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Structural Characterization of Mouse HAUSP, a Proteolysis Regulator of p53

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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*. *mHAUSP* cDNA consisted of 3,312 bp encodes 1,103 amino acids with a molecular weight of approximately 135 kDa containing highly conserved Cys, Asp (I), His, and Asn/Asp (II) domains. In this study, we carried out site-directed mutagenesis of 6 conserved amino acids (Cys224, Gln231, Asp296, His457, His465, and Asp482) in Cys box, QQD box, and His box. Interestingly, the conserved Gln 231 was not essential for the catalytic activity of mHAUSP. However, the other conserved amino acids were required for deubiquitinating activity of mHAUSP. We performed isopeptidase assay and confirmed that mHAUSP is able to remove ubiquitin from ubiquitinated substrates. In addition, we observed that mHAUSP induces apoptosis in HeLa cells.

Ubiquitination and deubiquitination have a precise role for selective protein degradation in the cell, and are important for the regulation of a number of intracellular processes, including cell cycle, apoptosis, transcriptional activation, signal transduction, antigen presentation, oncogenesis, preimplantation, and DNA repair (Ciechanover, 1998; Wilkinson, 2000).

Ubiquitin is a small polypeptide that is consisted 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 an E1 enzyme (Hershko et al., 1998). After activation, one of E2 enzymes transfers the ubiquitin from the E1 to an E3 enzyme, to which the substrate protein is specifically bound. The ubiquitin becomes attached to target proteins through isopeptide bonds, between the C-terminal Gly of ubiquitin and the ε-amino group of Lys of the target protein (Ciechanover, 1998; Layfield et al., 2001; Freiman et al., 2003). Additional Ub molecules form a branched poly-Ub chain. Polyubiquitinated proteins are targeted for degradation by the 26S proteasome in an ATP-dependent manner (Chau et al., 1989; Kim et al., 2003). Deubiquitination, a removal process of ubiquitins from ubiquitin-conjugated protein substrates, is mediated by deubiquitinating enzymes. They are different in length and functional domains, but contain highly conserved Cys, His, and Asp residues (Wilkinson, 2000; Baek et al., 2004; Lim et al., 2004).

The downregulation of p53 tumor suppressor activity through the ubiquitin-26S proteasome system in normal cells is mediated by Mdm2, which is the major p53 E3 ubiquitin ligase that controls the ubiquitination and degradation of p53. When DNA damage occurs, p53 is phosphorylated at variety of amino acid residues. This event prevents p53 association with Mdm2 and results in p53 stabilization. In addition, p53 is stabilized in response to oncogenic activation by the tumor suppressor p14^{arf} (ARF), which inhibits the activity of the Mdm2 ubiquitin ligase (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997; Appella et al., 2000; Salomoni et al., 2002). Recently, it has been reported that Yin Yang 1 (YY1) as a negative regulator of p53 promotes Hdm2 (human orthologue of Mdm2)-p53 physical interaction and stimulates Hdm2-mediated p53 ubiquitination and degradation. p14^{arf} (ARF) also interacts with YY1 and can disrupt the Hdm2-YY1 interaction (Sui et al., 2004). Other regulatory mechanism of p53 ubiquitination 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 (Li et al., 2002). It has been shown that the partial reduction of endogenous HAUSP levels destabilizes endogenous

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p53 (Li et al., 2002). However, nearly complete ablation of HAUSP stabilizes and activates p53 because HAUSP is also required for the stability of endogenous Mdm2, which is constitutively self-ubiquitinated and degraded *in vivo*. In the absence of HAUSP, Mdm2 appears to be extremely unstable, leading to indirect p53 activation. This feedback-mediated p53 stabilization is Mdm2 dependent (Cummins et al., 2004; Li et al., 2004). HAUSP belongs to the ubiquitin-specific processing protease (UBP) family of deubiquitinating enzyme and contains the characteristic Cys, Asp, and His motifs at the core enzymatic domain and localizes with the promyelocytic leukemia (PML) nuclear body (Everett et al., 1997; Lim et al., 2004).

In this study, we analyzed conserved amino acids of mouse HAUSP (mHAUSP) for deubiquitinating enzyme activity by site-directed mutagenesis. We confirmed that the Gln at amino acid 231 among 6 conserved amino acids (Cys 224, Gln 231, Asp 296, His 457, His 465 and Asp 482) was not essential for deubiquitinating enzyme activity of mHAUSP. In addition, we discovered that mHAUSP has isopeptidase activity and the overexpression of mHAUSP induces apoptosis in HeLa cells.

Materials and Methods

Cell culture and media

HeLa and NIH3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO BRL) supplemented with 10% fetal bovine serum (FBS, GIBCO BRL), penicillin (25 units/ml), and streptomycin (25 μg/ml) (GIBCO BRL).

