

Molecular cloning of *rHAUSP* encoding a deubiquitinating enzyme in rat testis

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Abstract. The tumor suppressor protein p53 is stabilized by the herpes-virus-associated ubiquitin-specific protease (HAUSP), a deubiquitinating enzyme. We previously isolated and characterized a mouse orthologue of *HAUSP*, *mHAUSP*. In this study, we have identified a rat orthologue of *HAUSP*, *rHAUSP*, from the rat testis by RT-PCR using primers used for cloning *mHAUSP*. *rHAUSP* cDNA encodes 3,312 bp and 1,103 amino acids with a molecular weight of approximately 135 kDa containing highly conserved Cys, Asp (I), His, and Asn/Asp (II) domains characteristic of the ubiquitin-specific processing proteases. *pI* value of *rHAUSP* is 5.31. *In vivo* and *in vitro* deubiquitinating enzyme assays demonstrated that *rHAUSP* has deubiquitinating enzymatic activity. The over-expression of *rHAUSP* induced cell death of cervical adenocarcinoma cells.

Introduction

Ubiquitin (Ub) is a small polypeptide of 76 amino acids and is conjugated to substrate proteins by a series of reaction catalyzed by several classes of enzymes. These include ubiquitin-activating enzymes (E1), ubiquitin-conjugating proteins (E2), and ubiquitin-protein isopeptide ligase (E3). Ubiquitin is activated at its C-terminus by adenylation and subsequent rearrangement to form a thiol ester with the E1. After activation, one of several E2 enzymes transfers ubiquitin from E1 to the E3, to which the substrate protein is specifically bound (1,2). The ubiquitin becomes attached to target proteins through isopeptide bonds, between the C-terminal Gly of ubiquitin and the amino group of Lys of the target protein (3,4). Additional Ub molecules form a branched poly-Ub chain. Poly-ubiquitinated proteins are targeted for degradation by the 26S proteasome in an ATP-dependent manner (1,2).

Deubiquitination, a removal process of ubiquitin from ubiquitin-conjugated protein targets, is mediated by deubiquitinating enzymes. They are different in length but contain highly conserved domains Cys, His, and Asp residues (5,6).

One of regulatory mechanisms for p53 stability is deubiquitination by HAUSP (herpes-virus-associated ubiquitin-specific protease, also known as USP7), which directly deubiquitinates and stabilizes p53. HAUSP also plays a crucial role in the regulation of p53-dependent apoptosis and the inhibition of cell growth (7). We previously isolated and characterized a mouse orthologue of *HAUSP*, *mHAUSP* (8). We report here that the rat *HAUSP* (*rHAUSP*), identified in rat testis, encodes a deubiquitinating enzyme, showing functional enzymatic activity.

Materials and methods

Cell culture and media. HeLa and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (FBS, Gibco BRL), penicillin (25 units/ml), and streptomycin (25 μ g/ml) (Gibco BRL).

Isolation of a full-length cDNA for *rHAUSP*. Total RNA was obtained from rat testis using TRIzol (Gibco BRL). cDNA was synthesized from total RNA according to the protocol of SuperScript Preamplification System (Gibco BRL). These RNAs were subjected to template for RT-PCR. The primer nucleotide sequences of these primers used for cloning of *mHAUSP* are: 5' primer (5'-CCGGATCCGACATGAA CCA-3') and 3' primer (5'-GACACTCGAGGGAAGTCA GTT-3'). The PCR amplified fragment with a predicted size was subcloned into pGEM-T Easy vector (Promega) and introduced into *Escherichia coli* DH5 α . *Escherichia coli* DH5 α harboring pGEM-*rHAUSP* clone was cultured to prepare supercoiled recombinant plasmid DNA using a Mini Prep kit (Bioneer). The DNA sequences were established by automated sequencing (ABI PRISM). Both strands were analyzed, confirming the fidelity of the sequence information.

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Plasmid constructs and site-directed mutagenesis. *rHAUSP* was subcloned into pGEX-4T-3 (Pharmacia), downstream of the glutathione S-transferase (GST) coding element.

