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Single Nucleotide Polymorphism That Accompanies a Missense Mutation (Gln488His) Impedes the Dimerization of Hsp90

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Running title SNP of Hsp90 that impedes dimerization

Abbreviations

SNP single nucleotide polymorphism

Hsp heat shock protein

Hsp90 90-kDa heat shock protein

hHsp90α and hHsp90β α and β isoforms, respectively, of human Hsp90

Grp94 94-kDa glucose-regulated protein/endoplasmic reticulum paralog of Hsp90

Trap1 mitochondrial paralog of Hsp90

yeast Hsp82 and Hsc82 82-kDa heat shock protein and heat shock cognate protein of yeast,

respectively

HtpG bacterial ortholog of eukaryotic Hsp90

PAGE polyacrylamide gel electrophoresis

SDS sodium dodecyl sulfate.

Abstract A single nucleotide polymorphism (SNP) that causes a missense mutation of highly conserved Gln488 to His of the α isoform of the 90-kDa heat shock protein (Hsp90α) molecular chaperone is observed in Caucasians. The mutated Hsp90α severely reduced the growth of yeast cells. To investigate this molecular mechanism, we examined the domain-domain interactions of human Hsp90α by using bacterial 2-hybrid system. Hsp90α was expressed as a full-length form, N-terminal domain (residues 1-400), or middle (residues401-617) plus C-terminal (residues 618-732) domains (MC domain/amino acids 401-732). The Gln488His substitution in MC domain did not affect the intra-molecular interaction with N-terminal domain, whereas the dimeric interaction mediated by the inter-molecular interaction between MC domains was decreased to 32%. Gln488Ala caused a similar change, whereas Gln488Thr, which exceptionally occurs in mitochondrial Hsp90 paralog, fully maintained the dimeric interaction. Therefore, the SNP causing Gln488His mutation could abrogate the Hsp90 function due to reduced dimerization.

1 Introduction

The 90-kDa heat shock protein (Hsp90) is a molecular chaperone either transiently or stably associated with more than 100 client proteins that are unstable unless chaperoned by Hsp90. Reflecting their importance in cellular function, the proteins belonging to the Hsp90-faimily members are distributed essentially among all cells of living organisms. Two isoforms of the cytosolic Hsp90, *i.e.*, Hsp90α and Hsp90β, are ubiquitously expressed in mammalian cells (Hickey, et al 1989; Rebbe, et al 1987). In yeast cytosol, 2 isoforms, designated as Hsp82 and Hsc82, are expressed (Farrelly and Finkelstein, 1984; Borkovich, et al 1989) and either one of the isoforms is essential for the growth of yeast cells at high temperatures (Borkovich, et al 1989).

Previously, a possible correlation between single nucleotide polymorphisms (SNPs) in the human Hsp90β gene in patients with varicocele associated with infertility was investigated (Filho, et al 2005). As a result, three SNPs were found, but all of them were nonsense mutations. Thus, it remains unknown how the mutation of hsp90 gene is associated with human diseases. Passarino et al (2003) surveyed genetic polymorphism in Hsp90 isoforms in 73 Caucasians. Among the total of 29 genetic variants observed, 3 of them altered amino acid sequences. One possessed a frame-shift mutation, which may have produced a severely truncated product. The other 2 variants showed substitution of a single amino acid, Gln488 to His in Hsp90α and Val656 to Met in Hsp90β. Subsequently, it was reported that yeast Hsc82

carrying the substitution from Val656 to Met could proliferate indistinguishably to the wild type (MacLean, et al 2006). In contrast, yeast cells solely expressing Hsp82 with the substitution of Gln488 to His proliferated 5- to 10-fold less well, suggesting that Hsp90α with His488 was not or less functional. Indeed, Gln488 is highly conserved among most Hsp90-family members (Fig. 1), indicating the importance of Gln488 for the function of Hsp90-family molecular chaperones.

Yeast expression system was a powerful technique for evaluating the significance of SNPs observed in Hsp90 (MacLean, et al 2006). However, the technique does not provide much information on the molecular mechanism underlying the defect. Other approaches are thus necessary for elucidating the molecular aspects of this phenomenon. Here we examined the effect of the SNP on the domain-domain interactions of Hsp90.

