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HIDE, a Testis Specific Deubiquitinating Enzyme, Interacts with HSP90

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고환 특이적으로 발현되는 탈유비퀴틴효소 HIDE와 HSP90의 상호작용

포천중문 의과대학교 생명과학전문대학원, 세포유전자치료연구소, 차병원 여성의학연구소

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연구목적: 본 연구는 아직 그 기능이 파악되지 않은 탈유비퀴틴효소 중 하나인 HIDE에 대한 기본적인 생화학적 특징과 고환에서의 발현 양상을 파악하고 있다.

연구재료 및 방법: 인간의 HIDE 유전자를 클로닝하여 효소활성이 있는지 세포 외 실험을 통해 확인하였고, 아미노산 서열을 분석하여 진화상 보존된 부분을 찾아 그 기능을 파악한 다음 HSP90과의 상호작용을 공동면역침전반응으로 확인하였다. HIDE의 조직별 발현양상을 파악하기 위해서 인간과 쥐의 RNA 블롯과 쥐의 단백질 블롯을 이용하여 각각 노던 블롯팅과 웨스턴 블롯팅을 수행하여 고환에서 많이 발현된다는 것을 알았고 이 사실을 바탕으로 쥐의 고환을 절개하여 면역조직화학반응으로써 고환 내의 HIDE 단백질의 발현양상을 파악하였다.

결 과: HIDE는 세포 외에서 유비퀴틴 잔기를 제거하는 탈유비퀴틴 활성이 있으나 세포 내에서 전체적인 유비퀴틴 복합체를 줄여주는 효과는 없었다. HIDE는 HSP90이라는 분자 샤페론과 상호작용한다. HIDE의 전사체는 고환에서 가장 많이 발현되며 다른 조직에서도 소량 발현된다. HIDE의 단백질은 웨스턴 블롯상에서 고환에서만 확인되었다. 고환 내에서의 HIDE의 발현양상은 왕성한 감수분열을 하는 정모세포에서 높았으며 지지세포나 정조세포에는 발현되지 않았다.

결 론: HIDE는 분자 샤페론 HSP90과 상호작용하며 고환 내의 감수분열 중인 세포에서 많이 발현되는 것으로 보아 감수분열이나 정자형성에 관여하는 것으로 보인다.

Key Words: Co-chaperone, CS domain, Deubiquitination, DUB, HIDE, Hsp90, Spermatogenesis, Ubiquitin

The ubiquitin specific proteases cleave ubiquitin moieties from substrates.[1,2] The hydrolysis reaction is tightly regulated by deubiquitinating enzymes.[1] Recently, several deubiquitinating enzymes are known to participate in various signaling processes including signal transduction,[3] genomic integrity,[4] transport,[5] and protein degradation.[6]

Hsp90 is one of highly conserved molecular

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chaperones. Hsp90 functions as a chaperone,[7] a signaling center,[8] and a transporter.[9] There exist a number of clients and co-chaperones of Hsp90. Co-chaperones of Hsp90 regulate chaperoning function of Hsp90,[10] and clients use Hsp90 as a chaperone. Hsp90 functions as a signal transducer and loss of function causes reduction of important signal regulators. Several inhibitors of Hsp90 including geldanamycin, radicicol, and hypericin were known to down-regulate ATPase activity of Hsp-90 or reduce the level of Hsp90.[10,11] It is known that Hsp90 is ubiquitinated by hypericin but the pathway is unknown. Although the ubiquitination pathway is largely unknown, it is possible that ubiquitination of Hsp90 is one of important posttranslational modifications.

The co-chaperones of Hsp90 are divided into two groups, the N-terminal co-chaperone and the C-terminal co-chaperone. The C-terminal co-chaperones have 3 or more TPRs (tetratricorepeat), and interacts the EEVD sequence of at the C-terminus of Hsp90.[13] However, N-terminal co-chaperones are not well characterized. Recently, a number of N-terminal co-chaperones of Hsp90 were found to have the CS domain, sub-family of the Hsp20-like fold.[14] p23 activates ATPase activity of Hsp90 and helps proper loading of client proteins.[15] Sgt1 interacts with the N-terminus of Hsp90, and plays a role in kinetochore formation[16,17] and plant disease resistance.[18] In addition, Chp-1 recently was found to interact with the N-terminus of Hsp90,[19] but its function has not been characterized.

It is known that the expression of Hsp90 is important in the germ cell development of rodents.[20] Even though the exact function of Hsp90 in germ cell formation remains to be investigated, a point mutation of Hsp90 leads to microtubule disorganization and male sterility in *Drosophila*.[21,22] FKBP-59, a co-chaperone of Hsp90, shows stage specific expression in the testis and may function during spermatogenesis.[23] The functions of chaperones during spermatogenesis are poorly understood. However, several evidences including regulation of expression level of chaperones, relationship between male infertility and elevated temperature, and the roles of HSFs show importance of chaperones during spermatogenesis.[24]

MATERIALS AND METHODS

1. Scanning for conserved domains and sequence alignments

Scanning of conserved domains was performed with the rpsblast (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Other programs were also used including Superfamily (http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/), SMART (http://smart.embl-heidelberg.de/), Pfam (http://www.sanger.ac.uk/Software/Pfam/), InterPro (http://www.ebi.ac.uk/interpro/), and Prosite (http://us.expasy.org/prosite/). Alignments were performed with the MultiAlign (http://prodes.toulouse.inra.fr/multalin/).

