

# Antagonistic regulation of myogenesis by two deubiquitinating enzymes, UBP45 and UBP69

Kyung Chan Park\*<sup>†</sup>, Jung Hwa Kim\*<sup>†</sup>, Eun-Jung Choi\*, Sang Won Min\*, Sangmyung Rhee\*, Sung Hee Baek\*, Sung Soo Chung\*, Oksun Bang\*, Dongeun Park\*, Tomoki Chiba<sup>‡</sup>, Keiji Tanaka<sup>‡</sup>, and Chin Ha Chung\*<sup>§</sup>

\*National Research Laboratory of Protein Biochemistry, School of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea; and <sup>†</sup>Tokyo Metropolitan Institute of Medical Science, Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Tokyo 113, Japan

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**Protein modification by ubiquitin is a dynamic and reversible process that is involved in the regulation of a variety of cellular processes. Here, we show that myogenic differentiation of embryonic muscle cells is antagonistically regulated by two deubiquitinating enzymes, UBP45 and UBP69, that are generated by alternative splicing. Both enzymes cleaved off ubiquitin from polyubiquitinated protein conjugates *in vivo* as well as from linear ubiquitin-protein fusions *in vitro*. In cultured myoblasts, the level of UBP69 mRNA markedly but transiently increased before membrane fusion, whereas that of UBP45 mRNA increased as the cells fused to form myotubes. Both myoblast fusion and accumulation of myosin heavy chain were dramatically stimulated by the stable expression of UBP69 but strongly attenuated by that of the catalytically inactive form of the protease, suggesting that the mutant enzyme acts dominant negatively on the function of the wild-type protease. In contrast, stable expression of UBP45 completely blocked both of the myogenic processes but that of inactive enzyme did not, indicating that the catalytic activity of the enzyme is essential for its inhibitory effects. These results indicate that differential expression of UBP45 and UBP69 is involved in the regulation of muscle cell differentiation.**

The covalent attachment of ubiquitin to proteins is involved in the regulation of a variety of cellular processes, including cell-cycle progression, cell differentiation, and apoptosis (1–3). Ubiquitin (Ub), a highly conserved 76-amino acid protein, is ligated to a wide variety of proteins by a multienzyme system consisting of E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes. Most of the proteins ligated to multiple units of Ub are then degraded by the 26S proteasome (4–6). Reversal of ubiquitination catalyzed by deubiquitinating enzymes (DUBs) also plays important roles in numerous cellular processes (7, 8). These include eye development in fly (9), transcriptional silencing (10), cytokine response (11), and growth regulation (12, 13). DUBs also are required for generation of free Ub from its precursors, encoded in a form of a tandem repeat of Ubs (14, 15) or fusion of Ub with certain ribosomal proteins (16), as well as for the recycling of Ub from the branched polyUb chains (17). Genome sequence projects have identified more than 90 DUBs, making them the largest family of enzymes in the Ub-dependent pathway (8). However, little is known about the specific function of DUBs.

Development of skeletal muscle involves a well defined sequence of events, including irreversible withdrawal of proliferating mononucleated myoblasts from cell cycle and fusion of their plasma membrane to form multinucleated myotubes (18, 19). Concurrent with the morphological differentiation, a large number of muscle-specific proteins, such as  $\alpha$ -actin and myosin, accumulate during the myogenic process. Ubiquitination of regulatory proteins, including MyoD, Myf5, and Id proteins, plays an important role in regulation of the muscle cell differentiation (20–22). However, it remains unknown whether deconjugation of ubiquitin from proteins by DUBs, is also required for the myogenic process. In the present study, we report the role

of two DUBs, termed UBP45 and UBP69, in regulation of muscle cell differentiation.

## Experimental Procedures

**Cloning of UBP cDNAs.** The *E. coli* JM101 cells carrying both the rat cDNA plasmids and pACUb-R- $\beta$ -gal were plated onto LB containing 100  $\mu$ g/ml ampicillin, 34  $\mu$ g/ml chloramphenicol, and 40  $\mu$ g/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside. Then, they were incubated at 37°C for 14 h. Among  $3 \times 10^5$  transformants, 9 appeared as white colonies, whereas the others were stained blue. All of the control cells (i.e., those transformed with pBS vector only) developed blue color. From the white colonies, plasmids were isolated and again transformed into the *E. coli* JM101 expressing either Ub-R- $\beta$ -gal or Ub-M- $\beta$ -gal. Two of the nine turned into white colonies when transformed with Ub-R- $\beta$ -gal but not with Ub-M- $\beta$ -gal (i.e., became blue), confirming that the white colony contains the plasmid carrying the rat cDNA specifying the UBP activity against the Ub- $\beta$ -gal proteins (23, 24). The plasmids were isolated, sequenced, and referred to as pBS-ubp45 and pBS-ubp69. Because UBP45 and UBP69 share an identical C-terminal region of about 40 kDa (see below), the nucleotide sequence for the N-terminal extension of UBP69 was deleted and the abbreviated cDNA was referred to as pBS-ubp $\Delta$ N.

