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Protein Stability of Mitochondrial Superoxide Dismutase SOD2 is Regulated by USP36

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ABSTRACT

SOD2 is a key mitochondrial antioxidant enzyme and its perturbation leads to oxidative cell death, which results in various disorders. In this study, we identified a deubiquitinating enzyme USP36 that regulates the protein stability of SOD2. The regulatory effect of USP36 on SOD2 was initially identified by 2-DE and MALDI-TOF/MS analyses. In addition, endogenous USP36 and SOD2 were shown to interact in an immunoprecipitation assay, which was verified using the yeast two-hybrid system. Furthermore, we demonstrated that SOD2 binds with ubiquitin molecules to form polyubiquitination chains and undergoes degradation through the ubiquitin-proteasomal pathway. Finally, USP36 was shown to be a specific deubiquitinating enzyme that reduces the ubiquitination level of SOD2 and was involved in SOD2 protein stability by extending its half-life. J. Cell. Biochem. 112: 498–508, 2011.

KEY WORDS: DEUBIQUITINATION; OXIDATIVE STRESS; ROS

Ubiquitination and deubiquitination play a key role in maintaining cell homeostasis and are involved in the regulation of a number of biological processes such as cell cycle control, transcriptional regulation, immune response, apoptosis, oncogenesis, pre-implantation, and intracellular signaling pathways [Ciechanover, 1998; Hershko and Ciechanover, 1998; Amerik and Hochstrasser, 2004; Baek, 2006; Ramakrishna et al., in press]. Protein ubiquitination is a critical event that is mediated by sequential enzymatic actions via ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2), and ubiquitin ligases (E3). A novel ubiquitination factor (E4) required for efficient polyubiquitination has been identified in yeast [Ciechanover, 1998; Koegl et al., 1999]. These polyubiquitinated proteins are then directed towards the 26S proteasome for ubiquitin dependent protein degradation [Pickart, 2001].

Deubiquitination, which is a process counteracting protein ubiquitination, is catalyzed by deubiquitinating (DUB) enzymes that are involved in the removal of ubiquitin by hydrolyzing the Ub-substrate isopeptide bond. DUB enzymes are primarily classified into 5 different classes: the ubiquitin-specific protease (USP), the ubiquitin C-terminal hydrolase (UCH), the ovarian tumor (OTU), the Josephin domain are papain-like cysteine proteases and the Jab1/MPN domain-associated metalloisopeptidase (JAMM) [Baek, 2002; Amerik and Hochstrasser, 2004].

USP36, an USP, is primarily localized to the nucleoli and is required to maintain normal nucleolar structure [Endo et al., 2009b]. USP36 was found to interact and stabilize nucleolar proteins nucleophosmin/B23 and fibrillarin by reducing its ubiquitination level and preventing it from protein degradation. The deubiquitinating activity of USP36 controls transcriptional regulation and ribosome biogenesis in response to the changes in environmental conditions. In addition, the siRNA of USP36-transfected cells showed slow a proliferation rate, indicating that this protein is involved in regulating normal cellular proliferation [Endo et al., 2009b]. Recently, it has been reported that nucleophosmin/B23 is responsible for recruiting USP36 in the nucleoli and regulating its deubiquitinating function on nucleolar proteins [Endo et al., 2009a]. Previously, we reported that HeLa DUB-1 was a C-terminal truncated form of USP36 and was shown to have PEST motifs and was polyubiquitinated [Kim et al., 2004, 2005]. Elevated expression

Abbreviations used: CBB, Coomassie Brilliant Blue; CHX, cyclohexamide; DUB enzyme, deubiquitinating enzyme; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; ROS, reactive oxygen species; SOD, superoxide dismutase; Ub, ubiquitin; UPP, ubiquitin-proteasomal pathway; USP, ubiquitin-specific protease.

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of USP36 was detected in ascites and serum of several ovarian cancer patients signifying its importance in cancer progression [Li et al., 2008].

The aim of this study was to identify potential protein substrates for USP36 and to elucidate its functional role in maintaining cell homeostasis. With this goal in mind, we used MALDI-TOF/MS analysis as a tool to screen for proteins that were modulated by the overexpression of USP36 in cervical adenocarcinoma cells. We demonstrated that the protein level of mitochondrial manganese superoxide dismutase (MnSOD or SOD2) was increased by 3-fold due to the overexpression of USP36. In addition, the strong interaction between SOD2 and USP36 was validated in an immunoprecipitation assay and verified using the yeast two-hybrid system. Here, we show that SOD2 was polyubiquitinated and the level of ubiquitination was reduced by the deubiquitinating activity of USP36. Finally, we demonstrated that USP36 tends to stabilize and extend the half-life of SOD2 protein by preventing it from protein degradation. Our results suggest that USP36 plays a vital role in stabilizing the SOD2 protein.

MATERIAL AND METHODS

CHEMICALS AND MATERIALS

Immobiline dry strips (IPG) pH 3–10 nonlinear 18 cm, and Pharmalytes pH 3–10 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden); Griess reagent, CHAPS, urea, DTT, Tris base, thiourea, glycine, ammonium persulfate, and SDS from Sigma (St. Louis, MO); and Coomassie Brilliant Blue (CBB) G-250 and TEMED from Bio-Rad (Hercules, CA).

CELL LINES AND CULTURES

HeLa, COS-7, and HEK293T were cultured in DMEM (Gibco BRL, Rockville, MD) supplemented with 10% FBS (Gibco BRL).

PLASMIDS

The cDNA encoding full length USP36, USP36 (C131S), and pCS4-HA-ubiquitin have been previously described [Kim et al., 2005]. The cDNA encoding the full length SOD2 was cloned into a pCS4-Flag tagged expression vector using standard molecular biology methods. The shRNA expression vector for human SOD2 was constructed using the vector pSilencer 1.0-U6 (Ambion, Austin, TX). The mRNA target sequence chosen for designing USP36-shRNA was as follows: USP36-shRNA: ACT TCA GAC TCG ACG GAA CTT C.

ANTIBODIES

Human anti-USP36 was obtained from Prof. Masa Komada (Tokyo Institute of Technology, Japan), human anti-SOD2 antibody (AbFrontier, Seoul, Korea), anti-9E10, anti-HA, and anti-Flag antibodies were purchased from Sigma–Aldrich (St. Louis, MO).

TWO-DIMENSIONAL ELECTROPHORESIS (2-DE)

pcDNA3-myc-vector or pcDNA3-myc-USP36 was transfected into HeLa cells for two-dimensional electrophoresis. One milligram of each lysate was mixed with a sufficient volume of the rehydration buffer containing 8 M urea, 2% CHAPS, 0.5% IPG buffer and 20 mM DTT to give a total of 350 μl and was loaded into the IPGphor system (Amersham Biosciences, Uppsala, Sweden) for isoelectric focusing. 2-DE and image analyses were performed as previously described [Kim et al., 2006]. The expression level was determined by the relative spot volume of the proteins in the gel compared with the volume of a single spot in the gel.