2. Plasmids and site-directed mutagenesis

The original *HIDE* (*H*sp90 *I*nteracting *D*eubiquitinating *E*nzyme) clone was kindly donated by Dr. T. Nagase at Kazusa DNA Research Institute in Japan. We subcloned its coding region into pGEX-4T-2 (Amersham Pharmacia) for prokaryotic expression, and pcDNA3-myc (Invitrogen) for eukaryotic expression. Human Hsp90β in pEGFP-C1 clone was kindly donated by Dr. Jun Kim at Korea University in Seoul, Korea. The *HIDE* (C506S) mutant was constructed using a QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to manufacture's instruction with two primers which have sequences of 5'-GCAACACCTCCTTCATGAAC-3' and 5'-GTTCATGAAGGAGGTGTTGC-3'.

3. Cell culture and transfection

293T human embryonic kidney cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). Cells were grown in the 5% CO_2 incubator. Transfection was performed by $CaCl_2$ precipitation method using BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) buffered saline.

4. Northern blot analysis

A multiple tissue blot containing RNA from 8 different human tissues was purchased from Clontech. A multiple tissue blot containing RNA from 13 different mouse tissues was purchased from Seegene. A partial fragment of human *HIDE* cDNA (from 1 to 450) and the full-length *β-actin* and *G3PDH* were labeled with deoxycytidine 5'-[α-^{32}P]-triphosphate by a random priming method when blotting a human tissue blot. A partial fragment of human *HIDE* cDNA (from 3082 to 4122) was also used when blotting a mouse tissue blot. The immobilized nucleic acids were hybridized at 68℃ for 1 hour in a bag containing 5× SSC, 1× Denhardt's solution, 100 μg/ml of denatured salmon sperm DNA, 0.1% SDS and 50% formamide, the membranes were then washed twice with 0.1× SSC, 0.2% SDS for 30 minutes. Autoradiography was carried out with Agfa CP-BU films for 24 hours at -80℃. The blots were subsequently reprobed with a radiolabeled *β-actin* DNA probe for the human blot or a *G3PDH* DNA probe for the mouse blot.

5. *In vitro* deubiquitinating enzyme activity assay

In vitro deubiquitinating activity assay, based on the cleavage of ubiquitin-β-galactosidase fusion proteins *in vitro*, has been described previously.[25] The wild-type *HIDE* cDNA and cDNA containing a missense mutant form, *HIDE* (C506S), were used. Ub-Met-β-gal was expressed from a pACYC184-based plasmid. Plasmids were co-transformed into *Escherichia coli* BL21 cells. Plasmid-bearing *E. coli* BL21 cells were induced with the final concentration of 0.2 mM IPTG (isopropyl-β-thiogalactopyranoside) after 1 hour of pre-incubation. After 4 hours of induction with the IPTG, they were lysed in lysis buffer (0.01 M phosphate [pH 7.4], 8 M urea, 1 % SDS, and 1% β-mercaptoethanol). The assay with these whole cell lysates was analyzed by immunoblotting with a rabbit anti-β-gal antiserum (ICN), a rabbit anti-GST antiserum (Upstate Biotechnology), and the enhanced chemiluminescence (Amersham Biosciences).

6. Immunohistochemistry

The adult mouse testes were surgically removed, and the samples were embedded in paraffin, sectioned at a thickness of 5 μm, and then fixed in 3.7% formaldehyde overnight at 4℃. For antibody staining, the sections were deparaffinized and placed into a 3% solution of hydrogen perxide for 5 minutes. And they were washed twice with PBST (133 mM NaCl, 10 mM sodium potassium phosphate, pH 7.4 containing 0.1% Tween 20), placed in a blocking solution with 0.5% BSA/PBST for 30 minutes. After incubation with the anti-HIDE antibody (1:100) for 1 hour followed by washing, biotinylated secondary antibody was applied for 30 minutes and streptavidin-horseradish peroxidase substrate, and counter staining was performed with hematoxylin.

7. Antibodies and antisera

Polyclonal antisera were generated in rabbits against the C-terminal region of HIDE which has the sequence of ASRIWQELEAEEEPVPEGSGP (Komed). Anti-Hsp90β, anti-GST, anti-β-galactosidase and anti-actin antibodies were purchased from Santa Cruz. Anti-myc antibody was obtained