**Purification of UBPs.** To facilitate the purification of UBP45 and UBP69 as well as of UBP $\Delta$ N, having only the identical C-terminal region, their cDNAs were ligated into pGEX vector. Then, they were transformed into BL21 cells and grown to mid-log phase in LB at 37°C. Expression of the enzymes was induced for 2 h by treating with 1 mM isopropyl- $\beta$ -D-thiogalactoside. After the treatment, the cells were harvested and suspended in PBS containing 3% (vol/vol) Triton X-100. The cell suspension then was disrupted by sonication and centrifuged at 100,000  $\times g$  for 2 h. The soluble extracts were applied to a glutathione-Sepharose column equilibrated with the same buffer. The column was washed with 0.5 M NaCl, and the proteins bound to the column were eluted with Tris-HCl (50 mM, pH 7.8) buffer containing 10 mM glutathione. The enzyme activity during purification was monitored by determining their ability to hydrolyze <sup>125</sup>I-labeled Ub-PESTc, as described (25).

**Construction of L6 Cell Lines Expressing UBPs.** The cDNAs for UBP45, UBP69, and UBP $\Delta$ N were ligated into the *Bam*HI site of pcDNA3.1 and tagged with Myc to its 5'-end (i.e., between *Nhe*I and *Hind*III sites) of the ORFs. The resulting plasmids and

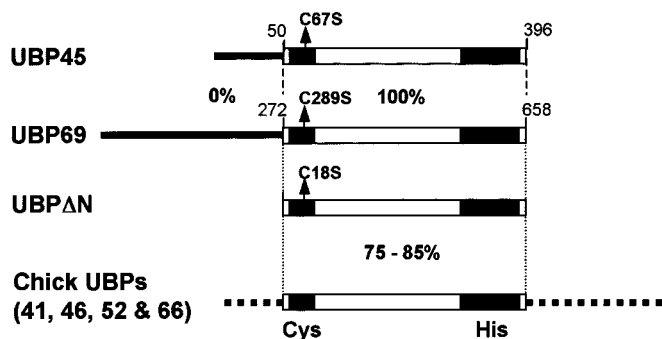
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Abbreviations: Ub, ubiquitin; DUB, deubiquitinating enzyme; UBP, Ub-specific processing protease; MHC, myosin heavy chain.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF106658 and AF106659).

<sup>†</sup>K.C.P. and J.H.K. contributed equally to this work.

<sup>§</sup>To whom reprint requests should be addressed. E-mail: chchung@snu.ac.kr.



**Fig. 1.** Primary structures of chick and rat UBPs. The thick and dotted lines indicate the variable regions. The white boxes of UBPA45 and UBPA69 (this study) and of chick UBPs (24, 27) denote the common regions, including the conserved Cys/His domains (the black boxes). UBPA $\Delta$ N was generated by deletion of the N-terminal region from UBPA69. Amino acid identities in the variable and common regions were expressed as percentages. The arrows indicate the conserved Cys residues in the common region, which were replaced by Ser.

the control vector containing Myc only were referred to as pcDNA/Myc-UBPA45, pcDNA/Myc-UBPA69, pcDNA/Myc-UBPA $\Delta$ N, and pcDNA/Myc, respectively. L6 myoblasts (between the 5th and 7th passages) were plated at  $5 \times 10^5$  cells per 60-mm dish in DMEM supplemented with 10% (vol/vol) FBS. After culturing for 24 h, the cells were transfected with the pcDNAs by using Lipofectamine Plus (GIBCO/BRL) by following the procedure recommended by the manufacturer. The transfected cells were cultured for 3 h, refed with the fresh culture medium supplemented with 10% (vol/vol) serum, and further cultured for 24–48 h. The cells then were cultured by changing the culture medium with DMEM containing 0.5 mg/ml of Geneticin at 3 days interval. The Geneticin-resistant transfectants were subcultured in 96-well microtiter plates, and single colonies were selected. The same approach was used to generate the L6 cells that stably express the mutant forms of the UBP enzymes (i.e., for UBPA45/C67S, UBPA69/C289S, and UBPA $\Delta$ N/C18S). As a result, we established 6–15 cell lines expressing each enzyme as well as the marker from empty vector.