MALDI-TOF/MS AND BIOINFORMATICS

For MALDI-TOF analysis, the excised spots were digested with trypsin and concentrated using a poros R2 and oligo R3 column (Applied Biosystems, Foster City, CA), as described previously [Cho et al., 2005; Kim et al., 2006]. Proteins were identified from the peptide mass maps using MASCOT (http://www.matrixscience.com) and MS-Fit (http://prospector.ucsf.edu) using monoisotopic peaks. To identify each acquired MS/MS spectrum, bioinformatics analysis and analytical methods were conducted as previously described [Cho et al., 2005; Kim et al., 2006].

MITOCHONDRIA AND CYTOSOL FRACTIONATION

For mitochondria and cytosol fractionation, 5×10^7 HeLa cells were resuspended using cytosolic buffer and homogenized for 50 strokes with the homogenizer. Lysates were spun at 800*g* for 20 min at 4°C. To remove any residual nuclei, a second spin was performed at 800*g* and 4°C for 10 min. The supernatant was then spun at 10,000*g* and 4°C for 20 min to pellet the mitochondria. The mitochondria pellet was washed with cytosolic buffer and spun at 10,000*g* and 4°C for 10 min and the supernatant was discarded. The mitochondria pellet was lyzed with mitochondria buffer and incubated on ice for 15 min. After incubation, the lysate was vortexed for 10 s to mix thoroughly and checked by Western blot analysis. The reagents used for mitochondrial and cytosol fractionation were obtained from the mitochondrial fractionation kit (Active motif, Tokyo, Japan).

YEAST TWO-HYBRID ASSAY

Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Palo Alto, CA, USA) was used for the binding assay between USP36 and SOD2. Human USP36 was cloned into the pGBT9 vector (Clontech) containing the GAL4 DNA-binding domain (BD). Human SOD2 was cloned into the pGAD424 vector (Clontech) containing the GAL4 activating domain (AD). As a positive control, pGBK-T7 and p53 (Clontech) were co-transformed into the AH109 yeast strain and plated on yeast drop-out minimal medium with low stringency medium plates (lacking tryptophan and leucine) and high stringency medium plates (lacking tryptophan, leucine, and histidine). Growth was observed at 30°C for 3–4 days.

WESTERN BLOT ANALYSIS

The transfected cells were lysed in a lysis buffer (1% Triton X, 150 mM NaCl, 50 mM Tris–HCl pH 7.5, 1 mM PMSF) for 20 min. Cell lysates were centrifuged at 13,000 rpm at 4°C, and supernatant was collected into a fresh Eppendorf tube. The protein concentration was determined by using the Bradford reagent (Sigma–Aldrich). The cell lysates were mixed with 6X SDS sample loading buffer (1M Tris–HCl [pH 6.8], 10% SDS, 30% Glycerol, 5% β-mercaptoethanol, 0.012% Bromophenol blue) and boiled for 5 min before loading onto SDS PAGE gel. Western blot analysis was conducted using enhanced chemiluminescence (AbFrontier).

IN VIVO CO-IMMUNOPRECIPITATION ASSAY

To examine in vivo ubiquitination at an endogenous level, pMT123-HA-ubiquitin was transfected into COS-7 cells. Forty-eight hours after transfection, co-immunoprecipitation was performed with an anti-SOD2 antibody (AbFrontier) and Western blot analysis was performed with an anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For in vivo ubiquitination by the overexpression of constructs, pCS4-Flag-SOD2 and pMT123-HA-ubiquitin were transfected into COS-7 cells. After 48 h of transfection, co-immunoprecipitation was performed with an anti-Flag antibody (Sigma–Aldrich) and Western blot analysis was performed with an anti-HA antibody (Santa Cruz Biotechnology). Similarly, a reciprocal blot was performed with the indicated antibodies.

IN VIVO DEUBIQUITINATION AND STABILIZATION ASSAYS

For the deubiquitination assay, pCS4-Flag-SOD2, pcDNA3-myc-USP36, pcDNA3-myc-USP36 (C131S), pSilencer-USP36-shRNA, and pMT123-HA-ubiquitin were co-transfected into COS-7 cells. After 48 h of transfection, co-immunoprecipitation was performed with an anti-SOD2 antibody (AbFrontier) and immunoblotted with an anti-HA antibody (Santa Cruz Biotechnology). Co-immunoprecipitation was also performed with an anti-HA antibody (Santa Cruz Biotechnology) and immunoblotted with an anti-SOD2 antibody (AbFrontier). pcDNA3-myc and pcDNA3-myc-DUB-1 were used as controls. To examine the effect of USP36 on stabilizing SOD2, pcDNA3-myc-USP36, pcDNA3-myc-USP36 (C131S), and pcDNA3-myc were co-transfected with pCS4-Flag-SOD2 into COS-7 cells. After 40 h of transfection, the cells were treated with 50 μg/ml cycloheximide (CHX; BIOMOL, Butler Pike, PA) for 0, 1, 2, 4, 6, and 8 h. Western blot analysis was then performed with an anti-Flag antibody (Sigma–Aldrich) and an equal amount of loading was confirmed using an anti-β-actin antibody (Santa Cruz Biotechnology).

RESULTS

IDENTIFICATION OF PROTEINS REGULATED BY USP36

We performed 2-DE and MALDI-TOF analyses in order to identify cellular proteins that were regulated by USP36. Protein spots were detected by CBB G-250 staining and protein spots that were up-regulated relative to the control sample were selected for further analysis (Fig. 1). In total, 12 upregulated spots and 4 downregulated spots from USP36-transfected HeLa cells were analyzed using MALDI-TOF/MS. Nine proteins that were upregulated proteins by USP36 overexpression are listed in Table I and Figure 1. These proteins corresponded lamin B1 (spot 725), protein phosphatase methylesterase-1 (spot 1273), tropomodulin 3 (spot 1379), heterogenous nuclear ribonucleoprotein A0 (spot 1720), RAN protein (spot 2142), SOD2 (spot 2249), laminin α-2 subunit precursor (spot 2334), germinal histone H4 (spot 2646), and thyroid receptor interactor (spot 2683). Of these, lamin B1 and SOD2 had a high score and were expressed 3-fold higher relative the control. We further validated the endogenous protein level of SOD2 in a mock control and USP36 transfected HeLa cells by Western blot analysis. The expression of USP36 resulted in a 3-fold increase in the expression level of endogenous SOD2 (Fig. 1C and D). SOD2, which is a manganese dependent SOD present in mitochondria, was shown to play a crucial role in the progression of cancer [Chan et al., 2009; Zejnilovic et al., 2009] and its gene inactivation results in several disorders [Melov et al., 1999]. A growing body of evidence has demonstrated that mitochondrial proteins such as mitochondrial creatine kinase, mitofusin, MnSOD, and mutant superoxide dismutase1 are associated with proteasomal degradation [Yen et al., 2008; Sun et al., 2009; Yonashiro et al., 2009; Kwon et al., 2010; Takabe et al., 2010; Ziviani et al., 2010; Livnat-Levanon and Glickman, in press]. Since the aim of the study is to identify substrates for USP36 in cancer cells, SOD2 was selected as a candidate for further study.

