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DUB-1, a Fate Determinant of Dynein Heavy Chain in B-Lymphocytes, Is Regulated by the Ubiquitin–Proteasome Pathway

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ABSTRACT

Ubiquitinaiton and deubiquitination of post-translational modification play counter roles in determining the fate of protein function in eukaryotic system for maintaining the cellular homeostasis. Even though novel family members of growth-regulating deubiquitinating enzymes (DUB-1 and DUB-2) have been identified, their target proteins and functions are poorly understood. *Dub* genes encoding DUB-1 and DUB-2 are immediate-early genes and are induced in response to cytokine stimuli rapidly and transiently. In order to explore the possible proteins regulated by DUB-1, we performed the matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis followed by immunoprecipitation. We confirmed that DUB-1 interacts with dynein heavy chain, which is known to regulate the movement of organelles and microtubule binding ability. In addition, structural and immunoprecipitation analyses revealed that DUB-1 contains a putative PEST motif and is polyubiquitinated, indicating that DUB-1 is also regulated by the ubiquitin–proteasome pathway. J. Cell. Biochem. 105: 1420–1429, 2008.

KEY WORDS: DEUBIQUITINATING ENZYME; PEST MOTIF; PROTEIN DEGRADATION; UBIQUITIN SPECIFIC PROTEASE

The post-translational modification of cellular proteins controlled by the ubiquitin–proteasome system (UPS) is a pivotal event in multicellular organisms in order to regulate cell cycle control, transcriptional regulation, immune response, apoptosis, oncogenesis, pre-implantation, and intracellular signaling pathways [Ciechanover, 1998; Pantaleon et al., 2001; Baek, 2003, 2006]. Ubiquitin (Ub) is a small polypeptide of 76 amino acids that serve as a death signal to be degraded [D'Andrea and Pellman, 1998; Glickman and Ciechanover, 2002]. Ubiquitin conjugation to protein targets is mediated by hierarchical enzymatic reactions. Ubiquitin activating enzyme E1 links ubiquitin in an ATP-dependent manner. Ubiquitin is then transferred to ubiquitin conjugating enzyme E2. This enzyme functions with ubiquitin ligase E3 to attach ubiquitin to the lysine residues in target proteins. Finally, the polyubiquitinated proteins are degraded by the 26S proteasome [Finley and Chau, 1991; Glickman and Ciechanover, 2002].

In contrast, a removal of ubiquitin from ubiquitin-conjugated proteins is mediated by a number of deubiquitinating (DUB) enzymes, which are cysteine proteases and consist of at least five families, the ubiquitin C-terminal hydrolases (UCH), the ubiquitin-specific processing proteases (UBP or USP), Jab1/Pab1/MPN-domain-containing metallo-enzymes (JAMM), Otu-domain ubiquitin aldehyde-binding proteins (OTU), and Ataxin-3/Josephin [Ciechanover, 1998; Baek, 2003]. One subfamily member of well-characterized USP enzymes in lymphocytes is murine cytokine-inducible DUB enzymes, DUB-1 and DUB-2 [Zhu et al., 1996a, 1997]. Interestingly, *Dub-1* and *Dub-2* genes are specifically expressed in B-lymphocytes in response to interleukin-3 (IL-3), interleukin-5 (IL-5) and granulocyte/macrophage colony-stimulating factor (GM-CSF), and in T-lymphocytes in response to interleukin-2 (IL-2), respectively [Zhu et al., 1996a, 1997]. Recently, cytokine-inducible DUB enzymes (DUB-3 and USP17)

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in human have been identified and characterized [Burrows et al., 2005; Shin et al., 2006].

The aim of our study was to find target proteins of DUB-1 and to investigate the regulation of degradation for DUB-1. Our structural and immunoprecipitation analyses demonstrated that the dynein heavy chain is one of target proteins for DUB-1, which contains a putative PEST motif and is polyubiquitinated. This indicates that DUB-1 as a deubiquitinating enzyme may regulate the movement of organelles and microtubule binding ability via the regulation of proteasomal degradation of dynein heavy chain, and is also regulated by the ubiquitin–proteasome pathway.

MATERIALS AND METHODS

CELLS AND CELL CULTURE

Ba/F3 cell is an IL-3 dependent murine pro-B cell line. Ba/F3 cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) with 10 pM IL-3 (R&D Systems Minneapolis, MN) and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (Gibco), respectively.

cDNA CONSTRUCTS AND TRANSIENT TRANSFECTION

The dynein heavy domain cDNA construct was a gift from Kazusa DNA Institute and was subcloned into pCS4-Flag expression vector. The full-length murine myc-tagged *Dub-1* was used for transfection. COS-7 cells were transfected using Exgen500 (Fermantas, Hanover, MD) according to the manufacturer's protocol. The transient transfection of Ba/F3 cells was performed as previously described [Baek et al., 2001].

IMMUNOPRECIPITATION ASSAYS AND SILVER STAINING

For immunoprecipitation assay, Ba/F3 cells were respectively transfected with 20 g of pcDNA3-myc and pcDNA3-myc- *Dub-1*. Cells were harvested after 48 h of transfection, and lysed in a lysis buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.4], 5 mM EDTA [pH 8.0], 1% NP-40 and protease inhibitor cocktail tablet (Roche, Mannheim, Germany) in PBS). Lysates were pre-incubated with an anti-Myc (9E10) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h at 4°C and combined with 30 l Protein A-Sepharose (Amersham Biosciences, Buckinghamshire, England) by rotating for 1 h at 4°C. The immunoprecipitates were washed with lysis buffer three times, and resolved by 7.5% SDS–PAGE. After running, the SDS–PAGE gel was stained with silver staining solution (Bioneer, Deajeon, Korea). In pcDNA3-myc-*Dub-1* transfected cells, the differentially expressed proteins were separated for MALDI-TOF-MS analysis.

MALDI-TOF-MS

MALDI-TOF-MS analysis was performed as described previously [Kim et al., 2006]. The MS/MS spectra were searched against the NCBInr human protein database using MASCOT algorithm (Matrix Science, Boston, MA) for peptide and protein identification. Peptide mass fingerprinting was carried out using the Mascot search engine included in the GPS Explorer software and mass spectra used for manual de novo sequencing were annotated with the Data Explorer software (Applied Biosystems, Foster City, CA). For protein identification, MASCOT ion score >30, was used as the criterion.