Plasmid constructs and site-directed mutagenesis

mHAUSP was subcloned into pGEX-4T-3 (Pharmacia), downstream of the glutathione S-transferase (GST) coding element. Site-directed mutagenesis on the conserved Cys224, Gln231, Asp296, His457, His465 and Asp482 amino acid residues in mHAUSP was carried out with QuikChange^{TM} 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 following. For Cys224 to Ser mutation, Cys(224)5'-3'; GGGAGCAACGAGTTACATGAATAGCTTGC and Cys(224)3'-5'; GCAAGCTATTCATGTAACTCGTTGCTCCC. For Gln231 to Glu mutation, Gln(231)5'-3'; GCTTGCTG-GAGACCTTGTTTTTCAC and Gln(231)3'-5'; GTGAAAA-ACAAGGTCTCCAGCAAGC. For Asp296 to Ala mutation, Asp(296)5'-'3; GCTTCATGCAACACGCTGTTCAAGAGC and Asp(296)3'-5'; GCTCTTGAACAGCGTGTTGCATGAAGC. For His 457 to Gln mutation, His(457)5'-3'; GCAG-TCTTGGTTCAGAGTGGAGATAATC and His(457)3'-5'; GATTATCTCCACTCTGAACCAAGACTGC. For His(465) to Gln mutation, His(465)5'-3'; GATAATCATGGTGGA-CAGTACGTGGTTTACC and His(465)3'-5'; GGTAAA-CCACGTACTGTCCACCATGATTATC. For Asp(482) to Ala mutation, Asp(482)5'-3'; GGTGTAAGTTCGCTG-ATGACGTGGTATCC and Asp(482)3'-5'; GGATACCAC-GTCATCAGCGAACTTACACC. The amplified cDNAs were confirmed by automated DNA sequencing. For the expression of *mHAUSP* and its mutant *mHAUSP* (C224S) in HeLa cells, cDNAs were subcloned into a eukaryotic expression vector, pcDNA3-myc vector.

Deubiquitinating enzyme assay in vitro

A deubiquitinating enzyme assay, based on the cleavage of ubiquitin-β-galactosidase fusion proteins, has been described previously (Lim et al., 2004). The wild-type *mHAUSP* cDNA and cDNA containing a missense mutant form, *mHAUSP* (C224S), *mHAUSP* (Q231E), *mHAUSP* (D296A), *mHAUSP* (H457Q), *mHAUSP* (H465Q), and *mHAUSP* (D482A) 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 one hour of pre-incubation. After 4 h 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). These whole cell lysates were analyzed by immunoblotting with a rabbit anti-β-gal antiserum (ICN), a rabbit anti-GST antiserum (Santa Cruz Biotechnology), and the enhanced chemiluminescence (Amersham Biosciences).

Isopeptidase activity assay

To confirm that mHAUSP has isopeptidase activity, the inhibitory effect of ubiquitin aldehyde on deubiquitinating activity of mHAUSP was analyzed. For the assay, NIH3T3 cells were transfected with 8 μg of pcDNA3-myc-*mHAUSP* using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. After 36 h, cells were harvested and washed with ice-cold phosphate-buffered saline before application of lysis buffer (20 mM Tris [pH 7.5], 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, and 1.5 mM $MgCl_2$) plus protease inhibitor mixture (Roche). Lysates were rotated at 4°C for 1 h followed by centrifugation at 13,000 rpm for 15 min. The supernatants were immunoprecipitated with an anti-myc antibody (Zymed) at 4°C for 6 h and combined with protein A plus G Sepharose (Amersham Biosciences), followed by rotation at 4°C for 1 h. Beads were then washed twice with lysis buffer containing 150 mM NaCl, once using lysis buffer containing 500 mM NaCl, and three times with reaction buffer (50 mM Hepes [pH 7.8], 0.5 mM EDTA, 0.01% Brij, and 3 mM dithiothreitol) before 50%

were used in hydrolysis reactions. To inhibit deubiquitinating activity, mHAUSP was preincubated in the presence of 2 μM ubiquitin aldehyde (Affiniti Research Products) for 30 min at 37°C. Mixed polyubiquitin chains (Ub_{2-7}) (Affiniti Research Products) were then used as substrates at a final concentration of 1 μM as previously described (Baek et al., 2004). Following hydrolysis, ubiquitins were detected by western blotting using an anti-ubiquitin antibody (Sigma), a horseradish peroxidase-conjugated secondary antibody (Zymed), and the ECL detection kit (Amersham Biosciences).