In our previous study, we defined the N-terminal (amino acids 1-400/N domain), middle (amino acids 401-617/M domain), and C-terminal domains (amino acids 618-732/C domain) based on the sites most susceptible to proteases (Nemoto, et al 1997). The N domain was found to contain an ATP/geldanamycin-binding region (amino acids 1-220/230) (Prodromou, et al 1997a; Stebbins, et al 1997). There are the intra-molecular interaction between the N and the M domains and the homo-dimeric interaction between the MC domains (amino acids 401-732) (Matsumoto, et al 2002; Nemoto, et al 1995). These interactions are thought to be essential for the function of Hsp90. We provide data herein showing that the SNP that caused

the Gln488His mutation occurring in Hsp90α is critical, because this mutation weakened the

inter-molecular dimeric interaction of Hsp90.

2 Materials and Methods

2.1   Materials

The materials used and their sources were the following: Hot Star PCR, from Qiagen Inc. (Chatsworth, CA, USA); restriction enzymes and DNA-modifying enzymes, from Nippon Gene (Tokyo, Japan); and KOD plus DNA polymerase, from Toyobo (Osaka, Japan).   All other reagents were of analytical grade.

2.2   Bacterial 2-hybrid system

Escherichia coli BTH101 [F^-, *cya-99, araD139, gal15, galK16, rpsL1 (Strl^r), hsdR2, mcrA1, mcrB1*] and plasmids pKT25^{kan} and pUT18^{amp} were provided by Drs. D. Ladant (Pasteur Institute, Paris, France) and L. Selig (Hybrigenics, S.A., Paris, France).   An improved version of the bacterial 2-hybrid system (Karimova, et al 2001) was employed to evaluate domain-domain interactions of Hsp90.   This method is based on the interaction-mediated reconstitution of an adenylate cyclase in the enzyme-deficient *E. coli* BTH101.   The PCR fragment carrying MC domain of Hsp90α was inserted into a *Pst*I/*Bam*HI site of pUT18^{amp} or pKT25^{kan} as described previously (Tanaka, et al 2001).   The PCR-amplified fragment of the full-length human Hsp90α was inserted into a *Pst*I/*Bam*HI of the two plasmids.   *E. coli* BTH101 was co-transformed with pKT25- and pUT18C-derived plasmids.   Complex formation between co-expressed proteins was distinguished by color development of the

colonies on the MacConkey indicator media agar plates (Karimova, et al 2001) containing 1%

(w/v) maltose, 50 μg/ml of ampicillin, and 50 μg/ml of kanamycin. Quantitative data were

obtained by the measurement of the β-galactosidase activity of their liquid cultures, as described

previously (Tanaka, et al 2001). The enzyme activity of bacterial cells carrying control

plasmids (pKT25 and pUT18C) was set to 0% and the activity of the cells carrying the plasmids

encoding the wild type of the full-length form or its domains was set to 100%. Statistical

significance was evaluated by using Student's *t*-test.

2.3   *In vitro* mutagenesis

Glutamine at position 488 was substituted to His by PCR-based site-directed mutagenesis.

Sense   primers   (5'-<u>CAC</u>AAACATATCTATTATATCACAGGTGAG-3'   for   His,

5'-<u>GCG</u>AAACATATCTATTATATCACAGGTGA-3'   for   Ala,   and   5'-

<u>ACC</u>AAACATATCTATTATATCACAGGTGAG-3' for Thr) were combined with an antisense

primer   (5'-TGTCTCCTTCATGCGAGAAACATACACT-3'). Mutated   nucleotides   are

indicated by underlines. PCR was performed with pKT25-hHsp90βMC, pUT18-hHsp90αMC,

or pUT18-hHsp90α as a template by use of KOD plus polymerase. Template DNA was then

digested by DpnI, and the PCR fragments were phosphorylated and ligated. E. c*oli* XL1-Blue

was transformed with the DNA. Mutagenesis was confirmed by DNA sequencing.

Thereafter, BTH101 competent cells were co-transformed with pUT18- and pKT25-derived

plasmids and then grown up as described above.   After a culture period of 3-5 days at 30°C, the

β-galactosidase activity of the colonies was quantified as described previously (Tanaka, et al

2001).