CO-IMMUNOPRECIPITATION FOR BINDING, UBIQUITINATION, AND DEUBIQUITINATION ASSAYS

For in vivo binding between dynein heavy chain and DUB-1, COS-7 cells were transfected with 6 g of pCS4-Flag-*dynein heavy chain* and/or pcDNA3-myc-*Dub-1*, respectively. After 48 h of transfection, co-immunoprecipitation was performed with an anti-Flag antibody (Sigma, St. Louis, MO) and an anti-Myc (9E10) antibody (Santa Cruz Biotechnology). Western blot analysis was performed with an anti-Myc (9E10) antibody (Santa Cruz Biotechnology) and an anti-Flag antibody (Sigma). For in vivo ubiquitination of dynein heavy chain and DUB-1, COS-7 cells were transfected with pCS4-Flag-*dynein heavy chain* or pcDNA3-myc-*Dub-1* and/or pMT123-HA-*ubiquitin*. After 48 h of transfection, co-immunoprecipitation was performed with an anti-Flag antibody (Sigma), an anti-Myc (9E10) antibody (Santa Cruz Biotechnology) and Western blot analysis was performed with an anti-HA antibody (Santa Cruz Biotechnology). For in vivo deubiquitination assay, COS-7 cells were transfected with pCS4-Flag-*dynein heavy chain*, pcDNA3-myc-*Dub-1*, pcDNA3-myc-*Dub-1C60S*, pcDNA3-myc-*USP7* and/or pMT123-HA-*ubiquitin*, respectively. In order to confirm the deubiquitination of dynein heavy chain, COS-7 cells were transfected with pCS4-Flag-*dynein heavy chain* pMT123-HA-*ubiquitin*, and pcDNA3-myc-*Dub-1* in a dose-dependent manner. After 48 h of transfection, co-immunoprecipitation was performed with an anti-Flag antibody (Sigma) and Western blot analysis was performed with an anti-HA antibody (Santa Cruz Biotechnology). For investigation of dynein heavy chain and DUB-1 stabilization, COS-7 cells were co-transfected pCS4-Flag-*dynein heavy chain*, pcDNA3-myc-*Dub-1*, and pMT123-HA-*ubiquitin* respectively. After 48 h of transfection, cells were treated with MG132 in a dose-dependent manner for 6 h. Immunoprecipitation was performed with an anti-Flag antibody (Sigma), or an anti-Myc (9E10) antibody (Santa Cruz Biotechnology) and Western blot analysis was performed with an anti-HA antibody (Santa Cruz Biotechnology). In order to confirm protein loading control, Western blot analysis was also performed with an anti-Actin antibody (Santa Cruz Biotechnology).

BIOINFORMATICS

Alignment of conserved amino acid sequences and the PEST motif for DUB enzymes derived from GenBank (Accession numbers: DUB-1 (Q61068) and DUB-2 (AAB95194)) was established using MegAlign software (clustal method) from DNASTAR (LASERGENE). In other to identify possible PEST sequences within DUB-1, the PEST algorithm (http://www.at.ambnet.org/embnet/tools/bio/PESTfind) was used [Spencer et al., 2004].

RESULTS

IDENTIFICATION OF PUTATIVE BINDING PROTEINS WITH DUB-1

Dub-1 is one of murine cytokine-inducible enzymes, and is specially expressed in B-lymphocytes in response to interleukin-3 (IL-3),

interleukin-5 (IL-5) and granulocyte/macrophage colony-stimulating factor (GM-CSF). DUB-1 plays a role in regulation of cellular growth via modulation of the ubiquitin-dependent proteolysis and the ubiquitination state of the unknown growth regulatory factors [Zhu et al., 1996a,b]. Therefore, it is very crucial to investigate the biological function of DUB-1 enzyme, which contributes to the cellular growth signaling in B-lymphocytes.

Since target proteins for cytokine-inducible DUB enzymes have not been identified yet, we screened the binding proteins with DUB-1, which is specially expressed in B-lymphocytes. We found differentially expressed proteins in DUB-1 overexpressed Ba/F3 cells by immunoprecipitaion (Fig. 1A). These differentially expressed proteins were analyzed by MALDI-TOF-MS. MASCOT results revealed a few candidate proteins including mKIAA1603, Col2a1 protein, and peripherin 1. One of proteins analyzed was identified as an mKIAA1603 protein (Fig. 1B), which is known to be dynein heavy chain. We further characterized the dynein heavy chain due to the best score number.