A
gggctggctgcggcggtctcgctcggctgtccgttccttgc
tggagaatttggccacaaagagctgccaagatagctgggccaggaagaaagcgccgcagccctgacccagacgctgttgccgaccccggggcactctggctgtcgaccaagcggctcaag
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M S G G A S A T G P R R G P P G L E D T T S K K K Q K D R A N Q E S K D G D P R 40 NLS
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K E T G S R Y V A Q A G L E P L A S G D P S A S A S H A A G I T G S R H R T R L 80
ttctttccttcatcgtcagggtcagcatccactcctcaagaggagcagaccaaagagggagcttgtgaagaccctcatgatctcttggctactcccactccagagttgttgctcgattgg
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aggcagagtgcagaagaggtgattgtcaagcttcgtgtgggagtaggtcccctgcagctggaggatgtagatgctgctttcacagatacagactgtgtggtgcggtttgcaggtggtcag
R Q S A E E V I V K L R V G V G P L Q L E D V D A A F T D T D C V V R F A G G Q 160
cagtggggtggtgtcttctatgctgagataaaaagctcttgtgctaaagtgcaaacccgcaaggcagtctcctgcacctgacactgcccaaaaaggtgcctatgctcacgtggccctcc CS1
Q W G G V F Y A E I K S S C A K V Q T R K G S L L H L T L P K K V P M L T W P S 200
ctcctggttgaggctgatgaacagctttgcataccaccgctgaactcccaaacctgcctcctgggctcagaggagaatttagccccttggcaggagagaaagcagtgcctcccgggaat
L L V E A D E Q L C I P P L N S Q T C L L G S E E N L A P L A G E K A V P P G N 240
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D P V S P A M V R S R N P G K D D C A K E E M A V A A D A A T L V D E P E S M V 280
aacctggcgtttgtcaagaatgactcgtatgagaagggcccggattcagtggtggtgcacgtgtacgtgaaggagatctgcagggacacctcaagagtacttttccgtgagcaggacttc
N L A F V K N D S Y E K G P D S V V V H V Y V K E I C R D T S R V L F R E Q D F 320
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T L I F Q T R D G N F L R L H P G C G P H T T F R W Q V K L R N L I E P E Q C T 360 CS2
ttctgtttcacggcttctcgcatcgacatctgccttcgtaagaggcagagtcagcgctgggggggcctggaggccccggctgcacgagtgggtggtgcaaaggttgccgtgccgacaggt
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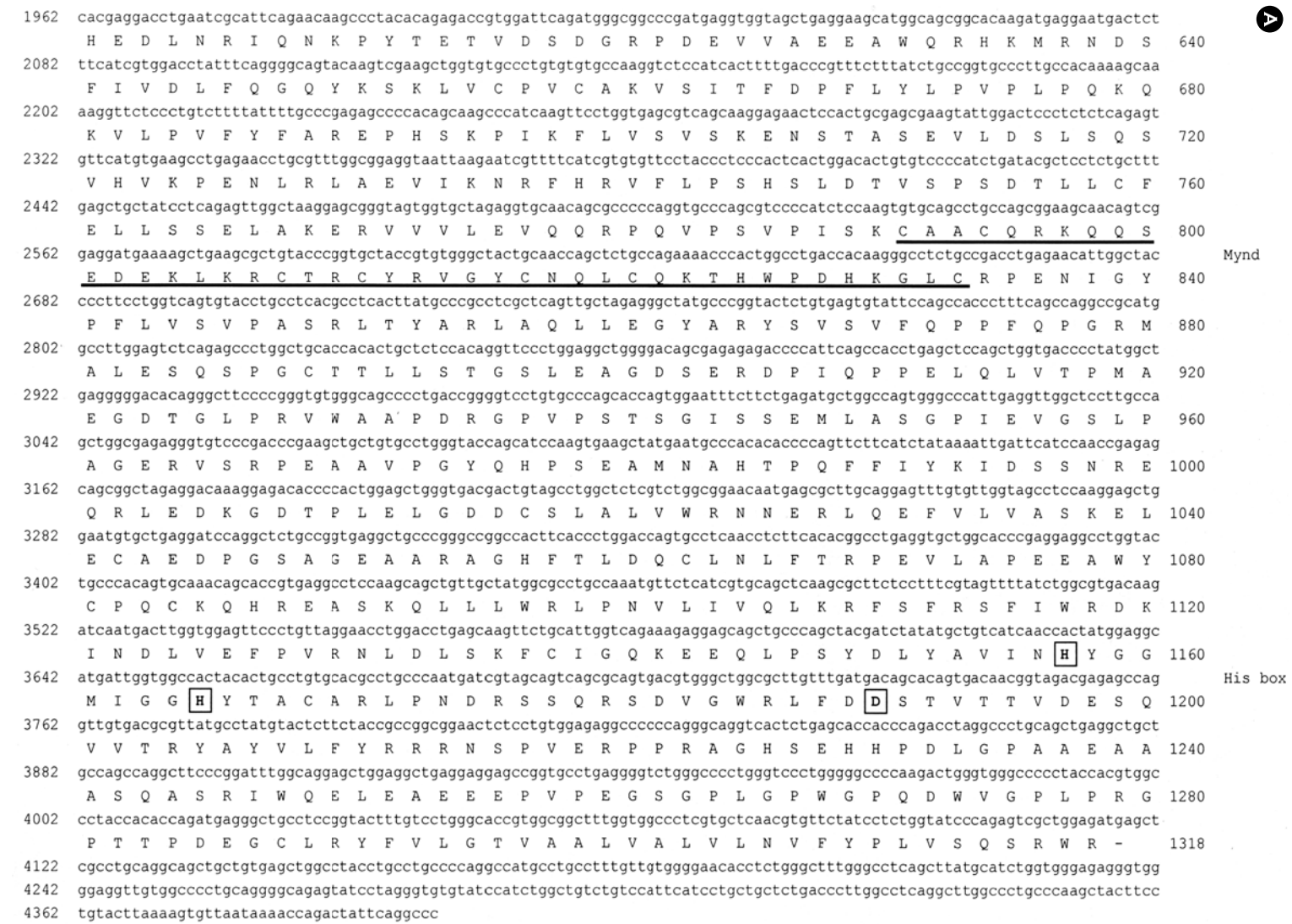
P T P L D S T P P G G A P H P L T G Q E E A R A V E K D K S K A R S E D T G L D 440
agtgtggcaacccgcacacccatggagcatgtaaccccaaagccagagacacacctggcctcgcccaagcctacatgcatggtgcctcccatgccccacagcccagttagtggagacagc
S V A T R T P M E H V T P K P E T H L A S P K P T C M V P P M P H S P V S G D S 480
gtggaggaggaggaagaggaagagaagaaggtgtgtctgccaggcttcactggccttgtcaatttaggcaacacctgcttcatgaacagcgtcattcagtctctgtccaacactcgggaa
V E E E E E E E K K V C L P G F T G L V N L G N T C F M N S V I Q S L S N T R E 520
ctccgggacttcttccatgaccgctcctttgaggctgagatcaactacaacaacccactagggactggtgggcgtctggccattggctttgccgtgctgcttcgggcgctgtggaagggc Cys box
L R D F F H D R S F E A E I N Y N N P L G T G G R L A I G F A V L L R A L W K G 560
acccaccatgccttccagccttccaagttgaaggccattgtggcgagtaaggccagccagttcacaggctatgcacagcatgatgcccaggagttcatggctttcctgctggatgggctg
T H H A F Q P S K L K A I V A S K A S Q F T G Y A Q H D A Q E F M A F L L D G L 600

Figure 1.


