expression may be done by a complex cellular reaction bringing up by heat shock and the heat shock proteins, but the exact mechanisms remains unknown.

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