The cells from each single colony were plated on plastic dishes at a concentration of  $2 \times 10^5$  cells per 100-mm dish in DMEM supplemented with 10% (vol/vol) FBS. To induce cell differentiation, the culture medium was changed to a differentiation medium containing 5% (vol/vol) horse serum 3 days after cell seeding. At the appropriate time of culture, cells were harvested for immunoblotting with the anti-MHC monoclonal antibody (MF-20) or stained with Giemsa solution for microscopic observation. The MF-20 antibody (26) was purchased from Developmental Studies Hybridoma Bank (Univ. of Iowa, Iowa City, IA).

## Results

**Deubiquitinating Activities of UBPA45 and UBPA69.** We have previously isolated cDNAs for UBPA41, UBPA46, UBPA52, and UBPA66 from chick muscle cDNA library by using an *E. coli*-based *in vivo* screening method (24, 27). These UBPs have nearly identical regions spanning the highly conserved Cys/His catalytic domains but with distinct N- and/or C-terminal extensions. However, the chick enzymes do not show any sequence similarity with known sequences of other UBPs, except the catalytic domains. Therefore, it has been suggested that these chick UBPs may represent a previously uncharacterized family of DUBs.

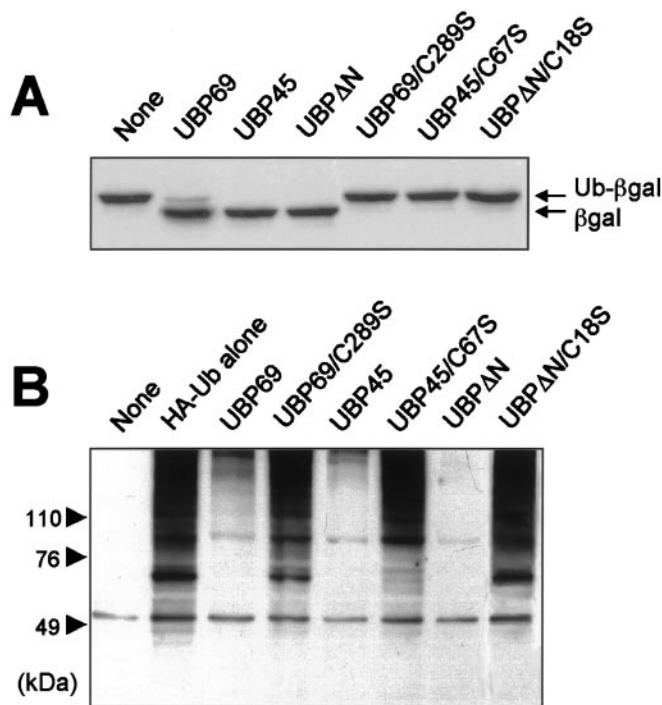
Further, by using the same screening method, we isolated two cDNAs that encode DUB proteins that have an identical C-terminal region spanning the highly conserved Cys/His catalytic domains but with distinct N-terminal extensions (Fig. 1). These

cDNAs were named *ubp69* (GenBank accession no. AF106659) and *ubp45* (GenBank accession no. AF106658), because they encode 69-kDa and 45-kDa proteins, respectively. UBPA45 and UBPA69 are identical to UBPA-t1 and UBPA-t2, respectively, that were previously reported by Wing and coworkers (28) to be “testis-specific.” After the sequence comparison with the CLUSTAL/W program, the identical region of UBPA45 and UBPA69 showed 82% identity in amino acid sequences and 79% identity in their nucleotide sequences with chick muscle UBPA41 (24, 27). With other UBPs, however, they had restricted similarity only at two short regions containing the conserved Cys/His domains. These results suggest that UBPA45 and UBPA69 also belong to the chick UBPA41 family. A search for the genomic DNA of the human homolog of rat UBPA45 and UBPA69 with the human genome database revealed the presence of a single gene at chromosome location 11q23, which consists of one exon each for the UBPA45- and UBPA69-specific regions and 12 exons for the identical region. Thus, it seems that UBPA45 and UBPA69 are derived from alternative splicing of the primary transcript.