1962 cacgaggacctgaatcgcattcagaacaagccctacacagagaccgtggattcagatgggcggcccgatgaggtggtagctgaggaagcatggcagcggcacaagatgaggaatgactct
H E D L N R I Q N K P Y T E T V D S D G R P D E V V A E E A W Q R H K M R N D S 640
2082 ttcatcgtggacctatttcaggggcagtacaagtcgaagctggtgtgccctgtgtgtgccaaggtctccatcacttttgacccgtttctttatctgccggtgcccttgccacaaaagcaa
F I V D L F Q G Q Y K S K L V C P V C A K V S I T F D P F L Y L P V P L P Q K Q 680
2202 aaggttctccctgtcttttattttgcccgagagccccacagcaagcccatcaagttcctggtgagcgtcagcaaggagaactccactgcgagcgaagtattggactccctctctcagagt
K V L P V F Y F A R E P H S K P I K F L V S V S K E N S T A S E V L D S L S Q S 720
2322 gttcatgtgaagcctgagaacctgcgtttggcggaggtaattaagaatcgttttcatcgtgtgttcctaccctcccactcactggacactgtgtccccatctgatacgctcctctgcttt
V H V K P E N L R L A E V I K N R F H R V F L P S H S L D T V S P S D T L L C F 760
2442 gagctgctatcctcagagttggctaaggagcgggtagtggtgctagaggtgcaacagcgccccaggtgcccagcgtccccatctccaagtgtgcagcctgccagcggaagcaacagtcg
E L L S S E L A K E R V V V L E V Q Q R P Q V P S V P I S K C A A C Q R K Q Q S 800
2562 gaggatgaaaagctgaagcgctgtacccggtgctaccgtgtgggctactgcaaccagctctgccagaaaacccactggcctgaccacaagggcctctgccgacctgagaacattggctac
Mynd
E D E K L K R C T R C Y R V G Y C N Q L C Q K T H W P D H K G L C R P E N I G Y 840
2682 cccttcctggtcagtgtacctgcctcacgcctcacttatgcccgcctcgctcagttgctagagggctatgcccggtactctgtgagtgtattccagccacccctttcagccaggccgcatg
P F L V S V P A S R L T Y A R L A Q L L E G Y A R Y S V S V F Q P P F Q P G R M 880
2802 gccttggagtctcagagccctggctgcaccacactgctctccacaggttccctggaggctggggacagcgagagagaccccattcagccacctgagctccagctggtgacccctatggct
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2922 gaggggacacagggcttccccgggtgtgggcagcccctgaccggggtcctgtgcccagcaccagtggaatttcttctgagatgctggccagtgggcccattgaggttggctccttgcca
E G D T G L P R V W A A P D R G P V P S T S G I S S E M L A S G P I E V G S L P 960
3042 gctggcgagagggtgtcccgacccgaagctgctgtgcctgggtaccagcatccaagtgaagctatgaatgcccacacaccccagttcttcatctataaaattgattcatccaaccgagag
A G E R V S R P E A A V P G Y Q H P S E A M N A H T P Q F F I Y K I D S S N R E 1000
3162 cagcggctagaggacaaaggagacaccccactggagctgggtgacgactgtagcctggctctcgtctggcggaacaatgagcgcttgcaggagtttgtgttggtagcctccaaggagctg
Q R L E D K G D T P L E L G D D C S L A L V W R N N E R L Q E F V L V A S K E L 1040
3282 gaatgtgctgaggatccaggctctgccggtgaggctgcccgggccggccacttcaccctggaccagtgcctcaacctcttcacacggcctgaggtgctggcacccgaggaggcctggtac
E C A E D P G S A G E A A R A G H F T L D Q C L N L F T R P E V L A P E E A W Y 1080
3402 tgcccacagtgcaaacagcaccgtgaggcctccaagcagctgttgctatggcgcctgccaaatgttctcatcgtgcagctcaagcgcttctccttcgtagttttatctggcgtgacaag
C P Q C K Q H R E A S K Q L L L W R L P N V L I V Q L K R F S F R S F I W R D K 1120
3522 atcaatgacttggtggagttccctgttaggaacctggacctgagcaagttctgcattggtcagaaagaggagcagctgcccagctacgatctatatgctgtcatcaaccactatggaggc
I N D L V E F P V R N L D L S K F C I G Q K E E Q L P S Y D L Y A V I N H Y G G 1160
3642 atgattggtggccactacactgcctgtgcacgcctgcccaatgatcgtagcagtcagcgcagtgacgtgggctggcgcttgtttgatgacagcacagtgacaacggtagacgagagccag
His box
M I G G H Y T A C A R L P N D R S S Q R S D V G W R L F D D S T V T T V D E S Q 1200
3762 gttgtgacgcgttatgcctatgtactcttctaccgccggcggaactctcctgtggagaggccccccagggcaggtcactctgagcaccacccagacctaggccctgcagctgaggctgct
V V T R Y A Y V L F Y R R R N S P V E R P P R A G H S E H H P D L G P A A E A A 1240
3882 gccagccaggcttcccggatttggcaggagctggaggctgaggaggagccggtgcctgaggggtctgggcccctgggtccctgggggccccaagactgggtgggccccctaccacgtggc
A S Q A S R I W Q E L E A E E E P V P E G S G P L G P W G P Q D W V G P L P R G 1280
4002 cctaccacaccagatgagggctgcctccggtactttgtcctgggcaccgtggcggctttggtggccctcgtgctcaacgtgttctatcctctggtatcccagagtcgctggagatgagct
P T T P D E G C L R Y F V L G T V A A L V A L V L N V F Y P L V S Q S R W R - 1318
4122 cgcctgcaggcagctgctgtgagctggcctacctgcctgccccaggccatgcctgcctttgttgtggggaacacctctgggctttgggcctcagcttatgcatctggtgggagagggtgg
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4362 tgtacttaaaagtgttaataaaaccagactattcaggccc

Figure 1.