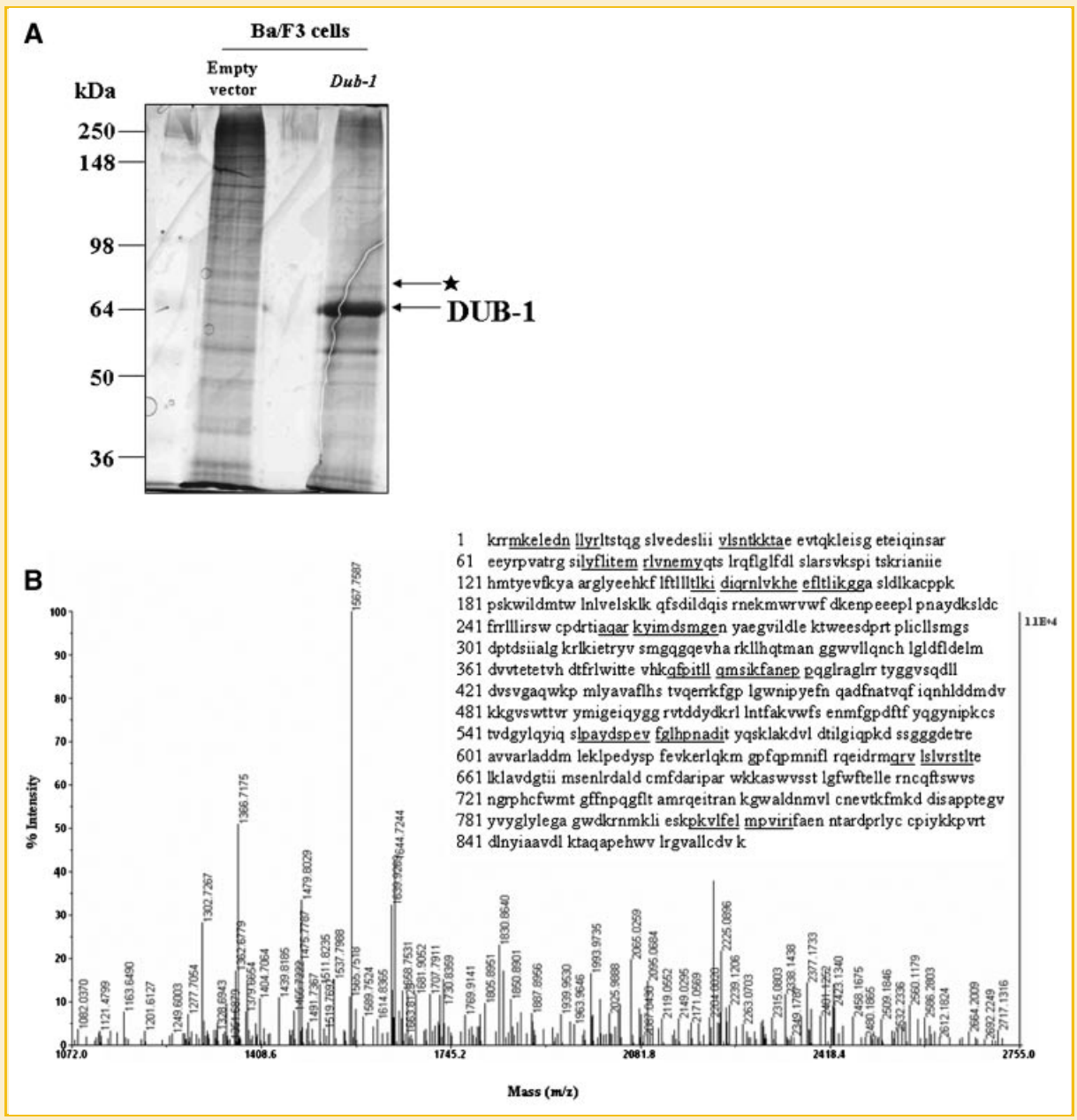

Fig. 1. Identification of binding proteins with DUB-1 using IP analysis. A: Ba/F3 cells were transfected with pcDNA3-myc or pcDNA3-myc-*Dub-1*, respectively. Each whole cell lysate (WCL) was incubated with an anti-Myc antibody. The immunoprecipitates were resolved in SDS-PAGE and stained with silver nitrate. A star indicates differentially expressed proteins in *Dub-1* overexpressed Ba/F3 cells. B: Mass spectrometric analysis of differentially expressed dynein heavy chain. MALDI-TOF peptide mass spectrum of the tryptic digest of dynein heavy chain is shown. Matched peptide sequences sourced from differentially expressed proteins were analyzed by MALDI-TOF-MS.