To determine whether the UBP enzymes have deubiquitinating activity, mutant forms of UBPA45 and UBPA69 were generated by substituting the conserved Cys residue (i.e., Cys-67 and Cys-289) with Ser; these mutants are referred to as UBPA45/C67S and UBPA69/C289S, respectively. We also replaced Cys-18 by Ser in UBPA $\Delta$ N, which has only the identical C-terminal region of the UBP enzymes. This mutant is referred to as UBPA $\Delta$ N/C18S. The UBP enzymes and their mutant forms were partially purified and incubated with the extracts of *E. coli* MC1000 transformed with pACUb- $\beta$ -gal, which encodes Ub- $\beta$ -gal fusion protein (23), followed by immunoblot analysis using anti- $\beta$ -gal antibody. UBPA45 and UBPA69, but not their mutant forms, could generate  $\beta$ -gal from Ub- $\beta$ -gal (Fig. 2A). UBPA $\Delta$ N also was capable of cleaving Ub- $\beta$ -gal, whereas UBPA $\Delta$ N/C18S could not. These results indicate that both UBPA45 and UBPA69 use the Cys residue as one of the catalytic residues as other known UBPs do. UBPA45, UBPA69, and UBPA $\Delta$ N, but not their active-site mutants, could also generate free Ub from a tandem pentameric repeat of Ub (His-Ub<sub>5</sub>; ref. 29) as well as from Ub fusion of carboxyl extension peptide of 80 amino acids (Ub-CEP80; ref. 30, and data not shown). Thus, both UBPA45 and UBPA69 can function in the generation of free Ub from its precursors, at least under *in vitro* conditions.

We also examined whether the UBP enzymes could cleave off Ub from polyUb chains linked to proteins via isopeptide bonds under *in vivo* conditions. HA-tagged Ub was transiently expressed in NIH 3T3 cells with and without UBPA45, UBPA69, UBPA $\Delta$ N, and their mutants, followed by immunoblot analysis of the cell extracts using anti-HA antibodies. As shown in Fig. 2B, polyubiquitinated protein conjugates were almost completely deubiquitinated in the cells expressing UBPA45, UBPA69, or UBPA $\Delta$ N, but remained accumulated in the cells expressing their mutant forms, in which the catalytic Cys residue was replaced by Ser. These results indicate that UBPA45 and UBPA69 show isopeptidase activity *in vivo*. These results also show that the N-terminal extensions of the UBP enzymes are not essential for their catalytic activity *in vivo* as well as *in vitro*.

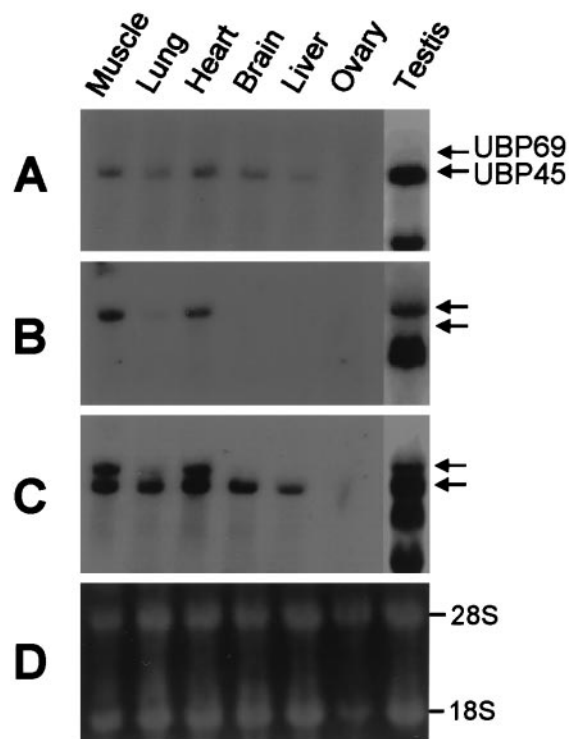
**Differential Expression of UBPA45 and UBPA69 Transcripts.** To determine the tissue-specific expression of the UBP mRNAs, Northern analysis was performed with three distinct probes for hybridization: the unique 5' region of *ubp45*, the unique 5' region of *ubp69*, and their identical region. When the unique 5' region of *ubp45* was used as a probe, the UBPA45 mRNA was detected with a size of about 3 kb in all tissues examined, except the ovary (Fig. 3A). However, the UBPA45 mRNA band also could be seen in the ovary when the same blot was exposed for a prolonged period (data not shown). Significantly, the UBPA69 mRNA with a size of about 3.4 kb was specifically expressed in the muscle-