3 Results

3.1 Evaluation of the domain-domain interactions of Hsp90α

In order to evaluate the effect of the SNP (Gln488His) on the function of Hsp90α, we examined the domain-domain interactions of Hsp90α. Hsp90α is present as a dimer mediated by the interaction of the MC domains (Nemoto, et al 1995). The dimeric interaction of Hsp90α produced by the two plasmids was reproduced by the bacterial 2-hybrid system (Fig. 2): The expression of either a full-length form of wild type or the Gln488His mutant produced from the pKT25-derived plasmids showed no activity (Fig. 2). When Glu488His mutant produced together with wild type of the full-length form, the inter-molecular interaction was decreased to 33% of the wild type. The inter-molecular interaction was 5.7-fold strengthened by using wild-type MC domains instead of the full-length form and was again decreased to 63% by the Gln488His substitution of one MC domain.

We previously demonstrated the intra-molecular interaction between the N and M domains of Hsp90α (Matsumoto, et al 2002; Tanaka, et al 2001). The amino acid substitution of Leu477 to Ala, which is near Gln488 (Fig. 1), caused the loss of the interaction of the MC domain of hHsp90α or that of HtpG, an *E. coli* ortholog of eukaryotic Hsp90, with the respective N domains. Moreover, yeast cells that solely expressed the mutant Hsc82 could not grow at higher temperature (37oC) (Matsumoto, et al 2002). Then, we here examined the effect of the Gln488His substitution on this intra-molecular interaction. As a result, the

intra-molecular interaction between the N domain and MC domains was maintained even after

the amino acid substitution (Fig. 3).

3.2   Dimeric interaction between MC domains having the Gln488His mutation

We here used the MC domain for the 2-hybrid system, because the interaction represented by

the β-galactosidase activity was several fold higher than that of the full-length form presumably

because of the enhanced expression of a shorter polypeptide in E. coli (Fig. 2).   The

dimer-forming potential of Hsp90αMC was decreased to 80% or 86% of the wild type by the

substitution in either subunit of the dimer (Fig. 4).   When the 2 mutated MC domains were

co-expressed, the dimeric interaction was further decreased to 32% of the wild-type Hsp90.   In

order to evaluate whether this effect was unique to His, we replaced the Gln488 of Hsp90αMC

with Ala and found that the Gln488Ala mutation yielded the results similar to the Gln488His

mutation (Fig. 4).   This finding indicated that Gln488 was indispensable for dimerization of

Hsp90α.   In fact, Gln488 is highly conserved in the Hsp90-family including yeast Hsp82

(Farrelly and Finkelstein 1984), yeast Hsc82 (Borkovich, et al 1989), human Grp94 (Maki, et al

1990), HtpG (Bardwell and Craig, 1987) and *Drosophila melanogaster* Trap1 (Felts, et al 2000).

However, exceptionally, Gln488 is substituted by Thr in human and mouse Trap1 (Song, et al

1996; Strasberg, et al 2002) (Fig. 1).

Because the Hsp90-family members so far studied form a dimeric structure (Welch and

Feramisco, 1982; Koyasu, et al 1986) that is tightly associated with the chaperone function of the Hsp90-family (Prodromou, et al 1997a, 1997b; Meyer, et al 2003), we speculated that, because human and mouse Trap1 have Thr at position 488 and maintain a dimeric conformation, Hsp90αMC having its Gln488 substituted by Thr might maintain a dimeric structure. We tested this idea and found that Hsp90αMC Thr488 indeed formed a homodimer as did wild type Hsp90αMC carrying Gln488 (Fig. 5).