DNA fragmentation assay

Genomic DNA was prepared from HeLa cells, *mHAUSP*-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 for 12 h. For the assay, HeLa cells were transfected with 8 μg of pcDNA3-myc-*mHAUSP* using LipofectAMINE according to the manufacturer's instructions. Briefly, cells were homogenized in 500 μl of cell lysis buffer (10 mM EDTA, 50 mM Tris-HCl pH 8.0, 0.5% SDS). The

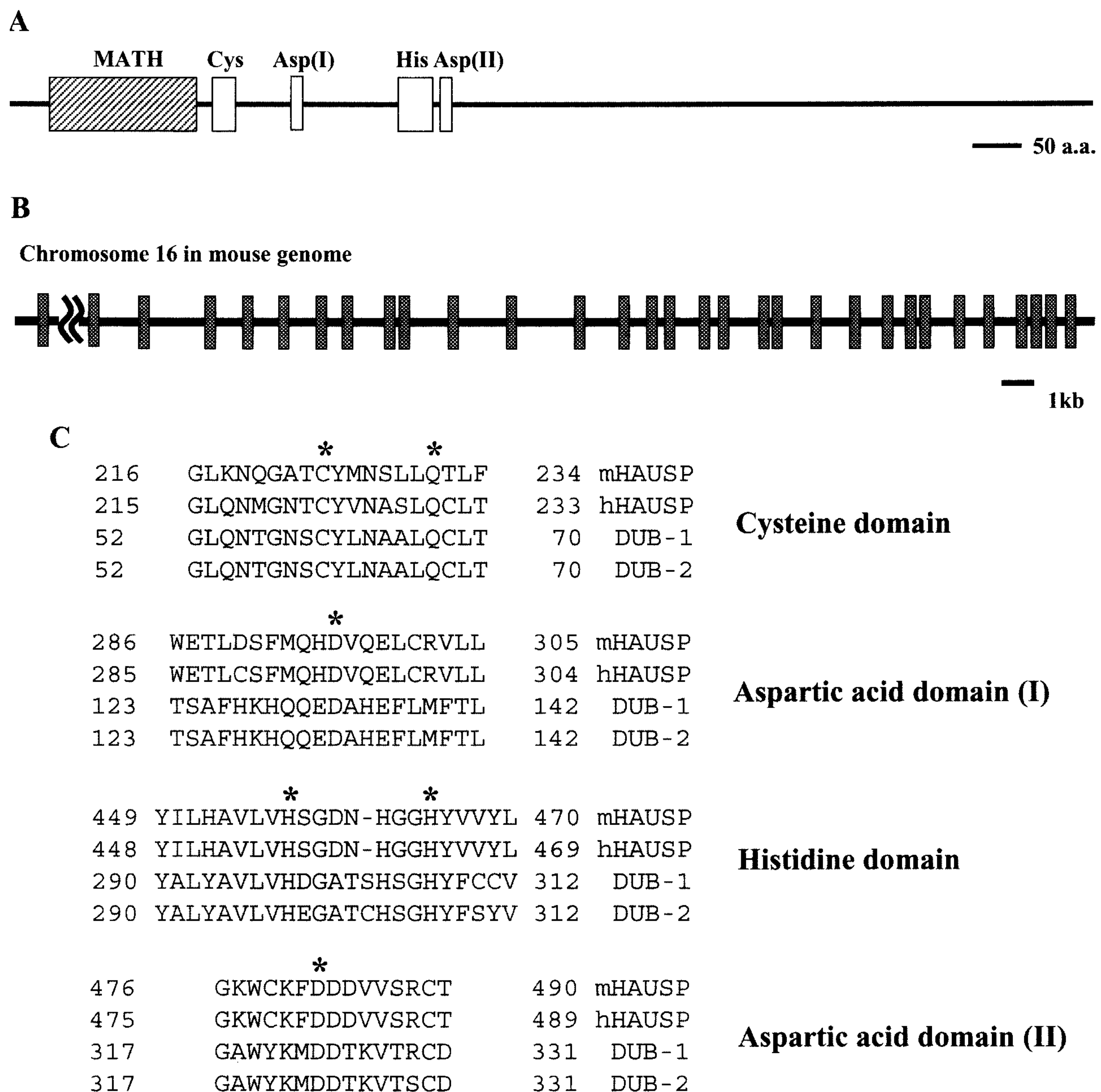

Fig. 1. A, A schematic representation of the mHAUSP: a.a., amino acids. B, Chromosomal location and exons of the mHAUSP. C, Alignment of Cys, Asp (I), His and Asp (II) domains for mHAUSP along with a human HAUSP (USP7), DUB-1 and DUB-2. Conserved amino acids (Cys, Asp (I), His, and Asn/Asp (II) domains) of deubiquitinating enzymes are indicated with an asterisk. The number indicates the first and last residues for each conserved domain.