**Fig. 2.** Deubiquitinating activities of UBP45 and UBP69. (A) The extracts (100  $\mu$ g) obtained from *E. coli* MC1000 cells expressing Ub-M- $\beta$ -gal were incubated alone or with 0.2  $\mu$ g of partially purified UBP69, UBP45, UBP $\Delta$ N, and their mutant forms for 2 h at 37°C. After incubation, the samples were subjected to SDS/PAGE, followed by immunostaining with anti- $\beta$ -gal antibody. (B) HA-tagged Ub was transiently expressed in NIH 3T3 cells without and with each of Myc-tagged UBP69, UBP45, UBP $\Delta$ N, and their mutant forms. The cells then were harvested, boiled, and subjected to SDS/PAGE, followed by immunostaining with anti-HA antibody. A duplicate gel, which was immunostained with anti-Myc antibody, showed that the expression of the UBP enzymes was nearly identical (data not shown).

type tissues, including skeletal muscle and heart, when the unique region of *ubp69* was used as a probe (Fig. 3B). When the identical region was used as a probe, the muscle-type tissues had both 3- and 3.4-kb transcripts, and the other tissues, except the testis, expressed only the 3-kb transcript (Fig. 3C). Of interest was the finding that the testis expressed four transcripts with different sizes (Fig. 3C), of which the 3- and 1.6-kb transcripts interacted with the UBP45-specific probe, whereas the 3.4- and 2.5-kb transcripts did with the UBP69-specific probe (Fig. 3A and B, respectively). Thus, it seems that two additional forms of the UBP mRNAs in the testis are also derived from alternative splicing of the same primary transcript.

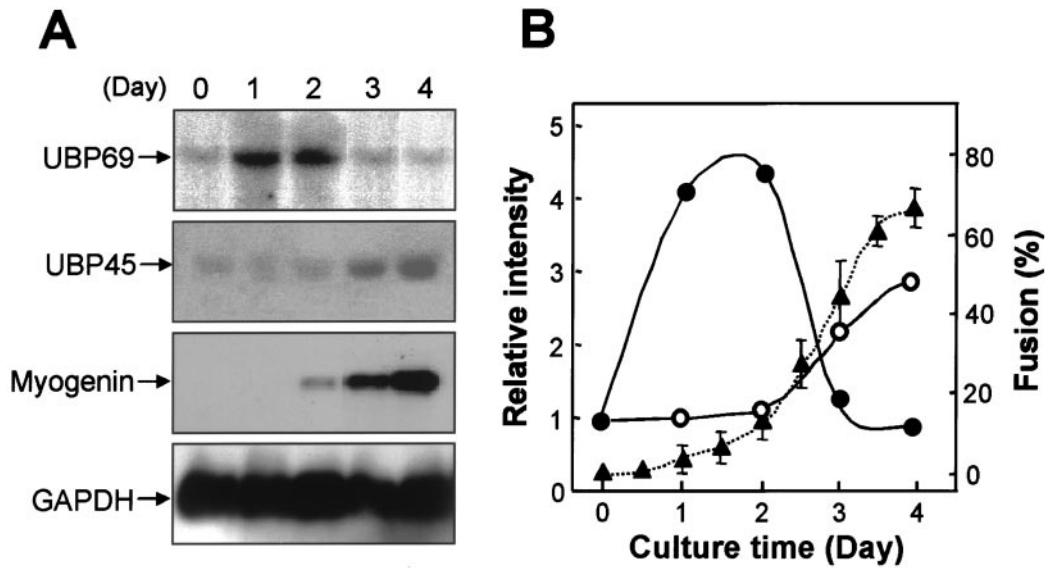
Because the UBP69 mRNA could be detected only in muscle-type tissues (with an exception in the testis), we examined whether the muscle-specific expression of UBP69 mRNA might be related with muscle cell differentiation. Total RNAs were prepared from L6 myoblasts that had been cultured for various periods followed by hybridization with the unique regions of *ubp45* and *ubp69*. The level of UBP69 mRNA dramatically increased before the onset of membrane fusion and of the expression of myogenin mRNA, and decreased as the myogenic processes proceeded (Fig. 4). In contrast, the UBP45 mRNA level remained low until the burst of membrane fusion but significantly increased thereafter. The mRNA level for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) remained constant throughout the culture period. These results strongly suggest that differential expression of UBP45 mRNA and UBP69 mRNA is related to muscle cell differentiation.