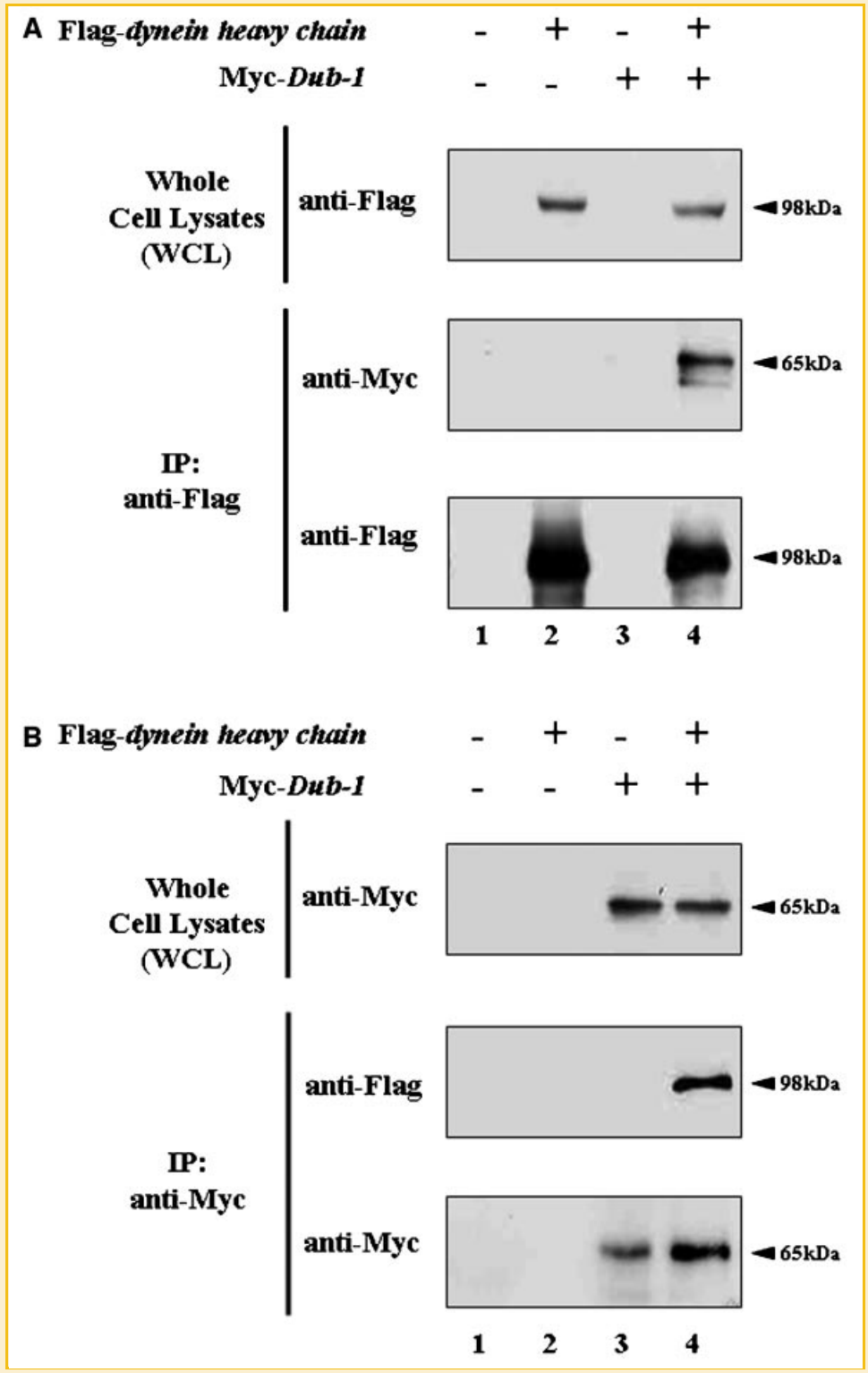

Fig. 2. In vivo binding assay between dynein heavy chain and DUB-1. COS-7 cells were transfected with pCS4-Flag-*dynein heavy chain* and/or pcDNA3-myc-*Dub-1*, respectively. WCL from COS-7 cells (lane 1), WCL from COS-7 cells transfected with pCS4-Flag-*dynein heavy chain* (lane 2), WCL from COS-7 cells transfected with pcDNA3-myc-*Dub-1* (lane 3), and WCL from COS-7 cells co-transfected with pCS4-Flag-*dynein heavy chain* and pcDNA3-myc-*Dub-1* (lane 4). Interaction of dynein heavy chain with DUB-1 was proved by co-immunoprecipitation with either an anti-Flag antibody (A) or an anti-Myc antibody (B).

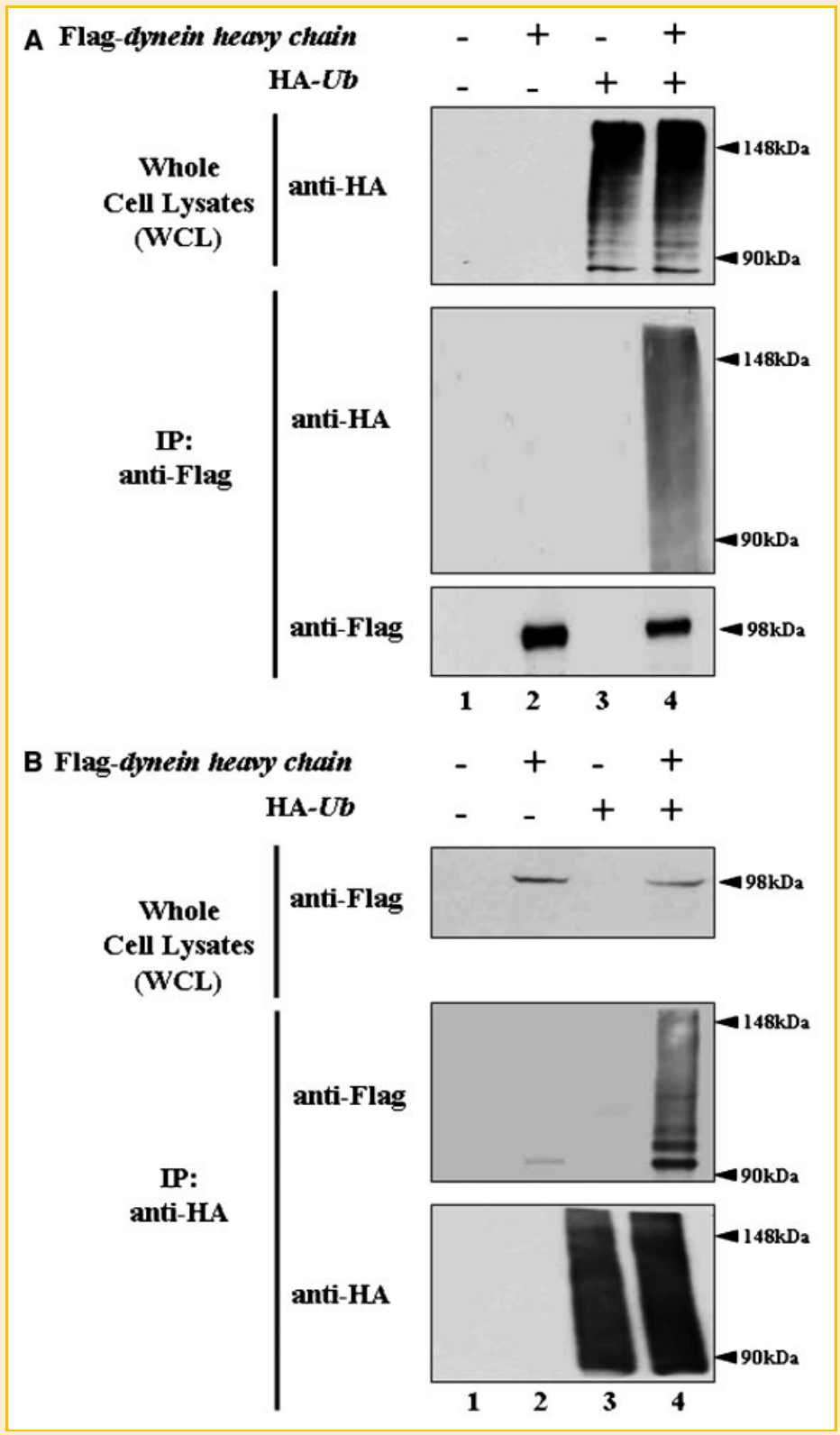

Fig. 3. In vivo ubiquitination of dynein heavy chain. COS-7 cells were transfected with pCS4-Flag-*dynein heavy chain* and/or pMT123-HA-*ubiquitin*. WCL from COS-7 cells (lane 1), WCL from COS-7 cells transfected with pCS4-Flag-*dynein heavy chain* (lane 2), WCL from COS-7 cells transfected with pMT123-HA-*ubiquitin* (lane 3), and WCL from COS-7 cells co-transfected with pCS4-Flag-*dynein heavy chain* and pMT123-HA-*ubiquitin* (lane 4). Ubiquitination of dynein heavy chain was proved by co-immunoprecipitation with either an anti-Flag antibody (A) or an anti-HA antibody (B).

DYNEIN HEAVY CHAIN INTERACTS WITH DUB-1 AND IS POLYUBIQUITINATED

Based on the observation that the dynein heavy chain binds DUB-1, we would like to elucidate whether the dynein heavy chain is a direct target of DUB-1. We carried out a binding assay in vivo and confirmed that the dynein heavy chain directly interacts with DUB-1 (Fig. 2A,B, lane 4, respectively). This suggests that DUB-1 may regulate the biological functions of dynein heavy chain by mediating the protein degradation.