USP36 INTERACTS WITH SOD2

Based on the MALDI-TOF/MS proteomic data, we further demonstrated correlation between USP36 and SOD2 by checking whether the two proteins interacted with each other by co-immunoprecipitation assays. For this purpose, Myc-tagged full length USP36 and Flag-tagged full length SOD2 were co-expressed in HEK293T cells and immunoprecipitated with either an anti-Myc or an anti-Flag antibody. Reciprocal co-immunoprecipitation of Myc-USP36 and Flag-SOD2 was also conducted to further validate that these two proteins interact. Our data showed a strong interaction between USP36 and SOD2 (Fig. 2A). We also demonstrated that endongenous USP36 and SOD2 from the HeLa cell lysate interact. We found that anti-SOD2 antibodies could co-precipitate endogenous USP36 (Fig. 2B). We further hypothesized that USP36 could be localized in the mitochondria. The isolation of mitochondrial and cytosolic fraction from HeLa cells was performed to check the expression of USP36 by Western blot analysis. The location of USP36 was found both in cytosolic and mitochondrial fractions and as a positive control SOD2 was detected only in mitochondrial fraction (Fig. 2C). The result of these experiments indicated that USP36 co-localizes with SOD2 in the mitochondria. Furthermore, we verified that USP36 and SOD2 interact by using the yeast two-hybrid system. Transformation of USP36 and SOD2 both individually and together was initially confirmed by growing the culture on SD medium lacking tryptophan (SD-Trp) and leucine (SD-Leu), respectively (Fig. 2D: a–c). Binding between USP36 and SOD2 was then analyzed by growing the culture on SD medium lacking tryptophan, leucine, and histidine (SD-Trp, -Leu, and -His), where only potential interactors can grow on this stringent medium (Fig. 2D: d, Part 3). The interacting proteins, tumor suppressor p53 and simian virus 40 (SV40) large T-antigen, were used as a positive control (Fig. 2D: d, Part 4). The culture was found to grow on this stringent media (SD-Trp, -Leu, and -His), strongly suggesting that USP36 interacts with SOD2.

POLYUBIQUITINATION OF SOD2

To determine the functional significance of the interaction between USP36 and SOD2, we further assessed whether the SOD2 protein was regulated by the ubiquitin-proteasomal pathway (UPP). We performed a ubiquitination assay on SOD2 and found that endogenous SOD2 undergoes polyubiquitination through the 26S proteasomal degradation pathway (Fig. 3A, lanes 2 and 3). We also confirmed by the immunoprecipitation assay that SOD2 was polyubiquitinated when the Flag-tagged SOD2 and HA-tagged ubiquitin constructs were co-transfected into COS7 cells (Fig. 3). This was demonstrated by the typical high molecular weight smear of the polyubiquitin chains in the Western blot (Fig. 3B and C, lanes 4 and 5),