ATGAACCACCAACAGCAGCAGCAGCA GCAAGAAAGCGGGAGCAG CAGCTGAGCGAGCCGAGGACATGGAGATGGAAG CTG GAGATACAGATGACCCACCA AGAATTACTCAGAAC 120
MNHQQQQQQQQQQKAGGEQQQLLSEPEDEMMEAGDTPPRITQN 40
CCCCTCATCAATGGAAATGAGCCTGAGTGGGACAGCAACGACAGGAGGACATGGAGGACACCCAGTTGGCGCTCCGAGGCAACCTTTAGTTCACCTGCGAGCGCTCAGC 240
PVIINGNVAVLSDGHSNAEEDMEDDTSWRS EATFQFTVERF S 80
AGACTGAGTGAGTCACTTACCGCTCGTGTGTCGAAATCGCCATGGAATTTGGTGTGCGCCGCTTTTATGGAGACAGCCGACCAAAAAGCGTAGGATTTCTTCTC 360
RLSESVLSPPPCFVVRNLPWKIMVMPRFYFPDRPHQKSVGFFL 120
CAGTCAATGCTGAATCTGACTCCAGCTCGTGGTCTGCCATGCACAAGCAGTGTGTAAGATAATAAATACAGGGATGACGACAAGTCACTCAGCCGGCGAATCAGCCACTGTTCTTC 480
QCNAAESDSTSWSC HAQAVLKIIN YRD DDKSFSR RISHLFF 160
CACAAAGAAAAGACTGGGATCTCTAATTTATGCGCTGGAGTGAAGTACTGATCCTGAGAAAGGATTTATAGATGACAAAAGTCACTTTGAGTTTGTACAGCGGATGCT 600
HKENDDWGF S N F M A W S E V T D P E K G F I D D D K V T F E V F V Q A D A 200
CCCCATGGAGTTCGCTGGGACTCAAAGAAGCATACTGGCTATGTGGCTTAAAGAATCAGGGAGCGAGTGTACATGAATAGCTGTGTCGAGACCTGTTTTTCAAAATCAGTTACGA 720
PHGVAWDSKKKHTTG YVGLKKNQ GATCYMNSLSL LQTLPFFT NQLR 240
AAGCTGTGTACATGTCACAGAGGGTGTATGATTCATCTAAAGCGTCCCTTTAGCATTGCAGAGAGTGTCTATGAGTTACAGCACAGCGATAAGCCTGTAGGAACAAAAGGCTC 840
KAVYMMMPTEEGDDSSSKSVPLALALQ R V F Y E L Q H S D K P V G T K K L 280
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TKENDFGWETLDSF M A W S E V T D P E K G F I D D D K V T F E V F V Q A D A 200
ATCCCAAGTTATCCGAGCAAAATGGTGTCTTATATTCACTGCAAAAGTAGACTCCGTTCTGATAGGAGAAAGATTATGATATCCAGTCAAGTATAAAGAAAGAAAGAAC 1080
IPKLF R G K M V S Y I Q C K E V D Y R S D R R E D Y Y D I Q L S I K G K K N 360
ATATTTGAATCAATTTGGGATTTATGTCGAGTGAACAATAGACCGGTGACAATAAATACGACCGTGGGAGCATGGCTGCAGGAAGCAGAGAAAGCGGTGAAGTTCTGACCTTGCCC 1200
IFESYFVAVVEQLDVGDNKYDAG EHG L Q E A E K G V K F L T L P 400
CCAGTCTTACACCTGACGCTTACGCGTTCATGTACGACCTCAGCGGACAAAACATCAAGATCAATGACAGTTTGGAGTTCCAGAACAGTTACCACTGTAGAAATTTTCAAAAA 1320
PVLHLQLMRMFMYD P Q T D N I K I N D R F E F L P D L P L D Q L Q K 440
ACGGACCTAAGGACCTGCAAAATATATTCTCCATGCAGTCTGGTTACAGTGGAGATAATCATGGTGGACATTACGTTGGTTTACCTAAACCCCAAGGGGATGCCAAATGTTGATAA 1440
TDPKDPAN Y I L H A V L V H S G D N H G G H Y V V Y L N P K G D G K W C K 480
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FDDDDVVS R C T K E E A I E H N Y G G H D D D L S V R H C T N A Y M L V Y I 520
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RRAATKLS E V A V T D H D A T I P Q C A G T T G V E R L Q E E K R I E A Q K R K E R Q 560
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E A H L Y M Q F V A E D D E E K V R Y T V F K V L K N 600
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S S L A E F V Q L S Q T M G F P Q D Q I R L W P M Q A R S N G T K R P A M L D 640
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NEADAGS K T M I E L S D N E N P W T I F L E T V D P E L A A S G A T L P K F 680
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DKDHDV M L F L K M Y D P K T R S L N Y C G H I Y T P I S C K I R D L L P V 720
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GACAAAACGATCCCTAAGACCTGGGTTTGTGTCACCTTATCAAATAGGATGAATATTTTCAGTTGCAAAAGCAGTTGCAAGAGGCTCAACACAGACCCATGTTGCTTCAAGTT 2520
DKTIPND P G F V V T L S N R M N Y F Q V A K T V A Q R L N T D P M L L F K 840
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FKSGYR D G P G N P L R H N Y E G T L R D L Q F F K P R Q P K K L Y Y Q 880
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DLLE E C K K A V E L G D E A S G R L R L L E I V S Y K I I G V H Q E E D E A L G 960
GAGTCTTATCTCTGCAACGAGTCCGACTTTAGAATAGAGGAAATACCTTTGGACRAGRTAAACATGATAAGGAAAACGAGATGCTAATCAGGCTGGGCAATTTCCACAAGAAAGTG 3000
ETC L T S P A T S R T F R I E I P L D Q V N I D K E N E M L I T T V A H F H K E V 1000
TTGGGACATTAGGAATCCGTTTGTCTGAGGATACACAGGGTGAAGTTCAGAGAAGTGTAGAGCGGATTCAGAGCCTGCTGGATATCCAGGAAAAGAGTTTGAAGTTTAAA 3120
FGTFPI P F G I L R I H G E H F R E V M K R I Q S L L D I Q S L D I Q E K E F E K F K 1040
TTCGCAATGTGATGAGGCGGACCAAGTACATCAATGAAGTGAAGTGAATTTGAAGGACTTTGAGCCACAACCTGGTAAAGTGTCTCATCTCGGCTTGGCTAGGGCTT 3240
FAIVMMGRH Q Y I N E D E Y E V N L K I E F E P Q P G N M S H P R P W L G L 1080
GACCACTTCAACAAAGCCCAAGAGGAGTGCCTACACTTACCTAGAAAAGGCAATTAATAATCCACAAGTGA 3312
DHF N K A P K R S R Y T Y L E K A I K I H N . 1103