Since the dynein heavy chain binds to DUB-1, it is possible that the dynein heavy chain is ubiquitinated and degraded by the ubiquitin–proteasome pathway. Therefore, we investigated ubiquitination of the dynein heavy chain in vivo. Immunoprecipitation analysis demonstrated that the dynein heavy chain is polyubiquitinated (Fig. 3). Due to the paucity of polyubiquitinated dynein heavy chain proteins in whole cell lysates, they were not detected by Western blotting. However, we observed the ladder of bands, which represent polyubiquitinated dynein heavy chains in co-trasnsfected cells through immunoprecipitation (Fig. 3A,B, lane 4), suggesting that the dynein heavy chain may be a target molecule for the ubiquitin–proteasome pathway.

DUB-1 DEUBIQUITINATES DYNEIN HEAVY CHAIN

Due to the fact that the dynein heavy chain binds DUB-1 and is polyubiquitinated, we investigated whether the dynein heavy chain is deubiquitinated by DUB-1, which regulates cellular growth by modulating the ubiquitin-dependent proteolysis [Zhu et al., 1996a,b]. When DUB-1 was overexpressed along with the dynein heavy chain, the ubiquitination level of dynein heavy chain was reduced significantly (Fig. 4, lane 4). However, when cells were treated with MG132, one of proteasome inhibitors, the ubiquitination level of dynein heavy chain was recovered (Fig. 4, lane 5). From these results, it is evident that dynein heavy chain is regulated by the ubiquitin–proteasome pathway and DUB-1 as a deubiquitinating enzyme controls the ubiquitination level of dynein heavy chain. As a control, we used a mutant construct of DUB-1 (C60S mutant form), which has inactivated deubiquitinating enzyme activity of DUB-1. In contrary to DUB-1 wild-type, the amount of polyubiquitinated dynein heavy chains was not reduced in transfected cells with the DUB-1 (C60S) mutant from (Fig. 4, lane 6). When USP7 was overexpressed as a negative control, the ubiquitination level of dynein heavy chain was not changed (Fig. 4, lane 7), indicating the specificity of DUB-1 to the dynein heavy chain. In addition, we investigated whether polyubiquitinated dynein heavy chain is deubiquitinated by DUB-1 in vivo. The assay demonstrated that DUB-1 has deubiquitinating activity for dynein heavy chain (Fig. 5, lanes 2–5). We also investigated whether the deubiquitination of dynein heavy chain is decreased by the treatment with MG132 in a dose-dependent manner. Deubiquitination of dynein heavy chain was gradually decreased with a higher dose of MG132 (Fig. 6, lanes 2–5). In addition, we confirmed that dynein heavy chain was stabilized with the treatment of MG132 in an immunoblot with anti-Flag antibody using whole cell lysates (Fig. 6). These results indicate that the degradation of dynein heavy chain is regulated by DUB-1, suggesting that dynein heavy chain is a target molecule for the ubiquitin–proteasome pathway. It has been known that the dynein heavy chain regulates the movement of organelles and vesicles along microtubules, and has ATPase activity [Shima et al., 2006]. Since the dynein heavy chain is known to

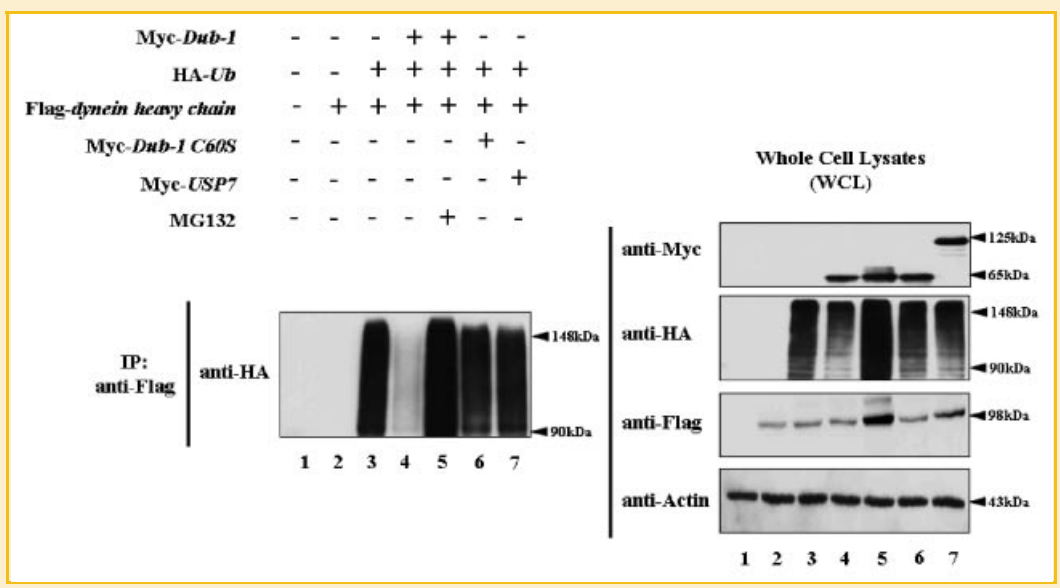

Fig. 4. In vivo deubiquitination of dynein heavy chain by DUB-1. COS-7 cells were transfected with pCS4-Flag-*dynein heavy chain*, pMT123-HA-*ubiquitin*, pcDNA3-myc-*Dub-1*, pcDNA3-myc-*Dub-1 (C60S)* and/or pcDNA3-myc-*USP7*. WCL from COS-7 cells (lane 1), WCL from COS-7 cells transfected with pCS4-Flag-*dynein heavy chain* (lane 2), WCL from COS-7 cells co-transfected with pCS4-Flag-*dynein heavy chain* and pMT123-HA-*ubiquitin* (lane 3), WCL from COS-7 cells co-transfected with pCS4-Flag-*dynein heavy chain*, pMT123-HA-*ubiquitin* and pcDNA3-myc-*Dub-1* (lane 4), WCL from co-transfected COS-7 cells treated with 10 M MG132 (lane 5), WCL from COS-7 cells co-transfected with pCS4-Flag-*dynein heavy chain*, pMT123-HA-*ubiquitin* and pcDNA3-myc-*Dub-1 (C60S)* (lane 6), and WCL from COS-7 cells co-transfected with pCS4-Flag-*dynein heavy chain*, pMT123-HA-*ubiquitin* and pcDNA3-myc-*USP7* (lane 7).