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 volts.

Results

Structural characterization of mHAUSP

We previously isolated and characterized a mouse orthologue of *HAUSP*, *mHAUSP*. In this study, we further characterized the structure and function of mHAUSP (Fig. 1). Interestingly, the structure showed the MATH domain toward the N-terminus and UCH domain including Cys, Asp (I), His, and Asn/Asp (II), possessing deubiquitinating activity in the middle (Fig. 1A). In addition, we found that the genomic *mHAUSP* is located to chromosome 16, and consisted of 31 exons (UCSC

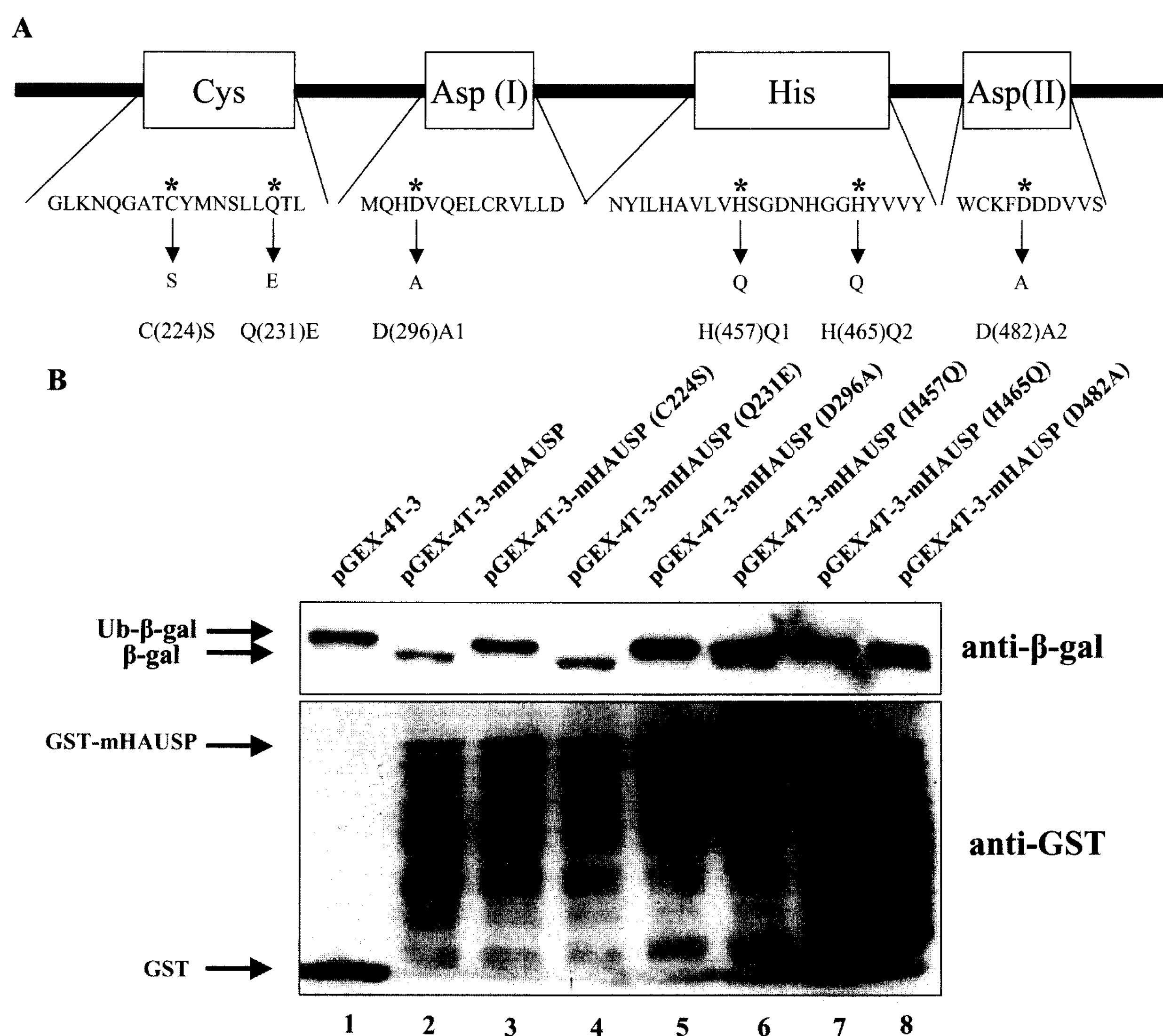

Fig. 2. A, The position of point mutation and converted amino acids by mutations. Conserved amino acids (Cys, Asp (I), His, and Asn/Asp (II) domains) of deubiquitinating enzymes are indicated with an asterisk. B, *In vitro* deubiquitinating activity assays for mHAUSP. A deubiquitinating enzyme assay based on the cleavage of ubiquitin-β-galactosidase fusion proteins by GST-Dub proteins co-expressed in BL21 *E. coli*. The co-expressed plasmids are pGEX-4T-3 (lane 1); pGEX-4T-3-*mHAUSP* (lane 2); pGEX-4T-3-*mHAUSP* (C224S) (lane 3); pGEX-4T-3-*mHAUSP* (Q231E) (lane 4); pGEX-4T-3-*mHAUSP* (D296A) (lane 5); pGEX-4T-3-*mHAUSP* (H457Q) (lane 6); pGEX-4T-3-*mHAUSP* (H465Q) (lane 7), and pGEX-4T-3-*mHAUSP* (D482A) (lane 8). The upper panel is a blot for anti-β-gal antibody and the lower panel is a blot for anti-GST monoclonal antibody.

Genome Bioinformatics site, http://genome.ucsc.edu) (Fig. 1B). We compared the predicted amino acid sequence of mHAUSP with human HAUSP, and murine DUB subfamily members DUB-1 and DUB-2 (Fig. 1C). This comparison suggests that the catalytic domain of mHAUSP is conserved as shown in other DUB subfamily members.

Essential amino acids for in vitro deubiquitinating activity of mHAUSP