Figure 1. Nucleotide and predicted amino acid sequences for the rHAUSP. This sequence was confirmed with RT-PCR followed by direct sequencing. The nucleotides and the amino acid residues are numbered on the right. Sequence analysis predicts that rHAUSP cDNA has 3,312 bp nucleotides encoding 1,103 amino acids.

Site-directed mutagenesis on the conserved Cys amino acid residue in *rHAUSP* was carried out with a QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). The PCR reaction was performed at 95°C for 30 sec, 55°C for 1 min, and 68°C for 15 min for a total of 15 cycles. For the PCR reaction, primers were designed as follows: 5' primer (5'-GGGAGCAACGA GTTACATCAATAGCTTGC-3') and 3' primer (5'-GCAAG CTATTTCATGTAACCTCGTTGCTCCC-3'). The amplified cDNA was confirmed by automated DNA sequencing.

Deubiquitinating enzyme assays. A deubiquitinating enzyme assay, based on the cleavage of ubiquitin-β-galactosidase fusion proteins, has been described previously (8,9). The wild-type *rHAUSP* cDNA and cDNA containing a missense mutant form, *rHAUSP* (CS), were used. They were inserted, in frame, into pGEX-4T-3 (Pharmacia), downstream of the glutathione S-transferase (GST) coding element. 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 0.1 mM IPTG (isopropyl-β-thiogalactopyranoside)

after 1 h of pre-incubation. After 4 h of induction with the IPTG induction, 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-galactosidase (ICN), a rabbit anti-GST antiserum (Santa Cruz Biotechnology), and enhanced chemiluminescence (Amersham Biosciences).

The effect of *rHAUSP* expression on the ubiquitin-proteasome system *in vivo* was analyzed by transfecting pcDNA3-myc-*rHAUSP* or pcDNA3-myc-*rHAUSP* (CS) along with pMT123-HA-ubiquitin into NIH3T3 cells. After 24 h of transfection, cells were harvested and lysed. Total protein (40 μg) was loaded in each lane of a 7.5% SDS-PAGE for immunoblotting analysis using an anti-HA (Santa Cruz Biotechnology). Equal loading was verified by immunoblotting against an anti-myc serum (9E10, Santa Cruz Biotechnology).