**Fig. 3.** Expression of the UBP69 and UBP45 mRNAs in various rat tissues. Northern analysis was performed by using the unique 5'-region of *ubp45*, which corresponds to the nucleotides from -234 to 150 (A), the unique 5'-region of *ubp69*, which is from 225 to 818 (B), and their common regions, which are from 450 to 1284 of *ubp45* (C), as probes. The same membrane was used for the hybridization but after deprobing by heating for 5 min at 94°C. Before transfer to demonstrate loading, the gel also was stained with ethidium bromide (D).

**Involvement of UBP45 and UBP69 in Regulation of Myogenesis.** To examine whether stable expression of UBP69 or UBP45 might influence the myogenic differentiation of the cultured myoblasts, L6 cell lines that constitutively express Myc-tagged UBP69 and Myc-tagged UBP45 were established by transfection with pcDNA/Myc-UBP69 and pcDNA/Myc-UBP45. The cells transfected with pcDNA/Myc vector only were also prepared as controls. These cells were incubated in the differentiation medium for various periods and stained with Giemsa solution. Fig. 5 shows that membrane fusion of the cells transfected with pcDNA/Myc-UBP69 is dramatically stimulated, compared with that with the empty vector. In contrast, the fusion of the cells transfected with pcDNA/Myc-UBP45 was almost completely blocked. These results suggest that UBP69 and UBP45 antagonistically function in regulation of the morphological differentiation of the cultured myoblasts.

To clarify further the antagonistic role of the UBPs in regulation of muscle cell differentiation, we also established L6 cell lines that constitutively express the mutant forms of the UBP enzymes, of which the catalytic Cys residues were replaced by Ser. Remarkably, membrane fusion of the cells expressing UBP69/C289S was strongly attenuated (Fig. 6A). This result suggests that UBP69/C289S dominant-negatively acts on the fusion-promoting function of the wild-type enzyme, such as by competing with the chromosomally expressed wild-type enzyme in interaction with polyubiquitinated target substrate(s). On the other hand, the cells expressing UBP45/C67S gained their ability in membrane fusion to an even better extent than that of the cells transfected with the empty vector. These results indicate that the

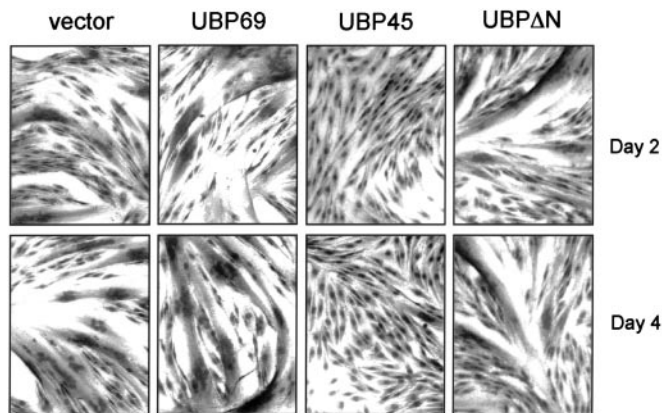


**Fig. 4.** Changes in the expression of the UBP69 and UBP45 mRNAs during myogenic differentiation of L6 myoblasts. (A) The L6 cells were cultured for the indicated periods as described under *Experimental Procedures*. Total RNAs were prepared from the cells and subjected to Northern analysis by using the unique regions of *ubp69* and *ubp45*. The cDNAs for myogenin and GAPDH also were used for the analysis. (B) The cultured cells also were stained with Giemsa solution. The extent of myoblast fusion ( $\blacktriangle$ ) was expressed as a percentage of the number of nuclei in the fused cells among the total number of nuclei in 10 randomly chosen fields under a microscope. Cells containing more than three nuclei were regarded as fused cells. The levels of the UBP69 ( $\bullet$ ) and UBP45 mRNAs ( $\circ$ ) were determined by scanning the bands in A and B, respectively, with a densitometer.