To determine whether the conserved amino acids of mHAUSP are essential for deubiquitinating activity, we carried out *in vitro* deubiquitinating enzyme assay using mutant forms of mHAUSP. It has been previously demonstrated that the conserved Cys at amino acid 224 is required for the deubiquitinating activity of mHAUSP (Lim et al., 2004). We have generated mutations at conserved amino acid residues throughout the polypeptide sequence, which were Q231E (glutamine to glutamic acid at amino acid 231), D296A (aspartic acid to alanine at amino acid 296), H457Q (histidine to glutamine at amino acid 457), H465Q (histidine to glutamine at amino acid 465), and D482A (aspartic acid to alanine at amino acid 482) (Fig. 2A). The deubiquitinating enzyme assay showed that mHAUSP has deubiquitinating enzyme activity as it cleaves the ubiquitin from Ub-Met-β-gal fusion proteins (Fig. 2B, lane 2). In this assay, the empty expression vector (pGEX-4T-3) was used as a control, which was not able to cleave the ubiquitin (Fig. 2B, lane 1). The mutant forms of mHAUSP containing C224S mutation, Q231E mutation, D296A mutation, H457Q mutation, H465Q mutation, and D482A mutation (Fig. 2B, lanes 3-8), were analyzed whether they could cleave the Ub-Met-β-gal fusion proteins. The mutant forms for Cys224, Asp296 and His465 showed no enzyme activity, indicating that these amino acids are critical for deubiquitinating enzyme activity (Fig. 2B, lanes 3, 5, 7). However, we observed that both H457Q and D482A mutants have partial deubiquitinating enzyme activity (Fig. 2B, lanes 6, 8). This result indicates that the structural disruption of enzyme by mutations at either His457 or Asp482 residue results in inefficient enzymatic activity of mHAUSP when compared with the mutation at Cys224, Asp296, or His 465. Interestingly, the mutation of Gln231, a putative amino acid required for oxyanion hole, did not inhibit the deubiquitinating enzyme activity of mHAUSP (Fig. 2B, lane 4), indicating that this amino acid is not essential for the deubiquitinating enzyme activity.

Isopeptidase activity of mHAUSP

In order to confirm that mHAUSP has isopeptidase activity, the effect of ubiquitin aldehyde, a specific inhibitor of deubiquitinating enzymes, on deubiquitinating activity of mHAUSP enzyme was investigated. In Fig. 3, we confirmed that mHAUSP has isopeptidase activity (Fig. 3, lane 3) when compared with isopeptidase T used