DNA fragmentation assay. Genomic DNA was prepared from normal HeLa cells, *rHAUSP*-transfected HeLa cells, and apoptosis-induced HeLa cells. In order to induce apoptosis as a control, HeLa cells were treated with 100 μg/ml mitomycin

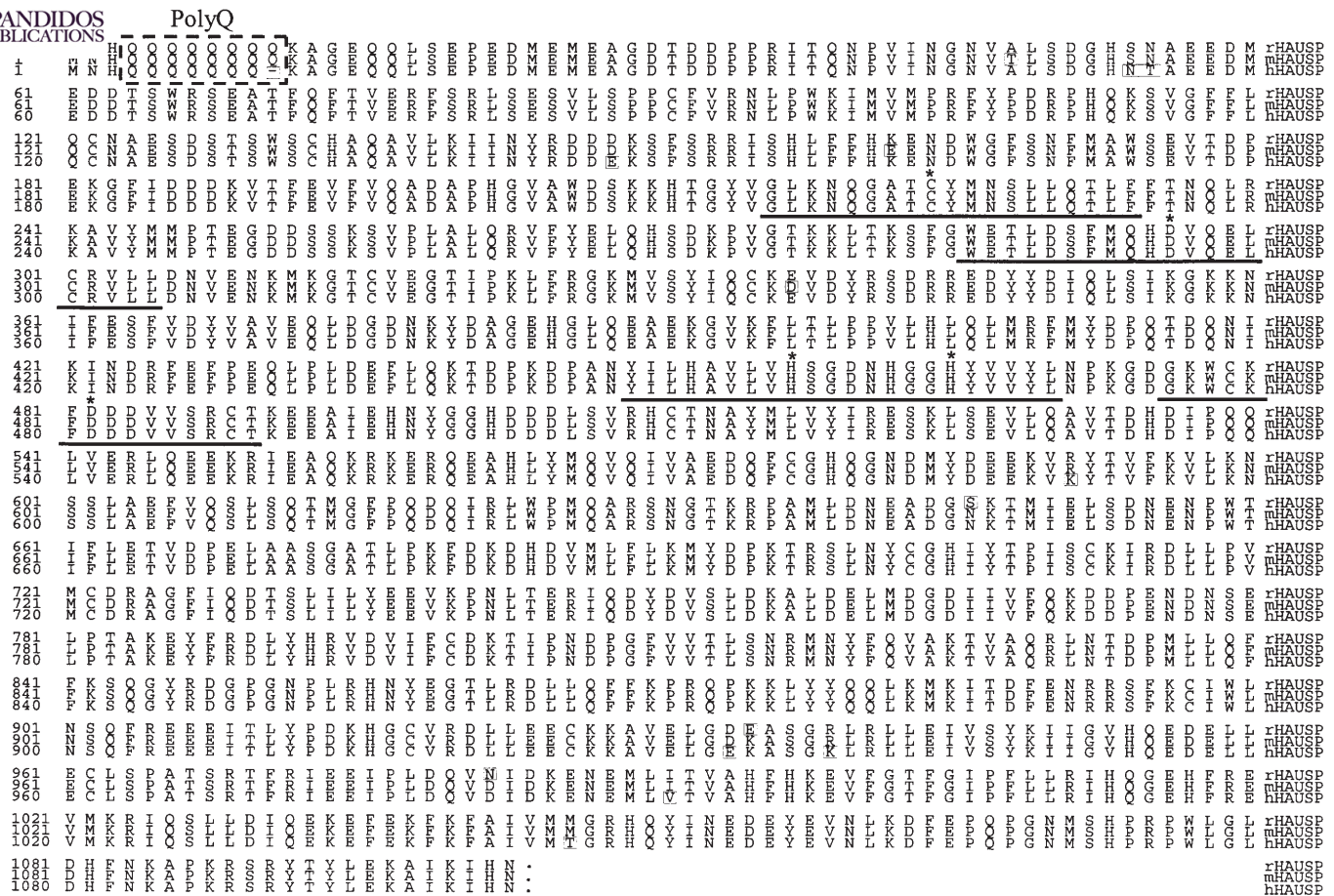


Figure 2. Alignment of amino acids for rat rHAUSP along with human hHAUSP (USP7) and with murine mHAUSP using MegAlign software (clustal method) from DNASTAR (LaserGene). Conserved amino acids [Cys, Asp (I), His, and Asn/Asp (II) domains] of deubiquitinating enzyme are indicated with an asterisk and black line. Also different amino acids among HAUSP family members are indicated by black boxes. PolyQ domain is indicated with a dotted box.