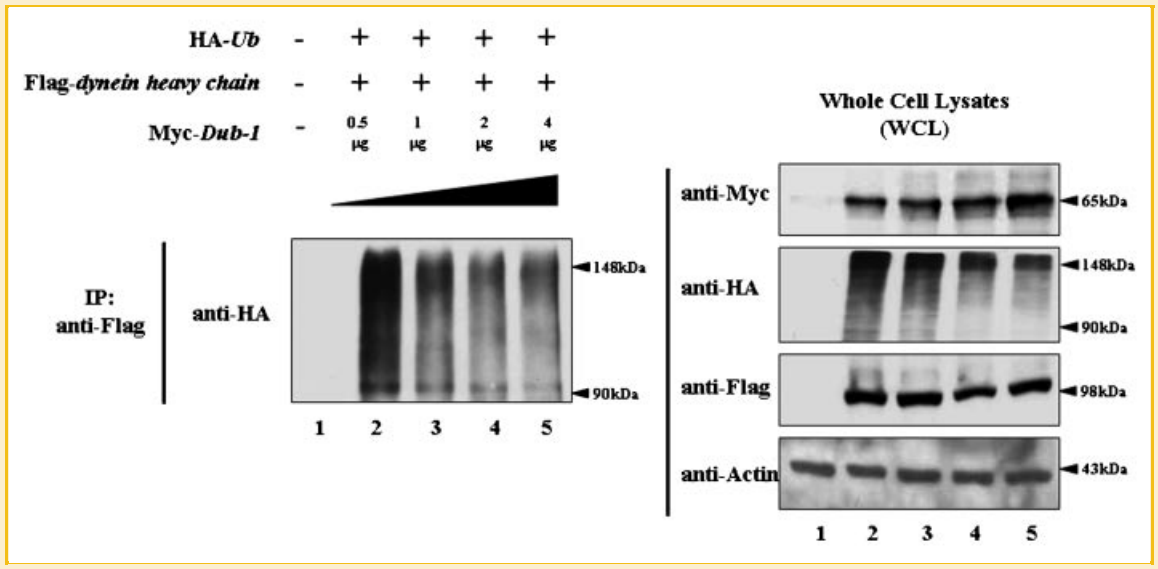

Fig. 5. Regulation of ubiquitination status for dynein heavy chain by DUB-1. The level of ubiquitination for deynein heavy chain is regulated by DUB-1 level. Each sample was transfected with pcDNA3-myc-*Dub-1* in a dose-dependent manner as follows: 0.5 g (lane 2), 1 g (lane 3), 2 g (lane 4), and 4 g (lane 5).

regulate nuclear segregation, mitosis, and transport of membranes, virus, and signaling molecules [Asai and Koonce, 2001; Vale, 2003], it is suggested that DUB-1 may regulate these processes via mediating the protein degradation of dynein heavy chain.

DUB-1 CONTAINS A CONSERVED PEST MOTIF AND IS ALSO POLYUBIQUITINATED

It has been previously reported that USP36 containing the PEST motif is polyubiquitinated and is involved in protein degradation pathway [Kim et al., 2005]. Therefore, we investigated whether the DUB-1 enzyme expressed in B-lymphocytes is also regulated by the ubiquitin–proteasome pathway. We first searched for the PEST motif, which is known to be involved in protein degradation, in murine DUB protein sequences using bioinformatics algorithm (http://emb1.bcc.univie.ac.at/). Interestingly, we found that murine DUB proteins contain a conserved PEST motif, suggesting that they may be degraded rapidly by the ubiquitin–proteasome pathway (Supplemental Material).

Since the PEST motif was identified in the sequence of DUB-1 protein, we carried out in vivo ubiquitination assay. As shown with

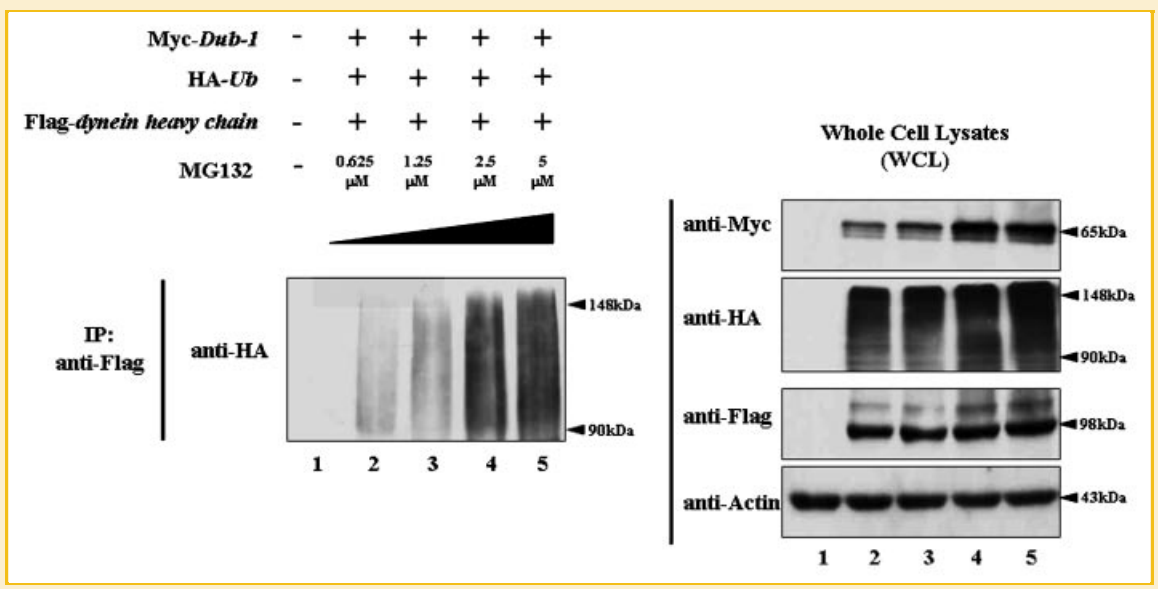

Fig. 6. The effect of MG132 on deubiquitination of dynein heavy chain by DUB-1. Deubiquitination of dynein heavy chain was gradually decreased with a higher dose of MG132. Each sample was treated with MG132 as follows: 0.625 M (lane 2), 1.25 M (lane 3), 2.5 M (lane 4), and 5 M (lane 5).