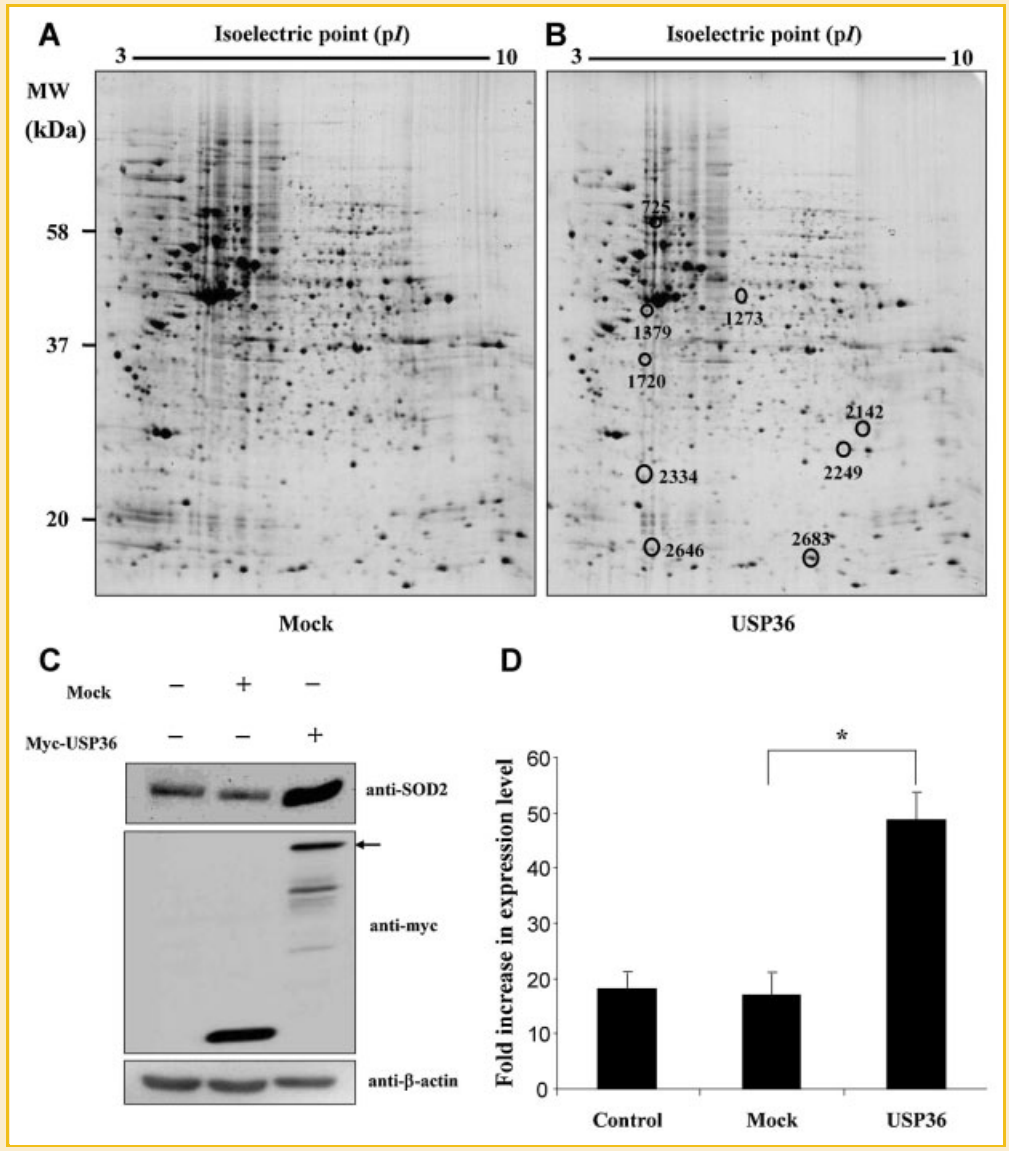

Fig. 1. Master image of a 2-DE stained with CBB G-250. A: 2-DE image of the control (pcDNA3-myc vector overexpressed HeLa cells; Mock). B: 2-DE image of USP36 overexpressed HeLa cells. Circles indicate overexpressed spots. spot 725: lamin B1, spot 1273: protein phosphatase methylesterase-1, spot 1379: tropomodulin 3, spot 1720: heterogenous nuclear ribonucleoprotein A0, spot 2142: RAN protein, spot 2249: SOD2, spot 2334: laminin α-2 subunit precursor, spot 2646: germinal histone H4, and spot 2683: thyroid receptor interactor. C: Western blot analysis of USP36 transfected HeLa cells. D: Fold increase in protein expression level of SOD2 from respective samples was statistically analyzed from three separate experiments. n = 3. *, $P < 0.05$.

which was particularly evident after MG132 treatment (Fig. 3B and C, lanes 5). Thus, our results indicate that the SOD2 protein is regulated by the ubiquitin-mediated proteasomal pathway.

DEUBIQUITINATION AND STABILIZATION OF SOD2 BY USP36

Based on the findings described above, we hypothesized that USP36 functions as a deubiquitinating enzyme (DUB enzyme) for SOD2 ubiquitination; thus, we performed RT-PCR to analyze the effect of USP36 on SOD2 at the transcriptional level and no significant change was observed at the mRNA level (data not shown). This result indicates that USP36 may regulate SOD2 at the post-translational level. This led us to investigate the level of polyubiquitination on SOD2 in the presence of USP36. Interestingly, we found that expression of USP36 decreased the level of polyubiquitination both on endogenous SOD2 (Fig. 4A, lane 4) and overexpressed SOD2 (Fig. 4B, lane 3). In contrast, a catalytic mutant USP36 (C131S) and mouse DUB-1, which were used as the negative controls, did not show any DUB enzyme activity on SOD2 (Fig. 4B, lanes 4 and 5). To test the specificity for USP36 function, cells were transfected with USP36-shRNA and Western blot analysis was performed. Transfection with USP36-shRNA reduced the endogenous expression level of USP36 (Fig. 4C). We then examined the ubiquitination status of the SOD2 protein in the presence of USP36-shRNA. Overexpression of USP36-shRNA resulted in a significant increase in SOD2 ubiquitination in cells (Fig. 4D, lane 6) relative to USP36 transfected cells (Fig. 4D, lane 4). Thus, our results indicate that

TABLE I. Protein Identities Determined by Mass Spectrometry

Spot ID	Accession number	Protein name	Score[a]	Matched peptides number	Sequence coverage (%)	Matched sequences	Theoretical MW	Fold change (USP36/mock)
725	gi|15126742	Lamin B1	118	19/60	34	LAQALHEMR, AGGPTTPLSPTR, ALYETELA DAR, LALDMEISAYR, LRE YEAALNSK, NQ NSWGTGEDVK, ALYETELADARR, LALDM EISAYRK, MATATPVPPRMGSR, SSEMNTS TVNSAR, SMYEEEINETRR, LYKEELEQTY HAK, SLETENSALQLQVTER, NTSEQDQPM GGWEMIR, NTSEQDQP MGGWEMIR, DQM QQQLNDYEQLLDVK, LKNTSEQDQPMGG WEMIR, SLETENSALQLQVTEREEVR, SLEG DLE DLKDQIAQLEASLAAAK	66,652	3.2
1273	gi|7706645	Protein phosphatase methylesterase-1	76	9/34	41	LLLLAGVDR, DHPYTWR, YWDGWFR, KD HPYTWR, VAEAVATFLIR, SL ENAIEWSVK, LDKDLTIGQMQGK, LPSRPPLPGSGGSQSG AK, SIVEGIIEE EEEDEEGSESISKR	42,687	2.2
1379	gi|18089010	Tropomodulin 3	67	9/55	37	EDYVPYTGEKK, QKPVQTFTEEK, DREDYV PYTGEK, SNDPVATAFAEMLK, TKENDAHL V EVNLNNIK, STTPFDREHLLSYLEK, YKD LDEDELLGNLSETELK, ILPVFDEPPNPTNV EESLKR, QLETVLDDLDPENALLPAGFR	39,741	2.5
1720	gi|13938287	Heterogenous nuclear ribonucleoprotein A0	71	9/57	39	FHPIQGHR, GGYGGGGGYGGSSF, SGGGG G GGGSSWGGR, EDSARPGAHAKVK, AVPKE DIYSGGGGGGSR, LFIGGLNVQ TSESGLR, GFGFVYFQNHDAADK, RGFGFVYFQNHDA ADK, GDVAEGDLIEHFSQFGTVEK	31,053	2.0
2142	gi|32425497	RAN protein	83	7/21	30	NVPNWHR, HLTGEFEK, HLTGEFEKK, NLQ YYDISAK, FNVWDTAGQEK, SNYNFEKPFL WLAR, YVATLGVEVHP LVFHTNR	25,378	2.2
2249	gi|15214595	SOD2 protein	85	8/35	34	RDFGSFDK, NVRPDYLK, GELLEAIKR, DFG SFDKF, GDVTAQIALQPALK, HHAAYVNNL NVTEEK, AIWNVINWENVTER, HHAAYVNN LNVTEEKYQEALA	22,290	3.75
2334	gi|56205913	Laminin alpha 2 subunit precursor	69	25/60	9	ARPEVNLK, ATYGNFMR, GVQPVSCPAN, T RIENADAR, GTYFDGTGFAK, ANVQGQRCD K, VNVEGIHCDR, GSYNNIVVNVK, EHSV HVERTR, CLPGFYGEPT, LPMSEELNDK, FS GAKCTECSR, HMAAPLIGQL TR.GENEEM EKDLR, LKPIKELEDNLK, CARGYTGYPD CK, HEIEMTEKEWK, DLAHEATKLATGPR, NLSMKARPEVNLK, TFSSSALLMYLATR, LS AIP NDTAAKLQAVK, YMQNLTVEQPIEVK, YCELCADGYFGDAVDAK,TFSSSALLMYLATRDLR, TTTTTGVLLGISSQ KMDGMGIEMI DEK	35,306	2.3
2646	gi|27686433	Germinal histone H4 gene	74	8/36	52	VFLENVIR, DAVTYTEHAK, SGLIYEETR, VTAMDVVYALK, DNIQGITKPAIR, RISGLIY EETR, VTAM DVVY ALKR	11,360	2.7
2683	gi|703098	Thyroid receptor inter actor	69	9/60	38	QEMLDDVQK, WMTGWLGGGSK, QEMLDD VQKK, KLMSLANSSEG, RTDVNPFLAP, DV RQEMLDDVQ, LSVHDMKPLDSPGR, HEVLR LMGSILGVR, LSVHD MKPLDS PGRR	21,938	2.3