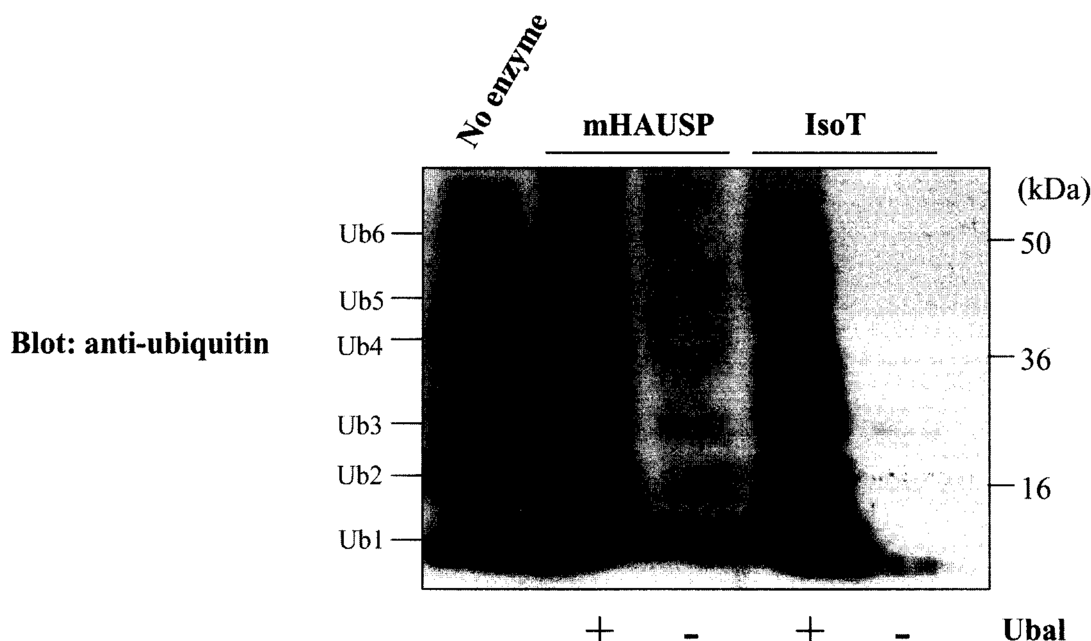

Fig. 3. The effect of ubiquitin aldehyde (Ubal) on mHAUSP enzyme activity in NIH3T3 cells. Equivalent quantities of purified mHAUSP or isopeptidase T (IsoT) were preincubated in the presence or absence of Ubal as indicated. An excess of branched polyubiquitin chains was then added. Branched polyubiquitin chains incubated alone were a negative control (no enzyme). Following hydrolysis, ubiquitin was detected by western blotting using an anti-ubiquitin antibody (Sigma).