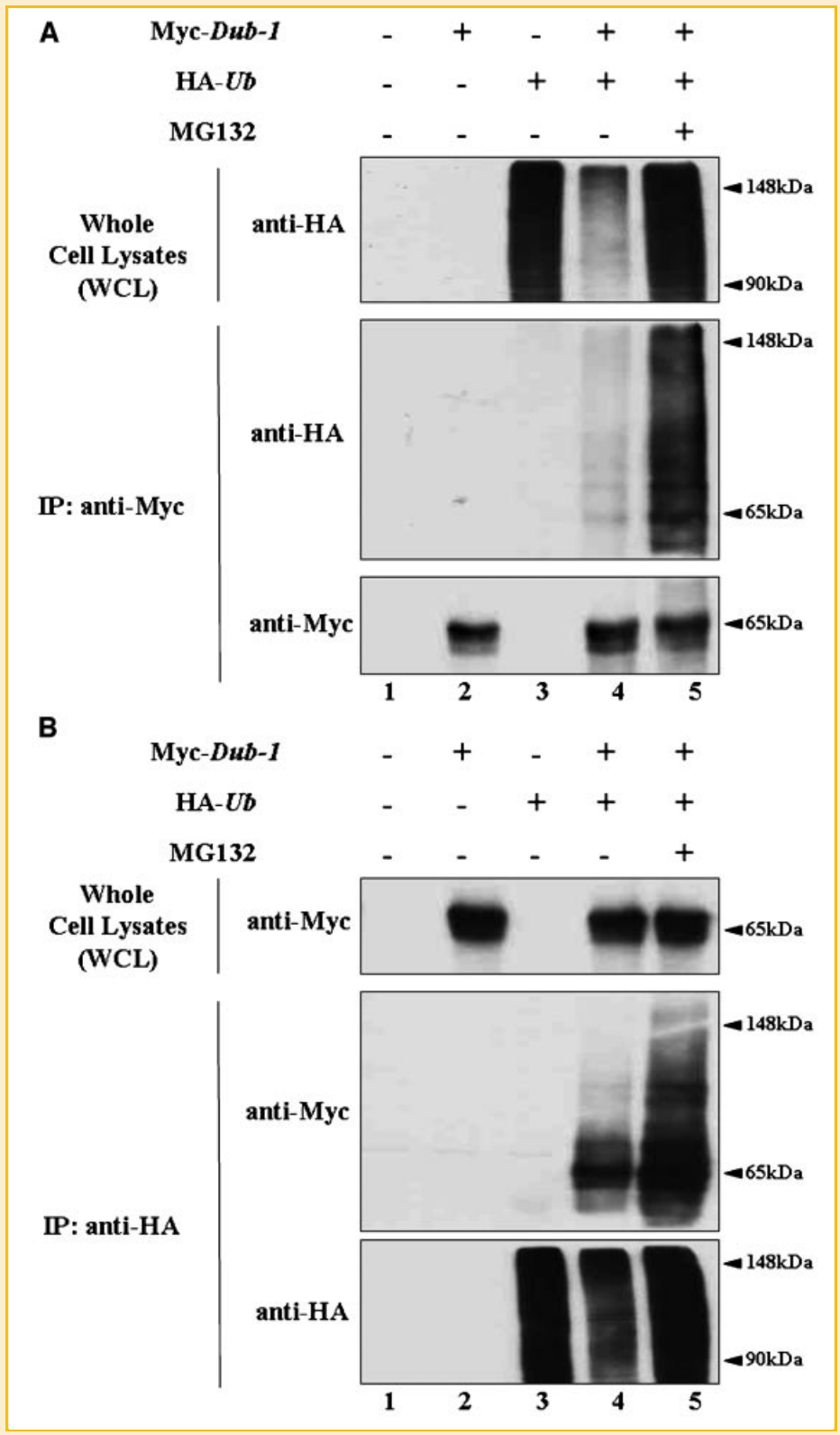

Fig. 7. In vivo ubiquitination of DUB-1. Ubiquitination of DUB-1 protein was demonstrated by co-immunoprecipitation assay. Each lane was loaded as follows: WCL from Ba/F3 cells (lane 1), WCL from Ba/F3 cells transfected with pcDNA3-myc-*Dub-1* (lane 2), WCL from Ba/F3 cells transfected with pMT123-HA-*ubiquitin* (lane 3), WCL from Ba/F3 cells co-transfected with pcDNA3-myc-*Dub-1* and pMT123-HA-*ubiquitin* (lane 4), and a proteasome inhibitor MG132 (10 M) has been added (lane 5). Ubiquitination of DUB-1 demonstrated by co-immunoprecipitation with an anti-Myc antibody (A) or an anti-HA antibody (B). MG132, a proteasome inhibitor, was used to inhibit DUB-1 protein from degradation.

USP36 [Kim et al., 2005], DUB-1 is also polyubiquitinated, indicating that the PEST motif in DUB-1 may be responsible for the DUB-1 degradation (Fig. 7A,B, lane 4). Next, we investigated the effect of MG132 on stability of DUB-1 protein. When MG132 was treated, we observed that DUB-1 was increased at a protein level (Fig. 7A,B, lane 5). We investigated whether ubiquitinated DUB-1 proteins are accumulated by the treatment with MG132 in a dose-dependent manner. Interestingly, we observed accumulation of ubiquitinated DUB-1 proteins with MG132 treatment in a dose-dependent manner (Fig. 8, lanes 2–5). These results indicate that the degradation of DUB-1 is also regulated by the ubiquitin–proteasome pathway. Our observation with in vivo ubiquitination assay suggests that the level of DUB-1 may be regulated by the ubiquitin–proteasome pathway through the interaction of the PEST motif as shown with USP36 [Kim et al., 2005]. Taken all together, it is expected that murine DUB enzymes are regulated at transcription and translational levels during proliferation of B-lymphocytes. It will be intriguing to determine the structural role of the PEST motif during proliferation of B-lymphocytes and investigate its relevance to immune diseases.