Figure 1. The human *HIDE* cDNA. **(A)** The nucleotide (upper line) and deduced amino acid (lower line) sequences of human *HIDE*. Each characteristic domain is underlined and designated on the right side. NLS, nuclear localization signal sequence; CS1 and CS2, CHORD-containing proteins and Sgt1 domain of HIDE; Conserved amino acid residues of the catalytic triad for HIDE are in boxes; Mynd, Mynd type zinc finger domain. **(B)** Comparison of the human, mouse, and rat HIDE domain topology. Characteristic domains and their ranges are shown. Note that CS1 and VR1 are reversed in mouse and rat HIDE (cross). VR1 and VR2, variable region 1 and 2, respectively. **(C)** Comparison of the CS1 and CS2 of HIDE with those of other CS domain proteins.

from the 9E10 hybridoma cell line. For the secondary antibody, HRP-tagged anti-mouse IgG (Zymed), HRP-tagged anti-rabbit IgG (Santa Cruz), and HRP-tagged anti-goat IgG (Santa Cruz) were used. Cells were lysed with lysis buffer (PBS, 1% Nonidet P40, Sigma). The lysate was precleared with 10 μl of protein G plus protein A agarose bead (Oncogene). And then, the precleared lysate was mixed with 1 μg of antibody for 3 hours. Next, the mixture was incubated with the bead for 1 hour. After 3 times of washing, immunoprecipitants were denatured with loading buffer.

RESULTS

While searching for full-length open reading

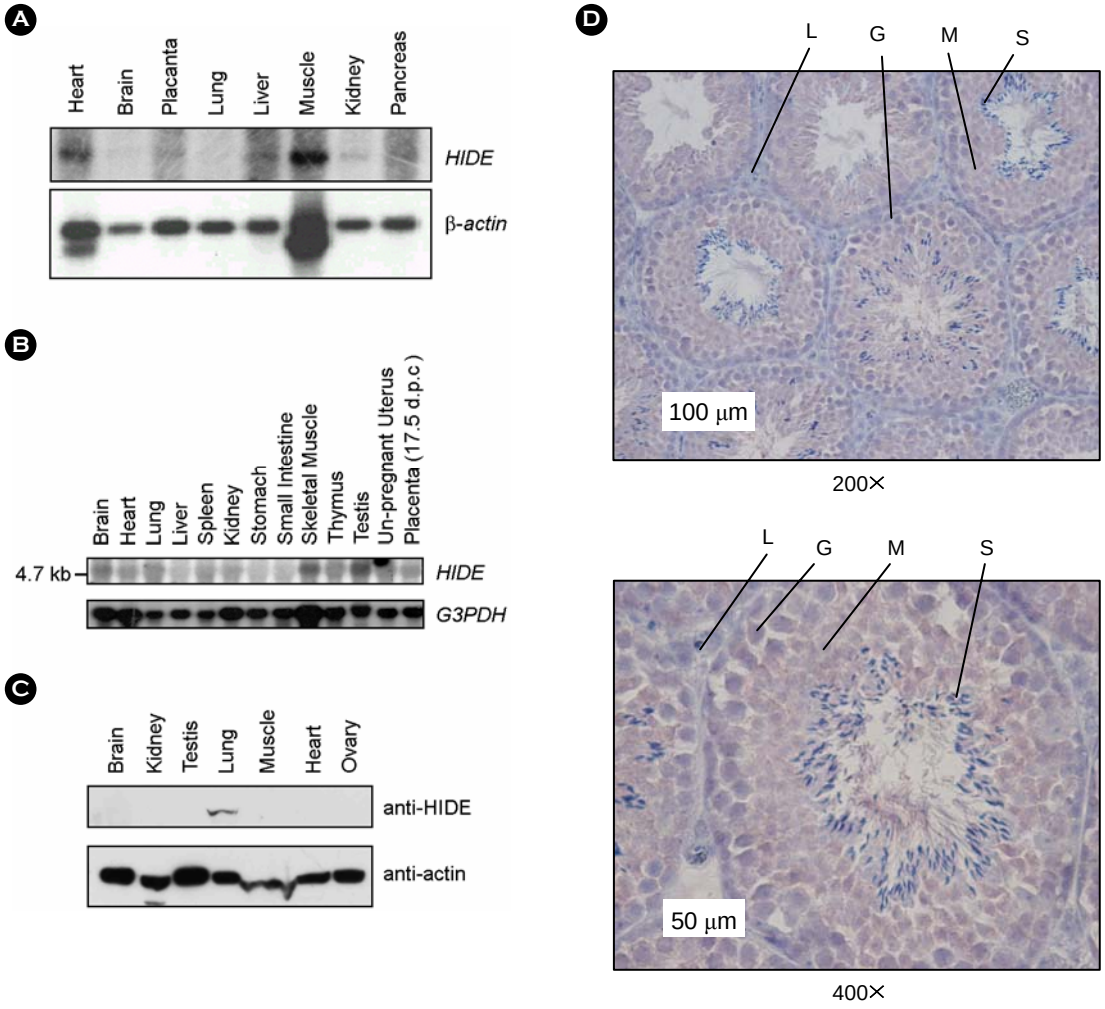

Figure 2. Expression analysis of *HIDE*. **(A)** Northern blot analysis was performed with a human *HIDE* partial fragment and a human tissue blot. A single *HIDE* transcript is visible (approximately 5 Kb). **(B)** Northern blot analysis was performed with another human *HIDE* partial fragment and a mouse tissue blot. Note that the highest expression in the testis. **(C)** Immunoblotting was performed with mouse tissue extracts and an anti-HIDE antibody, which was raised against a partial peptide of HIDE. Expression was detected only in the testis. **(D)** The mouse testis was surgically removed and stained with anti-HIDE antibody. The brown color in blue background of hematoxylin is HIDE proteins. As shown in the left (200×) and right (400×) panels, HIDE proteins are highly expressed in the meiotic spermatocytes (M). Expression level of HIDE is rare in spermatogonia (G), Leydig cells (L), and sperms (S).