4 Discussion

The present study demonstrated that the dimeric interaction of Hsp90 mediated by MC domain was affected by the SNP of CAA to CAC, causing Gln488His mutation. One might raise the possibility that the apparent difference in the inter-molecular interaction of the Hsp90α mutant might simply reflect the variation in expression levels between the wild and mutant types. The attempt to deny this possibility was not successful, because we could not immunologically quantify the expression levels of the wild type and mutated forms due to their low expression levels. Nevertheless, we postulated that the expression levels of wild and mutated types of Hsp90α were equivalent under our analytical conditions, because the intra-molecular interaction of MC domain with Gln488His with N domain was indistinguishable to that of the wild type (Fig. 3). Moreover, we demonstrated that the substitution from Gln488 to Thr in human Hsp90α, which naturally occurs in human and mouse Trap1, did not affect the

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dimeric interaction (Fig. 5).   This finding confirmed the importance of Gln488 for the dimeric

interaction.

The 3-dimensional structure of the M domain of yeast Hsc82 was obtained as a dimer

(Meyer, et al 2004).   In that structure, Gln488 is present at the edge of a loop

(Pro485-Glu-His-Gln488) between 2 α-helical regions (Fig. 1) and is not located at the interface

of the dimer.   The 3-dimensional structure of the HtpG dimer indicated that the configuration

is drastically modified by nucleotide species (ATP or ADP) associated with the dimer (Shiau, et

al 2006).   Gln427 of E. coli HtpG, which is equivalent to Gln488 of hHsp90α, is also located at

the edge of a loop (Lys424-Glu-Gly-Gln427) between α-helical and β-strand regions.

Importantly, this loop structure is located on the side opposite of the dimer interface of HtpG

both in the nucleotide-free and ADP-bound forms.   This is in contrast that amino acids that

affected the dimeric interaction of hHsp90α, such as Thr566, Ala629 (Kobayakawa, et al 2008),

Leu665, Leu666, Leu671 and Leu672 (Yamada, et al 2001), are located within the C-terminal

191 residues constituting the dimer.   Therefore, the effect of the Gln488His mutation on the

dimeric structure may be mediated in an indirect way.   For instance, the mutation might affect

the bending angle of the 3 domains, and therefore, sterically prevent the interaction between MC

domains.   A further study should be certainly needed to settle this issue.

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Legends for figures

Fig. 1 Comparison of the amino acid sequences of the Hsp90-family members around Gln488 of hHsp90α. mouse Hsp86; mouse Hsp84 (Moore, et al 1989); yeast Hsc82; yeast Hsp82; hGrp94, human Grp94; hTrap1, human Trap1; mTrap1, mouse Trap1; DmTrap1, *Drosophila melanogaster* Trap1; EcHtpG, HtpG from *E. coli*; and PgHtpG, HtpG from *Porphyromonas gingivalis* (Lopatin, et al 2000). Identical amino acids are indicated by hyphens. Leu477 and Gln488 were experimentally substituted to other amino acids in the previous (Matsumoto, et al 2002) and present studies, respectively. The α-helix and β-strand regions are indicated by underlines and broken lines, respectively, based on the crystal structures of yHsc82 (Meyer, et al 2003) and EcHtpG (Shiau, et al 2006).

Fig. 2 Dimeric interaction of hHsp90α carrying the Gln488His mutation. The dimer-forming potentials of the full-length form and the MC domain of Hsp90α having Gln488His were quantified by the use of the 2-hybrid system. Plasmid combinations for columns are indicated at the top. Values of the combination of controls (pUT18C and pKT25) and the combination of pUT18C-hHsp90α and pKT25-hHsp90α were set to 0% and 100%, respectively. Values are means $\pm$ S.D. (n=3). *p<0.05, n.s., not significant.

Fig. 3 Intra-molecular interaction between MC domain having Gln488His mutation and N

domain. The intra-molecular interaction between N domain and MC domain that carries Gln488His mutation of Hsp90α was quantified by using the 2-hybrid system. Plasmid combinations are indicated at the top. Values are means $\pm$ S.D. (n=3).

Fig. 4 Dimeric interaction of MC domain having the Gln488His or Gln488Ala mutation. The dimeric interaction between the MC domain of Hsp90α having Gln488His or Gln488Ala mutation was determined by the use of the 2-hybrid system. Plasmid combinations are indicated at the top. Values are means $\pm$ S.D. (n=3). $*p<0.05$, $**p<0.001$.