DISCUSSION

Ubiquitinaiton and deubiquitination process is one of post-translational modifications, and is connected with the eukaryotic system for maintaining cellular homeostasis. Investigation of deubiquitinating (DUB) enzymes, which mediate a removal of ubiquitin from ubiquitin-conjugated proteins, is very crucial to understand cellular signaling and homeostasis. Among DUB enzymes, DUB-1 is the one of murine cytokine-inducible enzymes, and the expression level of DUB-1 is strongly regulated by cytokines such as IL-3, IL-5, and GM-CSF [Zhu et al., 1996a,b]. DUB-1 may act as a key regulatory molecule for cytokine-inducible cell proliferation. However, further experiments will be important in determining the role of DUB-1 on cytokine-inducible cell proliferation. In order to understand DUB-1 signaling in B-lymphocytes, we investigated screened binding proteins with DUB-1. Immunoprecipitation analysis and silver staining experiments indicated the presence of differentially expressed proteins in DUB-1 expressed Ba/F3 cells, and candidates for putative binding proteins with DUB-1 including dynein heavy chain was identified by MALDI-TOF-MS analysis (Fig. 1). We confirmed that DUB-1 binds with dynein heavy chain (Fig. 2), indicating that dynein heavy chain is indeed a substrate for DUB-1. We demonstrated that dynein heavy chain is polyubiquitinated in vivo and is deubiquitinated with DUB-1 but not with USP7, a deubiquitinating enzyme for p53 (Figs. 3 and 4). Taken together, these results indicate that polyubiquitinated dynein heavy chains are degraded by the ubiquitin–proteasome pathway and DUB-1 enzyme has deubiquitinating activity for dynein heavy chain and acts as a regulatory molecule for dynein heavy chain.

Dynein protein mainly consists of dynein heavy chain, dynein light chain, and its intermediates. These proteins are in a complex by different protein isoforms, depending on the functional significance. Dynein heavy family, which has two major classes of axonemal and cytoplasmic proteins, mainly associate with microtubule-motor complex [David and Michael, 2001], and has a number of biological functions in the cell, including nuclear segregation, mitosis, and transport of membranes, virus, signaling molecules [Asai and Koonce, 2001; Vale, 2003]. It has also been known that the dynein heavy chain regulates the movement of organelles and vesicles along microtubules, and has ATPase activity [Shima et al., 2006]. *Mdnah5*$^{-/-}$ knock-out mice have been generated to investigate biological functions of dynein heavy chain [Ibanez-Tallon et al., 2002]. Disruption of the *Mdnah5* gene results in a mouse phenotype that replicates most of the features of classic Kartagener's syndrome. In addition, *Mdnah5*$^{-/-}$ mice have *hydrocephalus*, growth retardation and died perinatally. Another report demonstrated that the disruption of *Mdnah5* gene leads to primary ciliary dyskinesia [Ibanez-Tallon et al., 2004]. It is suggested that dysfunction of axonemal dynein heavy chain reveals a novel mechanism for *hydrocephalus* formation. Furthermore, in mice imbalance in

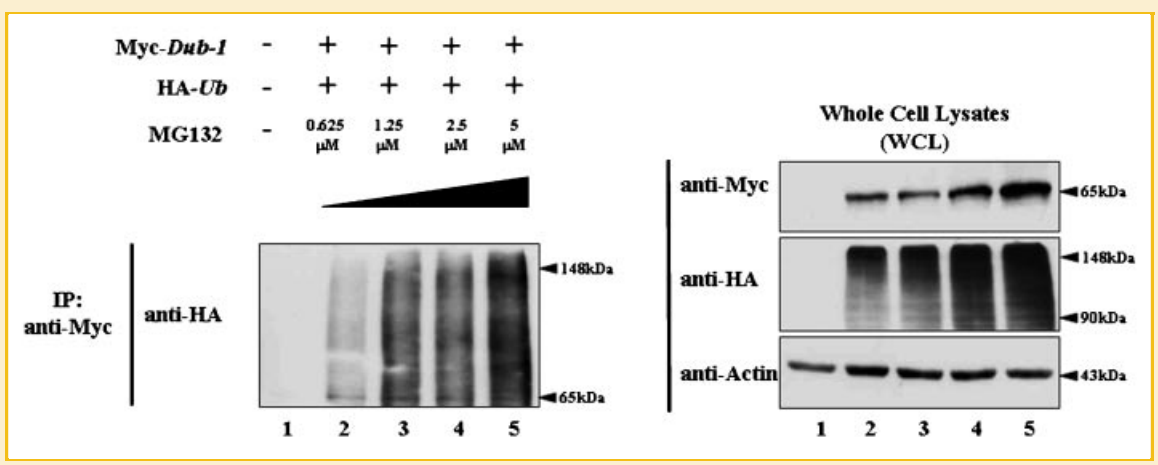

Fig. 8. The effect of MG132 on ubiquitination of DUB-1. Ubiquitinated DUB-1 proteins were gradually accumulated with a higher dose of MG132. Each sample was treated with MG132 as follows: 0.625 M (lane 2), 1.25 M (lane 3), 2.5 M (lane 4), and 5 M (lane 5).

amount of dynein heavy chain leads to aberrant accumulation and/or deregulation of dynein heavy chain. Based on the observation that *Mdnah5*$^{-/-}$ mice have growth retardation and died perinatally, we hypothesized that the functions of dynein heavy chain are crucial for growth and proliferation of cells. These functions mediated by the dynein heavy chain may be modulated by DUB-1 in B-lymphocytes since DUB-1 is a key regulator for the expression level of dynein heavy chain.

Some of deubiquitinating enzymes not only deubiquitinate target substrates, but also get ubiquitinated by the ubiquitination system. It has been recently reported that USP36 is polyubiquitinated and is degraded by ubiquitin–proteasome pathway [Kim et al., 2005]. USP36 has the PEST motif, which is known to be involved in protein degradation [Kim et al., 2005] and we found that murine DUB proteins, DUB-1 and DUB-2 contain a conserved PEST motif (Supplemental Material). Among the murine DUB proteins, DUB-1 expressed in B-lymphocytes is polyubiquitinated, and is degraded rapidly by the ubiquitin–proteasome pathway (Figs. 7 and 8), indicating that the turnover of DUB-1 is critical for cytokine response.

We investigated for the first time to identify specific target proteins for DUB-1. DUB-1 deubiquitinates dynein heavy chain, and is also ubiquitinated itself, suggesting that it is degraded by the ubiquitin–proteasome pathway. Our immunoprecipitation and MALDI-TOF-MS analyses suggest that DUB-1 is a key regulatory molecule for the functions of dynein heavy chain in regulating the cellular homeostasis. Due to the paucity of information concerning E3 ligases or deubiquitinating enzymes for DUB-1, important directions for future studies should include the analysis of mechanisms that regulate expression of DUB-1 in B-lymphocytes for cellular signaling.

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