(Sigma) for 12 h. For the assay, HeLa cells were transfected with 8 μ g of pcDNA3-myc-rHAUSP using Lipofectamine (Gibco BRL) according to manufacturer's protocol. Briefly, cells were homogenized in 500 μ l of DNA lysis buffer (10 mM EDTA, 50 mM Tris-HCl pH 8.0, 0.5% SDS). The homogenate was incubated at 50°C for 1 h. After incubation, proteinase K (0.5 mg/ml) and RNase (0.15 mg/ml) were added and incubated at 37°C for 30 min. Phenol:chloroform:isoamyl alcohol (25:24:1) extraction was repeated, and the supernatant was collected and mixed with 0.5 M NaCl and two volumes of ethanol at -20°C overnight. The genomic DNA was precipitated for 30 min at 14,000 rpm at 4°C. The final pellet was dried and resuspended in sterile water. Quantification of DNA content was carried out measuring at OD 260 nm using a spectrophotometer (DU530, Beckman Instruments). Genomic DNA was run on 1.5% agarose gel for 2 h at 50 V.

Results

cDNA cloning of rHAUSP. In order to identify the complete cDNA for rHAUSP, a rat orthologue of mouse and human HAUSPs (7,8), we first performed BLAST search with the amino acid sequence of mHAUSP (GenBank accession number AF548565). However, database from GenBank showed the first exon sequence of rat rHAUSP cDNA as unlisted. Therefore, we carried out RT-PCR with primers used for

Table I. The homology of amino acid sequences for rHAUSP with other orthologues, mHAUSP and hHAUSP.

	rHAUSP (%)	mHAUSP (%)	hHAUSP (%)
rHAUSP	100	99.5	98.7
mHAUSP		100	98.7
hHAUSP			100

cloning the full-length mHAUSP cDNA using rat testis. We found that rHAUSP consists of 3,312 bp and encodes 1,103 amino acids with a molecular weight of approximately 135 kDa (Fig. 1). pI value of rHAUSP is 5.31. rHAUSP contains the highly conserved Cys, Asp (I), His, and Asn/Asp (II) domains characteristic of the ubiquitin-specific processing proteases (Fig. 2). rHAUSP has 98.7% amino acid identity with hHAUSP and 99.5% amino acid identity with mHAUSP (Fig. 2; Table I). Interestingly, HAUSP family members contain a Glu-rich (PolyQ) domain at the N-terminal domain (Fig. 2).

In vitro and in vivo deubiquitinating enzyme assays. To investigate whether rHAUSP has deubiquitinating activity, we first carried out deubiquitinating enzyme assay *in vitro*.