fram (ORF) clones of human deubiquitinating enzyme to investigate their cellular functions, we found that the full-length cDNA for *HIDE*, which consists of 4,401 nucleotides. For this study, the cDNA clone[26] was obtained from Dr. T. Nagase at Kazusa DNA Research Institute in Japan. HIDE consists of 1,318 amino acids and the calculated molecular weight is approximately 146 kDa and isoelectric point (*p*I) is 5.89. We used rpsblast, Superfamily, SMART, Pfam, Interpro and Proite, as described in MATERIALS AND METHODS, to search conserved domains. The scanned results showed a nuclear localization signal, two CS domains, and the UCH (Ubiquitin carboxyl-terminal hydrolase) domain, which is flanked by Mynd type of zinc finger (Figures 1A and 1B). Between domains, low complexity regions, which have a number of repeated soluble residues, exist. Repetitive soluble residues frequently form a flexible loop, which connects one domain to another. It was known that the CS domain of p23, Sgt1, and Chp-1 interacts with the N-terminus of Hsp90.[15,16,19] rpsblast (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) scanning result shows that sgt1 and p23 domains have homology with CS1 and CS2 domains of HIDE (Figure 1C). Therefore, it is expected that HIDE might interact with Hsp90. Next, we compared amino acid sequences of human HIDE with mouse and rat HIDE. Interestingly, we found that CS1 and VR1 are reversed in mouse and rat HIDE proteins (Figure 1B).

We performed Northern blot analysis with a partial DNA fragment probe of *HIDE*. We examined mRNA level of *HIDE* using human and mouse tissue blots. The full length of transcripts was approximately 5 Kb, as shown in Figure 2A. mRNA level of *HIDE* showed the highest in heart and muscle, somewhat lesser in pancreas, liver, kidney and placenta, little in brain and lung in a human blot. The expression profile of mouse tissues is almost same as human tissues. However,

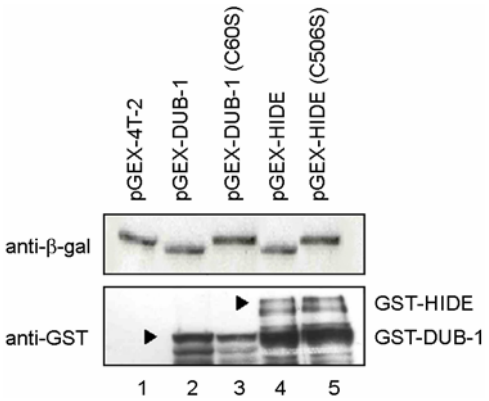

Figure 3. Deubiquitinating enzyme activity of HIDE. The upper panel is an immunoblot using an anti-β-gal antiserum. The co-expressed plasmids are pGEX 4T-2 (lane 1), pGEX-*DUB-1* (lane 2), pGEX-*DUB-1* (C60S) (lane 3), pGEX-*HIDE* (lane 4), pGEX-*HIDE* (C506S) (lane 5). The lower panel is an immunoblot using an anti-GST monoclonal antibody. HIDE cleaved the ubiquitin moiety, but HIDE (C506S) mutant did not cleave, which is a common property of cysteine proteases.

the expression level was the highest in the testis (Figure 2B). We examined the protein expression level of HIDE by immunoblotting analysis. Anti-HIDE antibody was raised against a partial peptide of HIDE, and a mouse tissue protein blot was constructed using adult mouse tissue extracts. Although the transcripts of *HIDE* were detected in various tissues, HIDE proteins were detected only in the testis (Figure 2C). The result of immunoblotting analysis led us to investigate the expression pattern of HIDE in the mouse testis. As shown in Figure 2D, the expression pattern of HIDE in seminiferous tubule revealed that it is highly restricted in the meiotic germ cells. In the seminiferous tubule, the HIDE proteins were rare in the Leydig cells, the spermatogonia, the elongated sperms, and the differentiated sperms. However, HIDE proteins were highly expressed in the primary, secondary spermatocytes, and the Sertoli cells. We suggest that HIDE might function in meiosis or germ cell development during spermatogenesis.

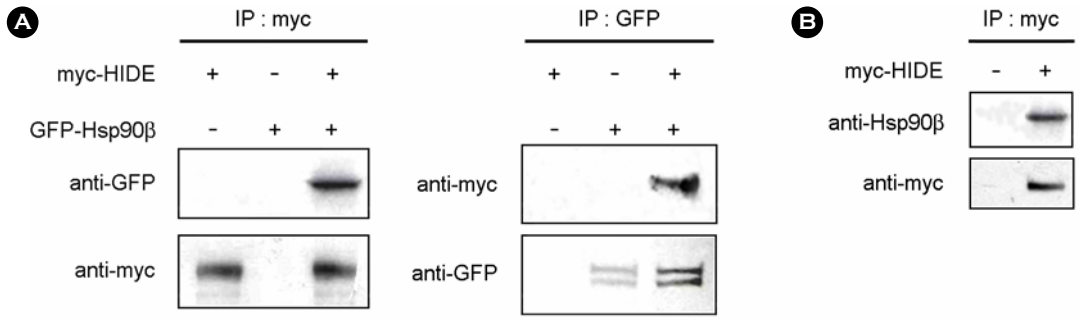

Figure 4. Interaction between HIDE and Hsp90. **(A)** HIDE interacts with Hsp90β *in vivo*. 293T cells were transfected with pcDNA3-myc-*HIDE* and pGFP-C1-*Hsp90β*, and then immunoprecipitated with an anti-myc antibody or an anti-GFP antibody. Precipitants were immunoblotted as described in MATERIALS AND METHODS. **(B)** In order to avoid the artificial interaction between HIDE and Hsp90β, we performed co-immunoprecipitation with an anti-myc antibody and an anti-Hsp90β antibody. pcDNA3-myc-*HIDE* was transfected and whole cell lysate was immunoprecipitated with an anti-myc antibody. Precipitants were immunoblotted as described in MATERIALS AND METHODS.