Fig. 5 Dimeric interaction of MC domain bearing the Gln488Thr mutation. The dimeric interaction between MC domains of Hsp90α having the Gln488Thr mutation was determined by using the 2-hybrid system. The interactions for Gln488His and Gln488Ala are shown for comparison. Plasmid combinations are indicated at the top. Values are means $\pm$ S.D. (n=3). $*p<0.001$.

Fig. 1

```
              477              488
               |                |
hHsp90α   VSLKDYCTRMKENQKHIYYITG
hHsp90β   T--SE-VS----T--S------
mHsp90α   ------------------F---
mHsp90β   T--SE-VS----T--S------
yHsc82    T--T--V---P-H--N------
yHsp82    T--T--V---P-H--N------
hGrp94    T--DQ-VE----K-DK--FMA-
hTrap1    T--SE-AS--RAGTRN---LCA
mTrap1    T--P--AS--QAGTRN---LCA
DmTrap1   M--EE-YNAVPAE-QN---LAA
EcHtpG    ---E--VS----G-EK-----A
PgHtpG    YTFDE-R-LVEGV-TDKDGQVV
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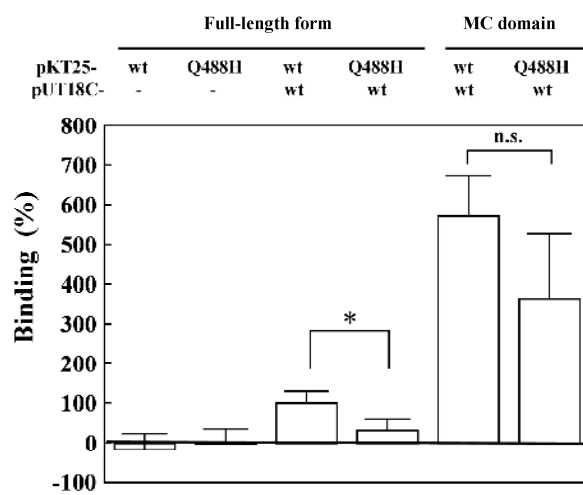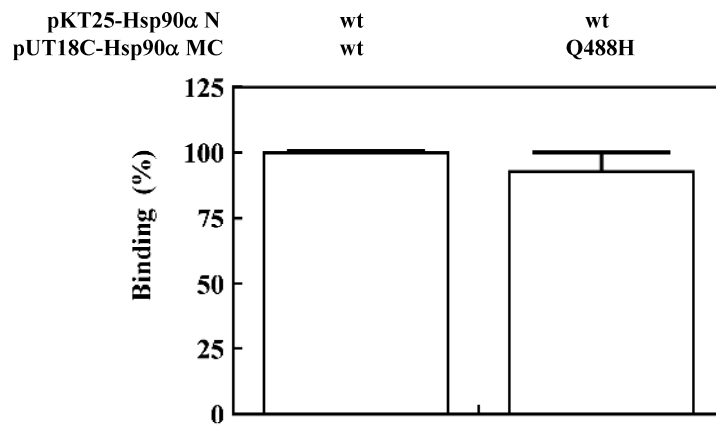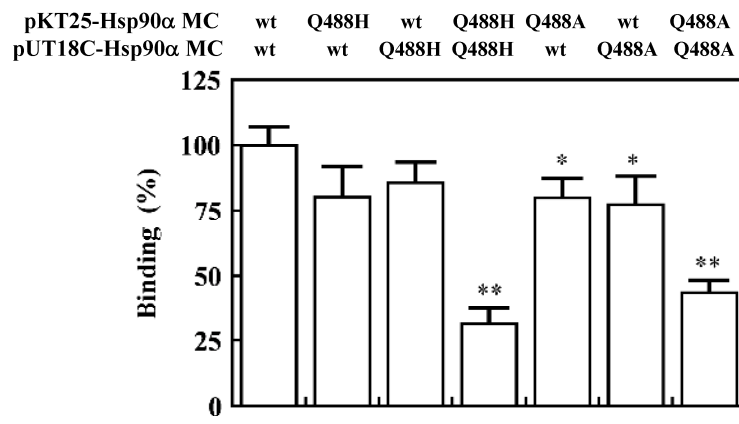
Fig. 2

Fig. 3

Fig. 4

Fig. 5