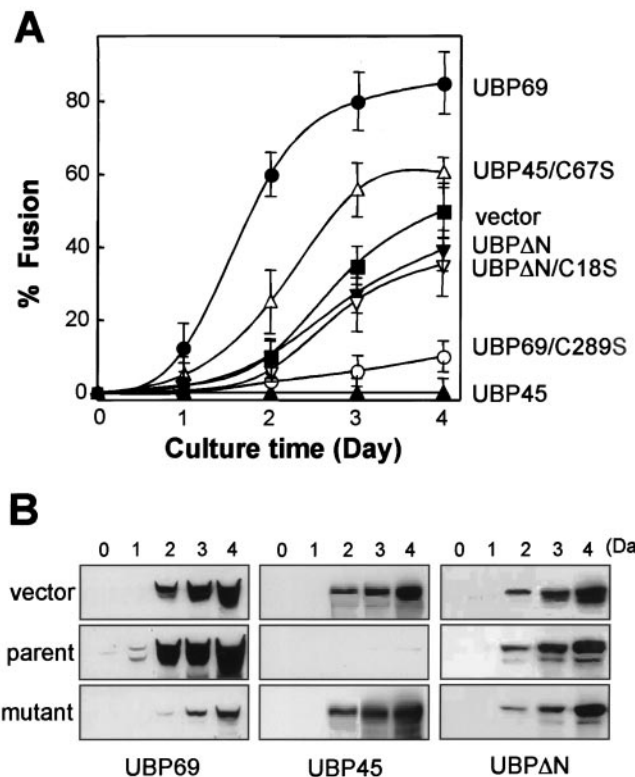
catalytic activities of UBP45 and UBP69 are essential for their regulatory function on the fusion.

To determine whether the differential effects of UBP45 and UBP69 on myoblast fusion are due to the presence of the distinct N-terminal regions, L6 cells that stably express only the identical C-terminal region of the UBP enzymes were generated by transfection with pcDNA/Myc-UBP $\Delta$ N and pcDNA/Myc-UBP $\Delta$ N/C18S. Significantly, the cells expressing either UBP $\Delta$ N or UBP $\Delta$ N/C18S could fuse to form myotubes, and the degree of membrane fusion in both cases was similar to that in the cells transfected to the empty vector (Figs. 5 and 6A). These results indicate that the distinct N-terminal regions of the UBP enzymes are required for their differential effects on cell fusion.

Accumulation of muscle-specific proteins, such as myosin heavy chain (MHC), is known to occur concurrently with



**Fig. 5.** Effects of stable expression of UBP45, UBP69, and UBP $\Delta$ N on membrane fusion of L6 myoblasts. The cells transfected with pcDNA/Myc-UBP69, pcDNA/Myc-UBP45, pcDNA/Myc-UBP $\Delta$ N, or pcDNA/Myc vector were cultured in the differentiation medium for the indicated periods, as described under *Experimental Procedures*. Then, they were stained and observed under a microscope.



**Fig. 6.** Effects of stable expression of UBP45, UBP69, UBP $\Delta$ N, and their mutant forms on myoblast fusion and accumulation of MHC. (A) The L6 cells transfected with pcDNA/Myc-UBP69, pcDNA/Myc-UBP45, pcDNA/Myc-UBP $\Delta$ N, or pcDNA/Myc vector were cultured for the indicated periods. They were then stained, and the extent of their membrane fusion was determined as in Fig. 4B. (B) Extracts were prepared from the cells cultured as above and subjected to immunoblot analysis by using the anti-MHC monoclonal antibody (MF-20).

membrane fusion during myogenic differentiation of embryonic muscle cells. Therefore, we examined whether stable expression of UBP69, UBP45, UBP $\Delta$ N, or their mutant forms might influence the accumulation of MHC in the cells. Extracts were prepared from the cells that had been cultured for various periods and subjected to immunoblot analysis by using a monoclonal antibody raised against MHC. The MHC protein level was dramatically increased in the cells expressing UBP69 but was markedly reduced in the cells expressing UBP69/C289S (Fig. 6B), demonstrating that UBP69 positively regulates the accumulation of muscle-specific proteins. This result also suggests that the mutant form of the enzyme dominant-negatively acts on the stimulatory effect of the wild-type enzyme on accumulation of muscle-specific proteins. In contrast, stable expression of UBP45 completely blocked the MHC accumulation, and that of UBP45/C67S resulted in normal accumulation of the protein but to an extent slightly higher than the control. On the other hand, accumulation of MHC normally occurred in the cells expressing either UBP $\Delta$ N or UBP $\Delta$ N/C18S during the myogenic period, as in the control cells transfected with the empty vector. These results show that UBP69 and UBP45 antagonistically function also in the regulation of accumulation of muscle-specific proteins, and that the distinct N-terminal regions of the UBP enzymes are required for their differential regulatory roles.