[a]Score is $-10 \times \mathrm{Log}(P)$, where P is the probability that the observed match is a random event; it is based on NCBInr database using the MASCOT searching program as MALDI-TOF data.

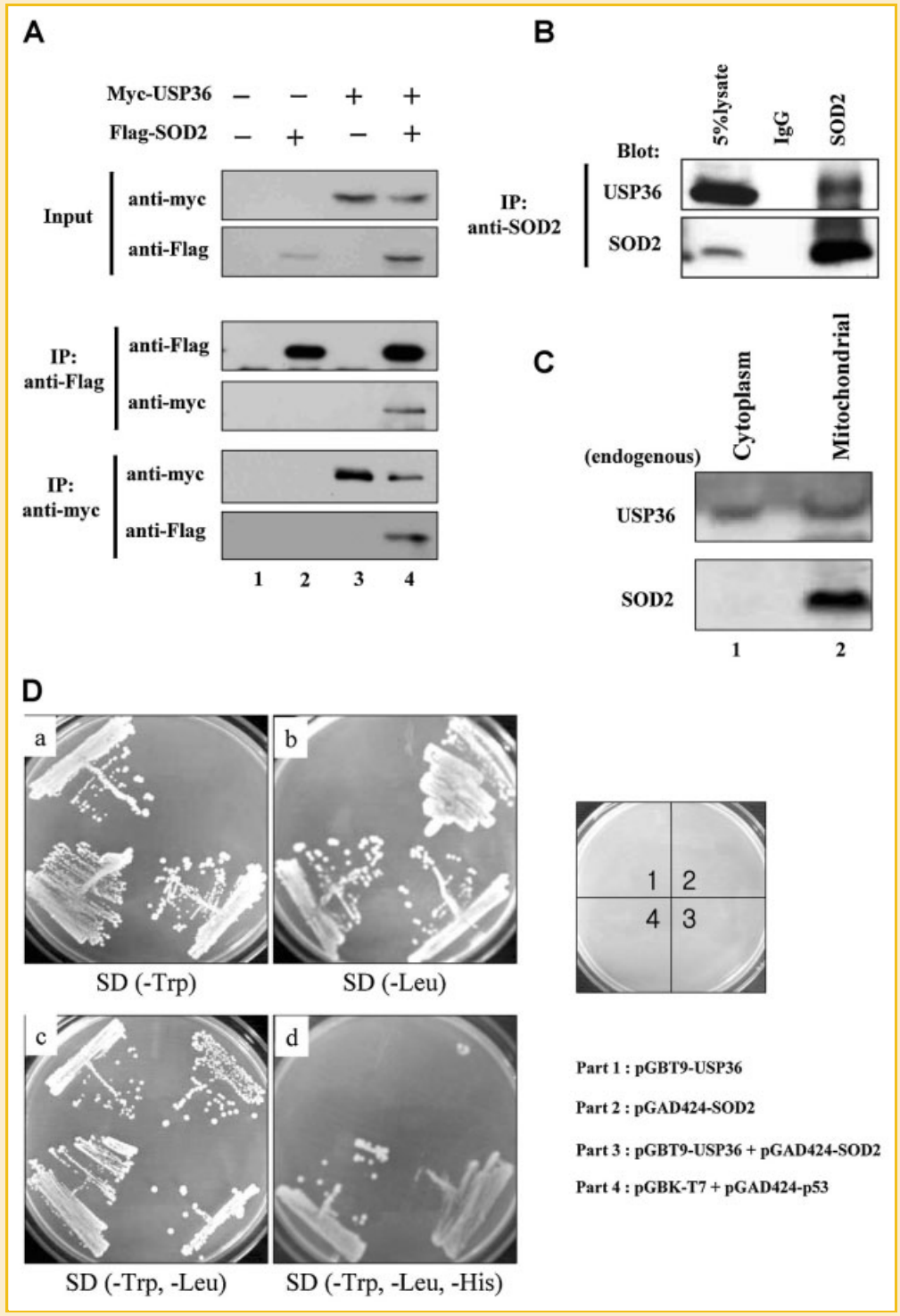

Fig. 2. USP36 interacts with SOD2. A: In vivo binding assay between USP36 and SOD2. HEK293T cells were transfected with pcDNA3-myc-USP36 and/or pCS4-Flag-SOD2, respectively. WCL from HEK293T cells (lane 1), WCL from HEK293T cells transfected with pCS4-Flag-SOD2 (lane 2), transfected with pcDNA3-myc-USP36 (lane 3), and co-transfected with pCS4-Flag-SOD2 and pcDNA3-myc-USP36 (lane 4). The interaction between USP36 and SOD2 was confirmed by co-immunoprecipitation with either an anti-Flag antibody or an anti-Myc antibody. B: Endogenous interaction between USP36 and SOD2 in HeLa cells. Cell lysates were immunoprecipitated with an anti-SOD2 antibody and blotted with an anti-USP36 antibody. C: The location of USP36 and SOD2 in HeLa cells. D: Binding assay using the yeast two-hybrid assay. Yeast cells (AH109) were transformed with the plasmids, pGBT9-USP36 and pGAD424-SOD2, and plated on SD (-Trp) to confirm BD-USP36 transformation (Part 1), SD (-Leu) to confirm AD-SOD2 transformation (Part 2), SD (-Trp and -Leu) to confirm co-transformation of BD-USP36 and AD-SOD2 (Part 3), and SD (-Trp, -Leu, and -His) to confirm interaction between BD-USP36 and AD-SOD2 (Part 3). Tumor suppressor p53 and simian virus 40 (SV40) large T-antigen, fused with the BD and the AD of GAL4, respectively (Part 4), were used as the positive control.