There has been known that several different types of deubiquitinating activity exist, and different deubiquitinating enzymes have different types of deubiquitinating activity. We characterized catalytic activities of HIDE by performing Ub-β-galactosidase assay. DUB-1, a known murine deubiquitinating enzyme, and its catalytically inactive mutant, DUB-1 (C60S),[27] were used as controls for the Ub-β-galactosidase assay (Figure 3, lanes 2 and 3). As shown in Figure 3, HIDE cleaved mono-ubiquitin moiety of Ub-β-galactosidase, but HIDE (C506S) mutant did not cleave. Interestingly, HIDE did not reduce global pool of polyubiquitin chains in *in vivo* deubiquitinating assay (data not shown). This indicates that the activities of HIDE are different from typical UBP family deubiquitinating enzymes. It is possible that substrates of HIDE are limited or HIDE has characteristic catalytic activity.

As described above, HIDE has two CS domains. The CS domain is an evolutionarily conserved domain from prokaryotes to mammals, but the consensus is low. The CS domain belongs to the Hsp20-like domain, which shows a characteristic beta-sandwich fold.[14] It is known that several Hsp20-like fold proteins including p23, Sgt1, and Chp-1 interact with the N-terminus of Hsp90.[15,16,19] Sgt1 and Chp-1 interact with Hsp90 independent with conformational change of Hsp90, but p23 interacts with Hsp90 when ATP is present. The interaction between HIDE and Hsp90 was confirmed by co-immunoprecipitation as shown in Figures 4A and 4B. Both ectopically expressed and endogenous Hsp90 interacted with HIDE. One of two bands in the right panel of Figure 4A may be posttrantionally modified Hsp90. However, binding regions between two proteins and ATP dependent interaction remain to be investigated.

DISCUSSION

Previous reports showed that chaperones function in the ubiquitination process. Among several chaperones, Hsp90 and its co-chaperones protect client proteins from degradation and mediate signals. CHIP (C-terminus of Heat shock protein 70-Interacting Protein) interacts with the C-terminus of Hsp90 and ubiquitinates misfolded clients. Hsp-90 is also mono-ubiquitinated during hypericin treatment in the dark.[11] The signaling for hypericin is not known, but it is possible that ubiquitination of Hsp90 exists. Hypericin reduces the level of Hsp90 via lysosomal degradation and the clients of Hsp90 are also reduced. It is known that tumor cells use Hsp90 more efficiently than normal cells.[12]

Therefore, both activation of ubiquitination or inhibition of deubiquitination are valuable drug targets. It is possible that HIDE deubiquitinates ubiquitinated clients of Hsp90 or Hsp90 itself. However, we were not able to find the ubiquitination of Hsp90 at the normal physiological condition.

HIDE has several known evolutionary conserved domains. The CS domain is known to interact with the N-terminus of Hsp90 and CHORD domain.[15,16,19] HIDE has a zinc finger motif in the middle of UCH domain (Figure 1B). The zinc finger motif in HIDE is the Mynd type of zinc finger, which is known as a protein-protein interaction domain. Deubiquitinating activity of HIDE is somewhat different from that of UBP family deubiquitinating enzyme, but similar to that of OTU family deubiquitinating enzymes. All known UBPs can reduce the global pool of polyubiquitin chains. However, OTUs have no global effect.[28] UBPs hydrolize lysine 48-linked polyubiquitin chains, but OTUs hydrolyze lysine 63-linked polyubiquitin chain.[28,29] The catalytic characteristics of HIDE remain to be investigated.

It is well known that spermatogenesis is controlled by several chaperones.[20~23] The functions of chaperones during spermatogenesis are largely unknown, but several evidences were suggested. First, the expression level and localization of chaperones in the testis during spermatogenesis are precisely controlled.[24] Second, it is critical for spermatogenesis that elevated temperature and malfunction of chaperone in the testis.[24] To maintain normal spermatogenesis, temperature within the testis is kept below 35℃. Third, several heat shock factors are expressed in the testis and deletion of these transcription factors cause male infertility.[24]

In conclusion, HIDE proteins were detected only in the testis by immunoblotting analysis (Figure 2C). Importantly, HIDE proteins were expressed largely in meiotic germ cells. It is well known that mutations of deubiquitinating enzymes cause malfunction in spermatogenesis. USP9Y (also known as DFFRY) is a deubiquitinating enzyme, which is located in the AZFa region of Y chromosome. Deletion of the AZFa region causes male infertility, and point mutation of *USP9Y* is found in several azoospermia patients.[30] Point mutations in the intron of *USP9Y* cause truncated USP9Y protein, and several azoospermic men express truncated USP9Y in their testis.[30] Therefore, investigation of precise biochemical and physiological functions of HIDE during spermatogenesis will help to unravel its developmental roles.