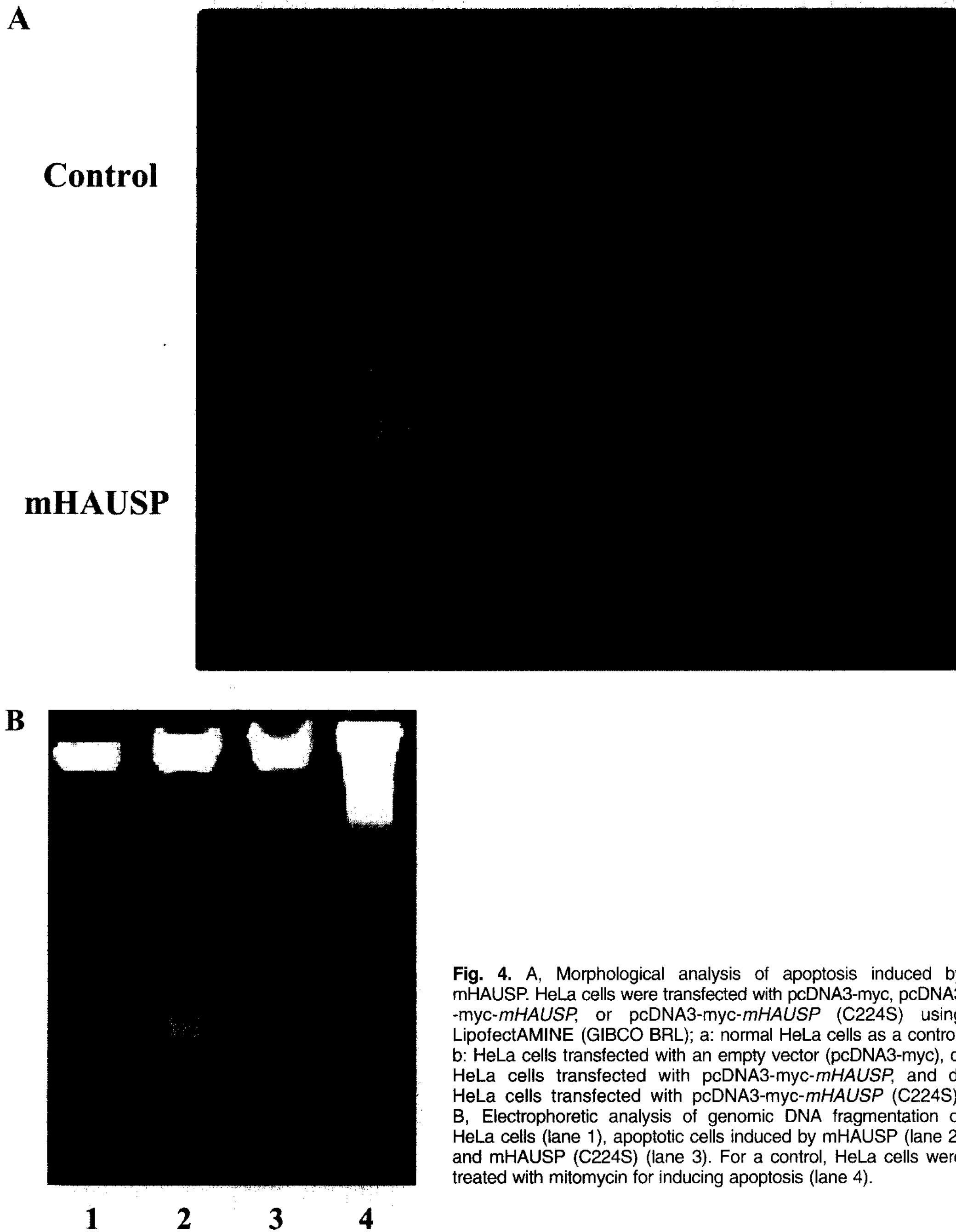

Fig. 4. A, Morphological analysis of apoptosis induced by mHAUSP. HeLa cells were transfected with pcDNA3-myc, pcDNA3 -myc-*mHAUSP*, or pcDNA3-myc-*mHAUSP* (C224S) using LipofectAMINE (GIBCO BRL); a: normal HeLa cells as a control, b: HeLa cells transfected with an empty vector (pcDNA3-myc), c: HeLa cells transfected with pcDNA3-myc-*mHAUSP*, and d: HeLa cells transfected with pcDNA3-myc-*mHAUSP* (C224S). B, Electrophoretic analysis of genomic DNA fragmentation of HeLa cells (lane 1), apoptotic cells induced by mHAUSP (lane 2) and mHAUSP (C224S) (lane 3). For a control, HeLa cells were treated with mitomycin for inducing apoptosis (lane 4).

as a control (Fig. 3, lane 5). In the presence of ubiquitin aldehyde, mHAUSP proteins expressed in NIH3T3 cells were not capable of hydrolyzing the ubiquitin from branched polyubiquitin chains (Fig. 3, lane 2), suggesting that mHAUSP has isopeptidase activity.

Apoptosis induced by mHAUSP in HeLa cells

Since it has been reported that HAUSP affected p53-mediated cell growth repression in H460 and MEF cells expressing p53 (Li et al., 2002), we therefore analyzed whether mHAUSP regulates cell growth repression in HeLa cells. In HeLa cells expressing wild-type p53, mHAUSP induced apoptosis (Fig. 4A). After the transfection of pcDNA3-myc-*mHAUSP*, HeLa cells underwent cell death completely within two weeks (Fig. 4A, panel c). Interestingly, HeLa cells transfected with pcDNA3-myc-*mHAUSP* (C224S) also died completely

within a week (Fig. 4A, panel d). This may due to the function of mHAUSP (C224S) as a dominant-negative mutant through interfering with endogenous mHAUSP-mediated deubiquitination of p53.

DNA fragmentation of apoptotic cells induced by mHAUSP in HeLa cells

We examined whether mHAUSP might result in apoptotic cell death in HeLa cells by measuring the pattern of DNA fragmentation, which represents DNA cleavage at the linker regions between nucleosomes. After extracting genomic DNA from HeLa cells and apoptotic HeLa cells induced by mHAUSP or mHAUSP (C224S), they were visualized as DNA ladders by conventional agarose gel electrophoresis. Apoptotic DNA standard as a control used was genomic DNA from HeLa cells that were treated with mitomycin in order to induce apoptosis (Fig. 4B, lane 4). In order to confirm the DNA fragmentation, apoptotic cells induced by mHAUSP or mHAUSP (C224S) were compared with the control cells undergoing apoptosis (Fig. 4B, lanes 2, 3).