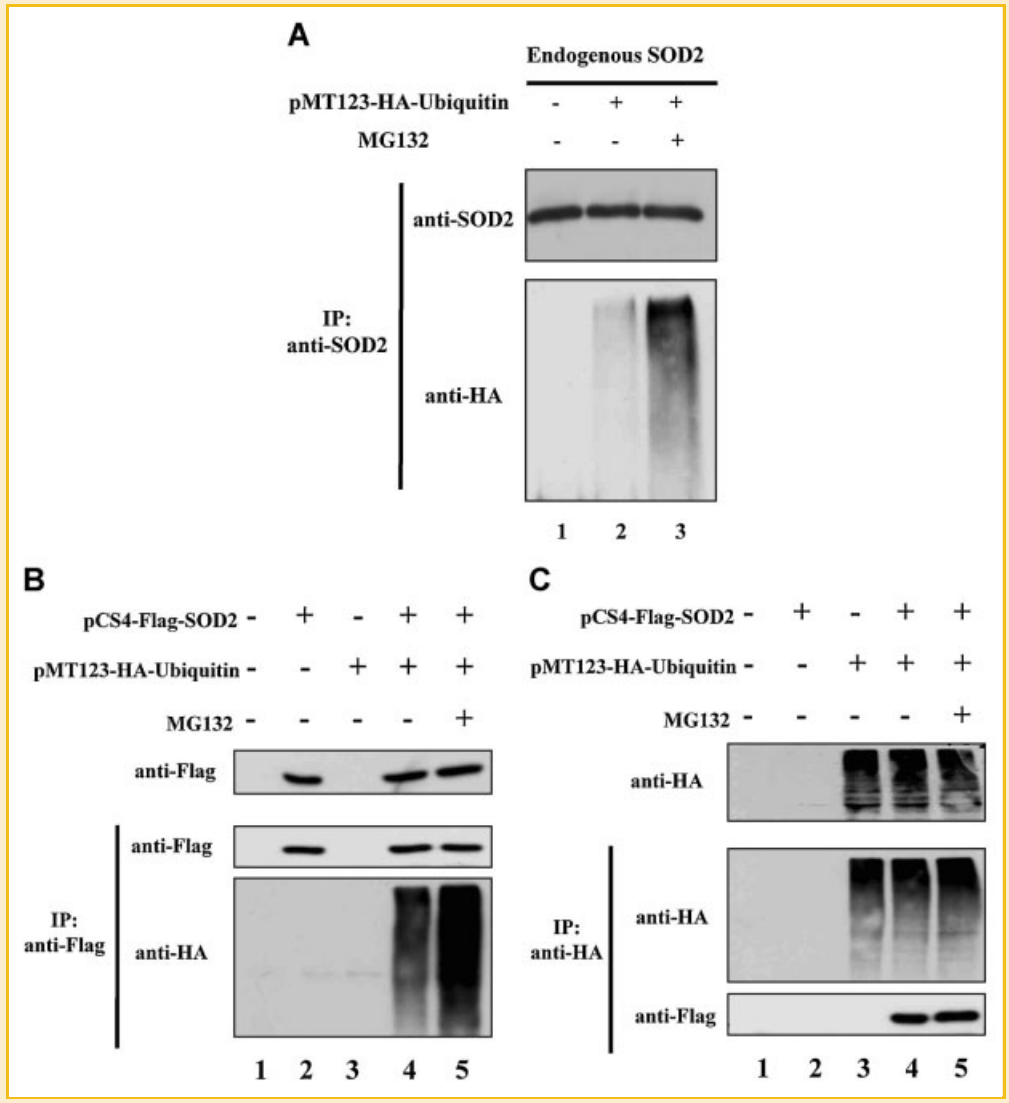

Fig. 3. Polyubiquitination of SOD2. SOD2 was polyubiquitinated and regulated by the UPP. A: Polyubiquitination of endogenous SOD2. COS-7 cells transfected with pMT123-HA-ubiquitin. WCL from COS-7 cells (lane 1), COS-7 cells transfected with pMT123-HA-ubiquitin (lane 2), and transfected with pMT123-HA-ubiquitin and treated with MG132 (lane 3). Ubiquitination of SOD2 was detected by co-immunoprecipitation with an anti-SOD2. B and C: Polyubiquitination of overexpressed SOD2. COS-7 cells transfected with pMT123-HA-ubiquitin and/or pCS4-Flag-SOD2. WCL from COS-7 cells (lane 1), COS-7 cells transfected with pCS4-Flag-SOD2 (lane 2), COS-7 cells transfected with pMT123-HA-ubiquitin (lane 3), COS-7 cells co-transfected with pCS4-Flag-SOD2 and pMT123-HA-ubiquitin (lane 4), and co-transfected with pCS4-Flag-SOD2 and pMT123-HA-ubiquitin and treated with MG132 (lane 5). Ubiquitination of overexpressed SOD2 was detected by co-immunoprecipitation with either an anti-Flag antibody or an anti-HA antibody.

USP36 is a specific DUB enzyme for SOD2. Next, we investigated whether the DUB enzyme activity of USP36 could stabilize SOD2 protein synthesis. HEK293T cells were transfected with a constant amount of Flag-tagged SOD2 alone or together with increasing amounts of Myc-tagged USP36. The protein expression level of SOD2 was dramatically increased by USP36 in a dose dependent manner (Fig. 5A), while the SOD2 mRNA expression level remained unchanged (data not shown). We then examined whether USP36 can extend the half-life of SOD2. To address this question, we overexpressed SOD2 along with USP36, USP36 (C131S), or a mock control separately and treated the cells with CHX, which inhibits protein synthesis. We then checked the protein level at regular time intervals by Western blot analysis. USP36 was found to stabilize and extend the half-life of SOD2 (Fig. 5B), whereas USP36 (C131S) and the mock control did not show any stabilization effect on SOD2 protein (Fig. 5C and D). Taken together, our results demonstrate that USP36 stabilizes and extends the half-life of the SOD2 protein.