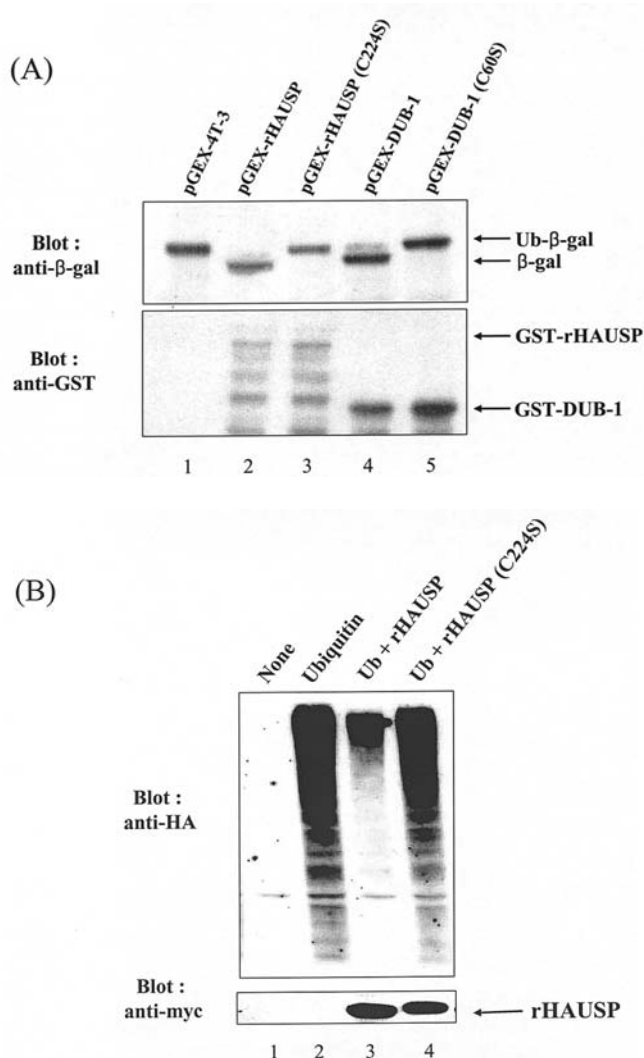


Figure 3. *In vitro* and *in vivo* deubiquitinating activity assays for rHAUSP. (A) rHAUSP has deubiquitinating enzyme activity *in vitro*. The upper panel is a blot for anti- β -gal antibody. The co-expressed plasmids are pGEX-4T-3 (lane 1), pGEX-rHAUSP (lane 2), pGEX-rHAUSP (C224S) (lane 3), pGEX-DUB-1 (lane 4), and pGEX-DUB-1 (C60S) (lane 5). (B) rHAUSP has deubiquitinating enzyme activity *in vivo*. HA-tagged ubiquitin was transiently expressed in NIH cells with or without myc-tagged rHAUSP and its mutant form rHAUSP (C224S), followed by immunoblot analysis of the cell extracts using an anti-HA antibody. An empty vector (lane 1), HA-tagged ubiquitin (lane 2), rHAUSP + HA-Ub (lane 3), and rHAUSP (C224S) + HA-Ub (lane 4).

The assay showed that rHAUSP has deubiquitinating enzyme activity as it cleaves the ubiquitin from Ub-Met- β -gal (Fig. 3). In this assay, DUB-1, DUB-1 (C60S) mutant, and the empty expression vector were used as controls (Fig. 3A, lanes 1, 4, and 5). The mutant form of rHAUSP, containing C224S (Fig. 3A, lane 3), failed to cleave the Ub-Met- β -gal. Thus, this result indicates that the conserved cysteine amino acid at position 224 is essential for deubiquitinating enzyme activity.

Next, we performed *in vivo* deubiquitinating enzyme assay for rHAUSP. We transfected HA-tagged ubiquitin, myc-tagged rHAUSP with HA-tagged ubiquitin, or its mutant form rHAUSP (C224S) along with HA-tagged ubiquitin into NIH3T3 cells, followed by immunoblot analysis of the cell extracts using an anti-HA antibody. The assay showed

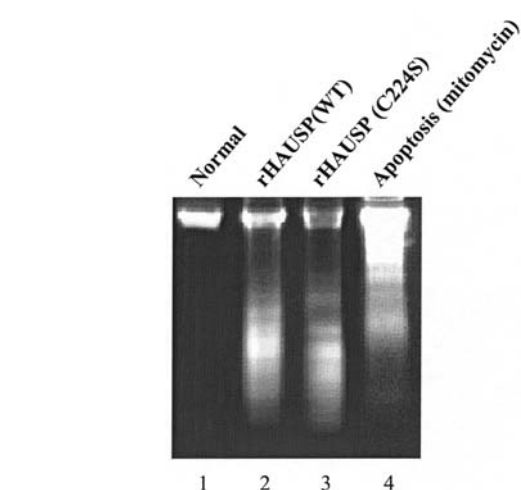


Figure 4. Electrophoretic analysis of genomic DNA fragmentation of HeLa cells (lane 1), apoptotic cells induced by rHAUSP (lane 2) and rHAUSP (C224S) (lane 3). For a control, HeLa cells were treated with mitomycin to induce apoptosis (lane 4).

that rHAUSP has deubiquitinating activity against polyubiquitinated proteins in those transfected cells (Fig. 3B, lane 3). The mutant form of rHAUSP (C224S) does not have deubiquitinating activity (Fig. 3B, lane 4). *In vivo* and *in vitro* deubiquitinating enzyme activity assays confirmed that rHAUSP has deubiquitinating activity both *in vivo* and *in vitro*.