Discussion

Deubiquitination, a reverse process of ubiquitination, is important for the regulation of a number of intracellular processes (Pickart et al., 2001, Glickman et al., 2002). Conjugation of ubiquitin to substrate proteins is catalyzed by several classes of enzymes involving ubiquitin-activating enzymes (E1), ubiquitin-conjugating proteins (E2), and ubiquitin-protein isopeptide ligase (E3). The polyubiquitinated proteins are targeted for degradation by the 26S proteasome (Ciechanover, 1998; Wilkinson, 2000). Deubiquitinating enzymes remove ubiquitins from ubiquitin-conjugated protein substrates. HAUSP, one of the deubiquitinating enzymes, separates ubiquitins from polyubiquitinated p53 and stabilizes p53 (Li et al., 2002; Lim et al., 2004). It has been demonstrated that the overexpression of HAUSP regulates p53-dependent apoptosis and inhibition of cell growth (Li et al., 2002). In addition, the partial reduction of endogenous HAUSP levels by using RNAi destabilized endogenous p53 (Li et al., 2004). The supporting study was performed for the role of HAUSP in apoptosis (Vugmeyster et al., 2002). They showed that the regulation by HAUSP is caspase-dependent proteolysis during apoptosis in thymocytes (Vugmeyster et al., 2002). The investigation of HAUSP with regard to apoptosis was carried out in human lung carcinoma cells (H460) (Li et al., 2002), mouse embryo fibroblasts (MEF) (Li et al., 2002), and fetal thymic organ (Vugmeyster et al., 2002).

Here, we examined mHAUSP-induced apoptosis in human cervical adenocarcinoma cells (HeLa). As expected, *mHAUSP*-transfected HeLa cells died completely within two weeks (Fig. 4A). 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 (Li et al., 2002). Therefore, our study supports a previous finding that the dominant negative effect of mHAUSP (C224S) leads to cell death of HeLa cells transfected with pcDNA3-myc-*mHAUSP* (C224S), which they died faster than HeLa cells transfected with pcDNA3-myc-*mHAUSP*.

We confirmed that HeLa cells transfected with pcDNA3-myc-*mHAUSP* and pcDNA3-myc-*mHAUSP* (C224S) became apoptotic cells by observing their morphological changes (Fig. 4A) and by measuring the pattern of DNA fragmentation (Fig. 4B), which represents chromosomal cleavage at the linker regions between nucleosomes. We showed that extracted genomic DNA from apoptotic HeLa cells induced by mHAUSP, mHAUSP (C224S), and mitomycin as a control visualized as DNA ladders by conventional agarose gel electrophoresis (Fig. 4B), indicating that these cells are apoptotic.

Deubiquitinating enzymes contain a catalytic triad, which is consisted of cysteine, aspartic acid, histidine, and aspartic acid residues. In addition, the previous study on the crystal structure of an UBP-family deubiquitinating enzyme, HAUSP, showed that it contains several conserved amino acid residues including C223, D295, H464, and D481 (Hu et al., 2002). It has been suggested that H464 forms a hydrogen bond with D481 instead of D295 on the distance. Thereby, it was reported that D481 is a new candidate for one of conserved amino acid residues for the catalytic triad (Hu et al., 2002). Quesada et al. (2004) showed the detailed sequence alignment among human deubiquitinating enzymes. The results revealed that the Cys, Asp, and His residues are highly conserved among all of these enzymes, which include a Gln residue located very close to the catalytic Cys amino acid. It has been suggested that this amino acid residue can be part of the oxyanion hole present in the structure of cysteine protease (Menard et al., 1991; Quesada et al., 2004).

Here, we investigated the functional role of Gln231, Asp296, His457, His465, and Asp482 residues in addition to Cys224 (Fig. 2B). Interestingly, the mutation at Gln231 did not inhibit the deubiquitinating enzyme activity of mHAUSP in our study (Fig. 2B, lane 4), indicating that this amino acid is not essential for the deubiquitinating enzyme activity. Even though Gln231 has been suggested as an important element of structural integrity for oxyanion hole constitution (Hu et al., 2002), the degree of contribution should be investigated depending on substrates. We also observed that mutants of mHAUSP at Cys224 (C224S), Asp296 (D296A), His457 (H457Q), His465 (H465Q), or Asp482 (D482A) disappeared deubiquitinating enzyme activity (Fig. 2B, lanes 3, 5-8), even though both His465 (H465Q) and Asp482 (D482A) have partial deubiquitinating enzyme activity (Fig. 2B, lanes 6, 8). These results indicate that each conserved

amino acid has different contribution for deubiquitinating enzyme activity. It is also possible that they may cooperate with each other even though Gln 231 may not be involved in deubiquitinating enzyme activity.

In summary, we found that the overexpression of mHAUSP or a putative dominant-negative mutant, mHAUSP (C224S), leads to cell death of HeLa cells, which confirms the previous finding that the disruption of catalytic core by mutating the conserved cysteine of HAUSP causes more rapid induction of apoptosis in a similar manner. The *in vitro* structural analysis revealed that each conserved amino acid of deubiquitinating enzymes, except the conserved glutamine, contributes to catalytic core for deubiquitinating enzymes. It is now clear that the activity of mHAUSP 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.

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