DISCUSSION

DUB enzymes play several roles in the ubiquitin pathway to regulate various biological processes [Reyes-Turcu et al., 2009]. The main role of DUB enzymes is to recycle ubiquitin molecules that may have

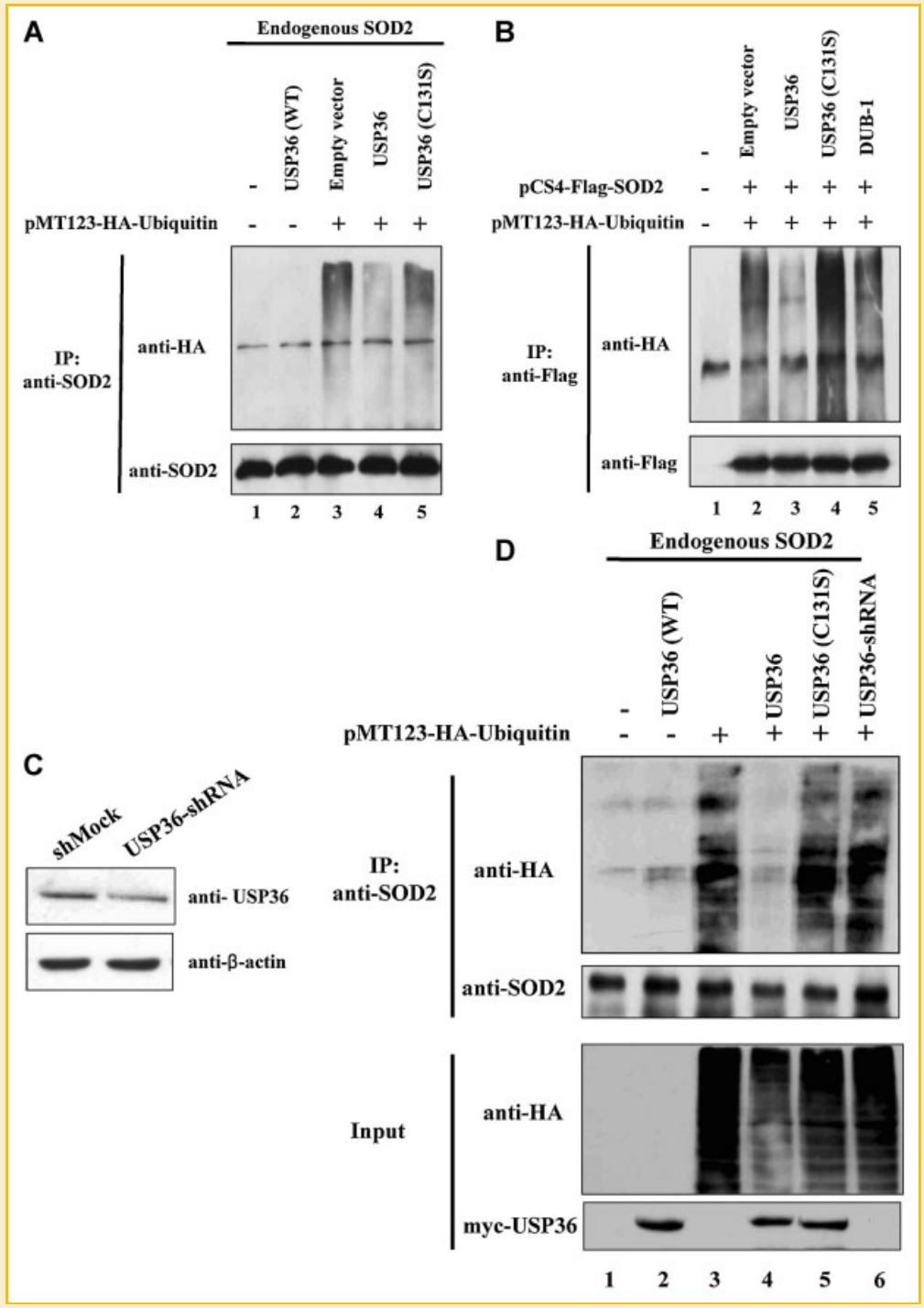

Fig. 4. Deubiquitination of SOD2 by USP36. A: The polyubiquitination level of endogenous SOD2 was reduced by the overexpression of USP36. COS-7 cells transfected with pMT123-HA-ubiquitin and pcDNA3-myc-USP36 or pcDNA3-myc-USP36 (C131S). Deubiquitination of endogenous SOD2 was demonstrated by immunoprecipitation with an anti-SOD2 antibody, which was blotted with an anti-HA antibody. B: COS-7 cells transfected with pCS4-Flag-SOD2, pMT123-HA-ubiquitin, pcDNA3-myc-USP36, pcDNA3-myc-USP36 (C131S), and pcDNA3-myc-DUB-1. Samples from COS-7 cells (lane 1), COS-7 cells co-transfected with pCS4-Flag-SOD2, pMT123-HA-ubiquitin, and pcDNA3-myc-vector (lane 2), COS-7 cells co-transfected with pCS4-Flag-SOD2, pMT123-HA-ubiquitin, and pcDNA3-myc-USP36 (lane 3), COS-7 cells co-transfected with pCS4-Flag-SOD2, pMT123-HA-ubiquitin, and pcDNA3-myc-USP36 (C131S), COS-7 cells co-transfected with pCS4-Flag-SOD2, pMT123-HA-ubiquitin, and pcDNA3-myc-DUB-1 as a negative control. Deubiquitination of SOD2 was confirmed by co-immunoprecipitation with an anti-Flag antibody, which was blotted with an anti-HA antibody. C: The knockdown efficiency of USP36-shRNA was checked by Western blot analysis in HeLa cells. D: HeLa cells transfected with pMT123-HA-ubiquitin, pcDNA3-myc-USP36, pcDNA3-myc-USP36 (C131S), and pSilencerUSP36-shRNA were immunoprecipitated using an anti-SOD2 antibody and blotted with an anti-HA antibody to check the specificity of the deubiquitinating activity of USP36 on endogenous ubiquitinated SOD2.