To determine whether the changes in the level of MHC protein upon expression of the UBP enzymes are due to alteration in the stability of MHC or to the expression of its mRNA, Northern analysis was performed by using a probe for a coding region of MHC. Under conditions that each of UBP69, UBP45, UBP $\Delta$ N, or their mutant forms was expressed, the accumulation pattern of MHC mRNA was almost exactly the same as that of MHC protein (see Fig. 6B and data not shown). Thus, it seems that both UBP enzymes act on certain regulatory protein(s) controlling the expression of MHC mRNA instead of directly acting on MHC that might have been ubiquitinated.

## Discussion

The data presented here indicate that deubiquitination-dependent mechanisms regulate myogenic differentiation of embryonic muscle cells. That is, differential expression of UBP45 and UBP69 is involved in the control of both membrane fusion and accumulation of muscle-specific proteins. Under conditions for normal cell proliferation, both UBP enzymes may be maintained to a low level. With any signal for withdrawal from cell cycle, such as serum deprivation, a dramatic increase in the expression of UBP69 may be induced (see Fig. 4) and play a critical role in the commitment step for myoblast differentiation. The expression of UBP45 until this stage remains low, but gradually increases as the myoblasts fuse to form myotubes for a possible role in prevention of asynchronous fusion of the cells that might not have yet been committed for differentiation. It also may be possible that in response to muscle damage, a similar change in the expression of the UBP enzymes may occur in the satellite cells, which constitute a reservoir of undifferentiated muscle precursor cells, leading to the cell differentiation and regeneration of skeletal muscle (31).

Skeletal muscle differentiation is regulated by a family of muscle-specific transcription factors, such as MyoD and Myf5, as well as by Id proteins, which act as negative regulators of the basic helix-loop-helix transcription factors by forming transcrip-

tionally inactive protein complexes (32–34). These regulatory proteins are subjected to covalent modification by ubiquitin, and targeted to proteasomes for rapid degradation (20–22). However, the ubiquitinated proteins also may be targeted to DUBs for deubiquitination. Therefore, multiple scenarios are possible for the function of UBP45 and UBP69. For example, UBP45 and UBP69 may act on any of the same or different positive or negative regulators of myogenic differentiation in a way that promotes or attenuates the proteasome-mediated degradation of the regulatory proteins, such as trimming polyUb chains conjugated to proteins and/or removing polyubiquitinated degradation remnants or inhibiting it by complete reversal of the ubiquitination reaction before interaction of ubiquitinated proteins with proteasomes (7, 35). In this respect, the distinct N-terminal extensions of UBP45 and UBP69 may differentiate their specific function or provide substrate specificity or preference, although both enzymes have a potential to act on any ubiquitinated protein without discrimination when they were overexpressed (see Fig. 2B).

It has been reported that the distinct N-terminal regions of UBP-t1 and UBP-t2 in testis, which are identical to UBP45 and UBP69, respectively, determine their subcellular localization (28). UBP-t1 was shown to locate in the nucleus, whereas UBP-t2 was primarily in perinuclear regions. Therefore, it also seems possible that the antagonistic function of UBP45 and UBP69 in regulation of myogenesis might be caused by their action in different cellular compartments.

Recently, Liquid facets (Lqf), an endocytic protein homologous to vertebrate epsin, has been identified as a specific substrate for Fat facets (Faf), a *Drosophila* DUB essential for patterning of the compound eye (36). Faf reverses the ubiquitination of Lqf, thus preventing the proteasome-mediated degradation of the protein. The p53 tumor-suppressor protein also has been identified as a specific target for deubiquitination by herpesvirus-associated ubiquitin-specific protease (HAUSP; ref. 37). HAUSP removes Ub from polyubiquitinated p53 and stabilizes the suppressor protein. Thus, it has been suggested that HAUSP also might function as a tumor suppressor *in vivo* through the stabilization of p53. Likewise, the identification of specific target substrate(s) should be essential for the elucidation of the mechanisms by which UBP45 and UBP69 can antagonistically regulate the myogenic differentiation of embryonic muscle cells.

Roles of UBP45 and UBP69 might not be limited to the regulation of muscle cell differentiation. The expression level of both UBP45 and UBP69 transcripts in testis is much higher than that in any of the muscle-type tissues tested, including skeletal muscle and heart (see Fig. 3). Moreover, it has been shown that the expression of UBP-t1 and UBP-t2 in testis is developmentally regulated (28), although the role of the enzymes during spermatogenesis is unknown. In addition, the UBP45 mRNA is broadly expressed in nonmuscle tissues. Thus, it seems possible that both UBP45 and UBP69 could function rather broadly in terminal differentiation, such as in spermatocyte differentiation.

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