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REFERENCES

1. Wing SS. Deubiquitinating enzymes-the importance of driving in reverse along the ubiquitin-proteasome pathway. Int J Biochem Cell Biol 2003; 35: 590-605.
2. Johnston SC, Riddle SM, Cohen RE, Hill CP. Structural basis for the specificity of ubiquitin C-terminal hydrolases. EMBO J 1999; 18: 3877-87.
3. Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, et al. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signaling. Nature 2004; 430: 694-9.
4. Nijman SM, Huang TT, Dirac AM, Brummelkamp TR, Kerkhoven RM, D'Andrea AD, et al. The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. Mol Cell 2005; 17: 331-9.
5. Schmitz C, Kinner A, Kolling R. The deubiquitinating enzyme Ubp1 affects sorting of the ATP-binding cassette-transporter Ste6 in the endocytic pathway. Mol Biol Cell 2005; 16: 1319-29.
6. Li M, Chen D, Shiloh A, Luo J, Nikolaev AY, Qin J,

et al. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature 2002; 416: 648-53.
7. Young JC, Moarefi I, Hartl FU. Hsp90: a specialized but essential protein-folding tool. J Cell Biol 2001; 154: 267-73.
8. Nollen EA, Morimoto RI. Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins. J Cell Sci 2002; 115: 2809-16.
9. Young JC, Barral JM, Hartl FU. More than folding: localized functions of cytosolic chaperones. Trends Biochem Sci 2003; 28: 541-7.
10. Murata S, Chiba T, Tanaka K. CHIP: a quality-control E3 ligase collaborating with molecular chaperones. Int J Biochem Cell Biol 2003; 35: 572-8.
11. Blank M, Mandel M, Keisari Y, Meruelo D, Lavie G. Enhanced ubiquitinylation of heat shock protein 90 as a potential mechanism for mitotic cell death in cancer cells induced with hypericin. Cancer Res 2003; 63: 8241-7.
12. Workman P. Altered states: selectively drugging the Hsp90 cancer chaperone. Trends Mol Med 2004; 10: 47-51.
13. Brinker A, Scheufler C, Von Der Mulbe F, Fleckenstein B, Herrmann C, Jung G, et al. Ligand discrimination by TPR domains. Relevance and selectivity of EEVD-recognition in Hsp70 × Hop × Hsp90 complexes. J Biol Chem 2002; 277: 19265-75.
14. Garcia-Ranea JA, Mirey G, Camonis J, Valencia A. p23 and HSP90/α-crystallin proteins define a conserved sequence domain present in other eukaryotic protein families. FEBS Lett 2002; 529: 162-7.
15. Obermann WM, Sondermann H, Russo AA, Pavletich NP, Hartl FU. *In vivo* function of Hsp90 is dependent on ATP binding and ATP hydrolysis. J Cell Biol 1998; 143: 901-10.
16. Lingelbach LB, Kaplan KB. The interaction between Sgt1p and Skp1p is regulated by HSP90 chaperones and is required for proper CBF3 assembly. Mol Cell Biol 2004; 24: 8938-50.
17. Bansal PK, Abdulle R, Kitagawa K. Sgt1 associates with Hsp90: an initial step of assembly of the core kinetochore complex. Mol Cell Biol 2004; 24: 8069-79.
18. Takahashi A, Casais C, Ichimura K, Shirasu K. HSP90 interacts with RAR1 and SGT1 and is essential for RPS2-mediated disease resistance in Arabidopsis Proc Natl Acad Sci USA 2003; 100: 11777-82.
19. Wu J, Luo S, Jiang H, Li H. Mammalian CHORD-containing protein 1 is a novel heat shock protein 90-interacting protein. FEBS Lett 2005; 579: 421-6.
20. Ohsako S, Bunick D, Hayashi Y. Immunocytochemical observation of the 90 KD heat shock protein (HSP90): high expression in primordial and premeiotic germ cells of male and female rat gonads. J Histochem Cytochem 1995; 43: 67-76.
21. Gruppi CM, Zakeri ZF, Wolgemuth DJ. Stage and lineage-regulated expressioin of two hsp90 transcripts during mouse germ cell differentiation and embryogenesis. Mol Reprod Dev 1991; 28: 209-17.
22. Zhang YQ, Matthies HJG, Mancuso J, Andrews HK, Woodruff III E, Friedman D, et al. The *Drosophila* fragile X-related gene regulates axoneme differentiation during spermatogenesis. Dev Biol 2004; 270: 290-307.
23. Sananes N, Baulieu EE, Goascogne CL. Stage-specific expression of the immunophilin FKBP59 messenger ribonucleic acid and protein during differentiation of male germ cells in rabbits and rats. Biol Reprod 1998; 58: 353-60.
24. Sarge KD, Cullen KE. Regulation of hsp expression during rodent spermatogenesis. Cell Mol Life Sci 1997; 53: 191-7.
25. Baek KH, Kim MS, Kim YS, Shin JM, Choi HK. DUB-1A, a novel subfamily member of deubiquitinating enzyme, is polyubiquitinated and cytokine inducible in B-lymphocytes, J Biol Chem 2004; 279: 2368-76.
26. Nagase T, Ishikawa KI, Suyama M, Kikuno R, Hirosawa M, Miyajima N, et al. Prediction of the coding sequences of unidentified human genes. XII. The

complete sequences of 100 new cDNA clones from brain, which code for large proteins *in vitro*. DNA Res 1998; 5: 355-64.

27. Zhu Y, Carroll M, Papa FR, Hochstrasser M, D'Andrea AD. DUB-1, a deubiquitinating enzyme with growth-suppressing activity. Proc Natl Acad Sci USA 1996; 93: 3275-9.
28. Evans PC, Ovaa H, Hamon M, Kilshaw PJ, Hamm S, Bauer S, et al. Zinc-finger protein A20, a regulator of inflammation and cell survival, has deubiquitinating activity. Biochem J 2004; 378: 727-34.
29. Kovalenko A, Chable-Bessia C, Cantarella G, Israel A, Wallach D, Courtois G. The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. Nature 2003; 424: 801-5.
30. Sun C, Skaletsky H, Birren B, Devon K, Tang Z, Silber S, et al. An azoospermic man with a de novo point mutation in the Y-chromosomal gene USP9Y. Nat Genet 1999; 23: 429-32.