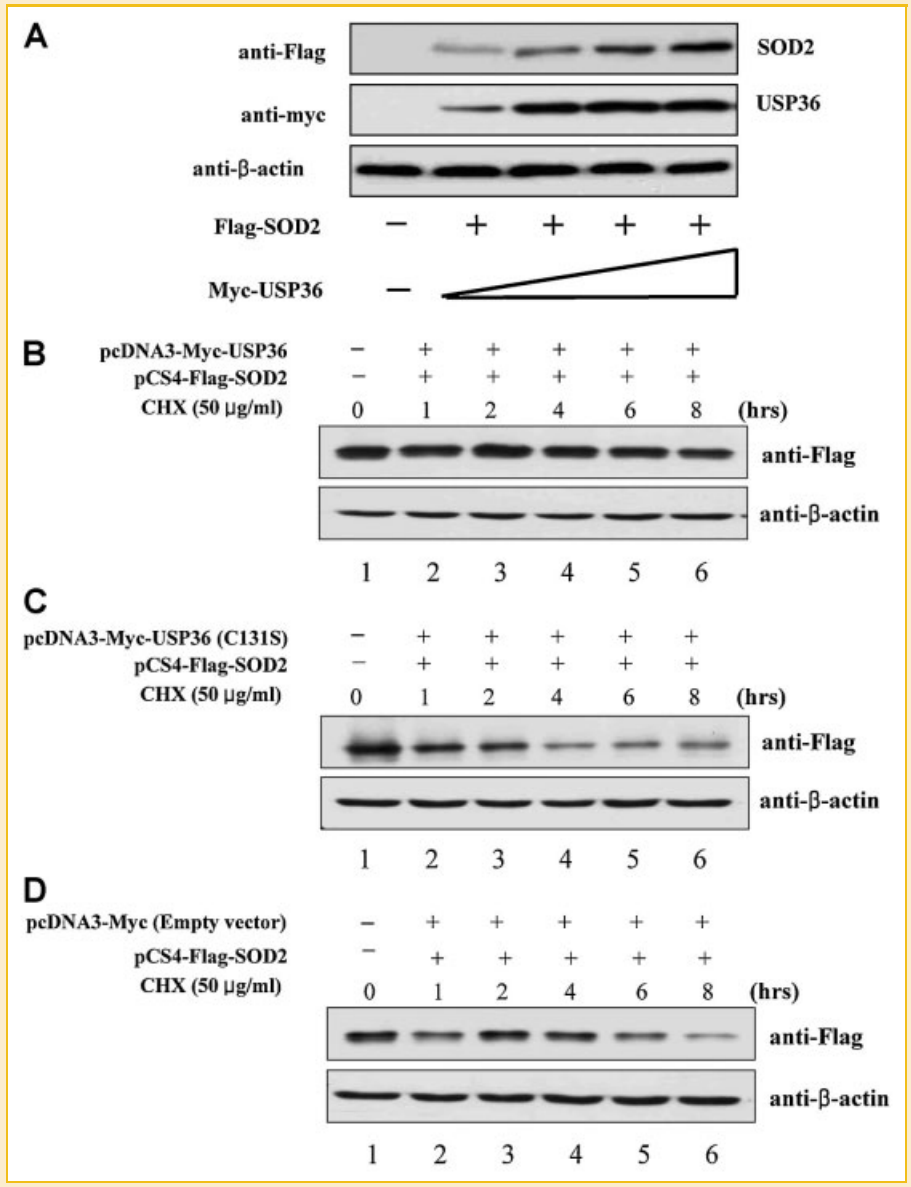

Fig. 5. Stabilization of SOD2 by USP36. A: USP36 increased the amount of SOD2. COS7 cells were co-transfected with a constant amount of pCS4-Flag-SOD2 (2 μg) together with or without increasing amounts of pcDNA3-myc-USP36 (1, 2, 4, and 6 μg). After 48 h of transfection, cell lysates were prepared, and subjected to immunoblotting with the indicated antibodies. B: COS-7 cells was co-transfected with pcDNA3-myc-USP36 and pCS4-Flag-SOD2 and treated with CHX at 50 μg/ml concentration for 0, 1, 2, 4, 6, and 8 h. The SOD2 protein was detected using an anti-Flag antibody. C: COS-7 cells were co-transfected with pcDNA3-myc-USP36 (C131S) and pCS4-Flag-SOD2 and treated with CHX. D: COS-7 cells were co-transfected with pcDNA3-myc vector and pCS4-Flag-SOD2 and treated with CHX as described above.

been involved in the ubiquitination of target proteins [Reyes-Turcu et al., 2009]. Thus, DUB enzymes are critical in regulating the stability and function of their substrate proteins to maintain cellular homeostasis. The aim of the present study was to identify the cellular proteins that were regulated by USP36. Based on the importance of USP36 in the regulation of cell proliferation and biochemical pathways relevant to cancer [Endo et al., 2009a,b], we sought to identify putative target cellular proteins that were regulated by USP36 in cervical cancer cells. By identifying the substrates of various DUB enzymes, we can better understand their actual cellular roles in biological systems. In this regard, a proteomics approach could be a powerful tool for screening potential substrates for DUB enzymes.

Our proteomic analysis revealed a number of proteins that were upregulated as well as downregulated when USP36 was over-expressed in HeLa cells (Fig. 1). Among all proteins identified, SOD2 was demonstrated to be a promising substrate for USP36 since its expression was 3-fold relative to the control.

Superoxide dismutase (SOD) is one of the most predominant antioxidant enzymes in eukaryotes and it catalyzes the conversion of superoxide anions to hydrogen peroxide. SOD2 is a key mitochondrial antioxidant enzyme encoded by the *SOD2* gene

[Finkel and Holbrook, 2000; Barnham et al., 2004; Storz et al., 2005]. Several studies have reported its association with single nucleotide polymorphisms and cancer progression [Chan et al., 2009; Zejnilovic et al., 2009]. Disruption of SOD2 results in heart and liver disorders, metabolic acidosis, and early neonatal death [Melov et al., 1999]. Thus, SOD2 is mainly involved in oxidative stress-induced cell death [Andersen, 2004; Lin and Beal, 2006]. The stability of these proteins is essential for normal cellular processes. Recently, several reports have demonstrated that mitochondrial proteins such as creatine kinase, mitofusin, MnSOD, and mutant superoxide dismutase1 are associated with proteasomal degradation [Yen et al., 2008; Sun et al., 2009; Yonashiro et al., 2009; Kwon et al., 2010; Takabe et al., 2010; Ziviani et al., 2010; Livnat-Levanon and Glickman, in press]. Therefore, our primary interest was to determine whether SOD2 undergoes ubiquitination and to investigate the regulatory role of USP36 on SOD2 protein degradation.

Initially, we performed localization studies and confirmed that SOD2 is present in the cytoplasm when pCS4-Flag-SOD2 was expressed in HeLa cells (data not shown), and we previously reported that USP36 (HeLa DUB-1) was localized to both the cytoplasm and nucleus [Kim et al., 2004], but predominantly expressed in the nucleoli [Endo et al., 2009a,b]. Here we demonstrated that USP36 was also located in the mitochondria of the cell, which is different from the earlier reports on cytoplasm and nucleoli localization. We showed that USP36 interacts with SOD2 endogenously through a co-immunoprecipitation assay and binding was further verified using the yeast two-hybrid system (Fig. 2).

The functional interaction of USP36 with SOD2 could affect the stability of the SOD2 protein by preventing its protein degradation. We demonstrated that SOD2 undergoes polyubiquitination through the 26S proteasomal degradation pathway (Fig. 3). Furthermore, we showed that USP36 deubiquitinates SOD2 and prevents it from being ubiquitinated, which was demonstrated in the reduced ubiquitin smear of SOD2 (Fig. 4). We also demonstrated that deubiquitination of SOD2 by USP36 increases its protein stability and extends its half-life (Fig. 5). Taken together, the intermolecular interaction between USP36 and SOD2 may play an important role in the regulation of SOD2-mediated signaling under oxidative stress.

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