DNA fragmentation induced by rHAUSP in HeLa cells. Since we found that mHAUSP affected p53-mediated cell growth repression (8), we examined whether rHAUSP leads to apoptotic cell death in HeLa cells by measuring the pattern of DNA fragmentation, which represents DNA cleavage at the linker regions between nucleosomes. First, we extracted genomic DNA from normal HeLa cells and apoptotic HeLa cells induced by rHAUSP and visualized DNA ladders by conventional agarose gel electrophoresis. Genomic DNA from HeLa cells that were treated with mitomycin in order to induce apoptosis was used as a control (Fig. 4, lane 4). In order to confirm the DNA fragmentation, apoptotic cells induced by rHAUSP were compared with the control cells undergoing apoptosis (Fig. 4, lanes 2 and 3). Interestingly, HeLa cells transfected with either pcDNA3-myc-rHAUSP or pcDNA3-myc-rHAUSP (C224S) died, indicating that a catalytically inactive form of rHAUSP (C224S) may interfere with endogenous rHAUSP-mediated deubiquitination of p53.

Discussion

Deubiquitination, a reverse process of ubiquitination, is catalyzed by deubiquitinating enzymes and is important for the regulation of a number of intracellular processes (10,11). Deubiquitinating enzymes remove ubiquitins from ubiquitin-conjugated protein substrates. HAUSP, one of the deubiquitinating enzymes, detaches ubiquitins from polyubiquitinated p53 and stabilizes p53 (8,12). It has been demonstrated that the over-expression of HAUSP regulates p53-dependent apoptosis and inhibition of cell growth (7,8). In addition, the partial reduction of endogenous HAUSP



using RNAi destabilized endogenous p53 (13). A g study was performed for the role of HAUSP in apoptosis. They showed that the regulation by HAUSP is caspase-dependent proteolysis during apoptosis in thymocytes (14). The investigation of HAUSP with regard to apoptosis was carried out in human lung carcinoma cells (H460) (7), mouse embryo fibroblasts (MEF) (7), and fetal thymic organ (14).

In this study, we identified *rHAUSP*, a rat orthologue of mouse and human *HAUSPs* and confirmed that rHAUSP acts as a deubiquitinating enzyme. *rHAUSP* consists of 3,312 bp and encodes 1,103 amino acids with a molecular weight of approximately 135 kDa (Fig. 1). rHAUSP has 98.7% amino acid identity with hHAUSP and 99.5% amino acid identity with mHAUSP (Table I). Interestingly, the first exon sequence of *rHAUSP* cDNA was not shown in database from GenBank. Therefore, we carried out RT-PCR with primers used for cloning the full-length for cDNA *mHAUSP* using rat testis. It was similar to the first exon sequence of *mHAUSP* and *hHAUSP*. One of the interesting domains in HAUSP family member is a Glu-rich region (PolyQ), showing 7 Glu residues in hHAUSP, and 8 Glu residues in both mHAUSP and rHAUSP (Fig. 2).

Here, we examined *rHAUSP*-induced apoptosis in human cervical adenocarcinoma cells (HeLa). As expected, *rHAUSP*-transfected HeLa cells died completely within two weeks. It has been shown that HAUSP (C224S), a mutant form of HAUSP, increased the level of p53 ubiquitination leading to decreased amount of endogenous p53 protein (7). Therefore, our study supports a previous finding that the dominant negative effect of rHAUSP (C224S) leads to cell death of HeLa cells transfected with pcDNA3-myc-*rHAUSP* (C224S), which died faster than HeLa cells transfected with pcDNA3-myc-*rHAUSP*. We confirmed that HeLa cells transfected with pcDNA3-myc-*rHAUSP* and pcDNA3-myc-*rHAUSP* (C224S) became apoptotic by observing their morphological changes and the pattern of DNA fragmentation, which represents chromosomal cleavage at the linker regions between nucleosomes. Extracted genomic DNA from apoptotic HeLa cells induced by *rHAUSP*, *rHAUSP* (C224S), and mitomycin as a control visualized as DNA ladders by conventional agarose gel electrophoresis, indicating that these cells are apoptotic (Fig. 4).

It is clear that the activity of rHAUSP enzyme is essential for regulating p53 deubiquitination and stabilization. Therefore, identifying detailed molecular mechanisms of p53 regulation by the ubiquitin-proteasome pathway offer an excellent opportunity for pharmacological approaches to regulate cell proliferation in tumorigenic and embryonic stem cells. In addition, finding its substrates besides p53 and the molecular mechanism of deubiquitination by this enzyme will further clarify its biological roles.

Acknowledgements

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