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Authors

Cui, Ziyou
Hwang, Soyun Michelle
Gomes, Aldrin V

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Identification of the Immunoproteasome as a Novel Regulator of Skeletal Muscle Differentiation

Ziyou Cui,[a] Soyun Michelle Hwang,[a] Aldrin V. Gomes[a,b]

Department of Neurobiology, Physiology, and Behavior[a] and Department of Physiology and Membrane Biology,[b] University of California, Davis, California, USA

While many of the molecular details of myogenesis have been investigated extensively, the function of immunoproteasomes (i-proteasomes) in myogenic differentiation remains unknown. We show here that the mRNA of i-proteasome subunits, the protein levels of constitutive and inducible proteasome subunits, and the proteolytic activities of the 20S and 26S proteasomes were significantly upregulated during differentiation of skeletal muscle C2C12 cells. Knockdown of the i-proteasome catalytic subunit PSMB9 by short hairpin RNA (shRNA) decreased the expression of both PSMB9 and PSMB8 without affecting other catalytic subunits of the proteasome. PSMB9 knockdown and the use of i-proteasome-specific inhibitors both decreased 26S proteasome activities and prevented C2C12 differentiation. Inhibition of the i-proteasome also impaired human skeletal myoblast differentiation. Suppression of the i-proteasome increased protein oxidation, and these oxidized proteins were found to be more susceptible to degradation by exogenous i-proteasomes. Downregulation of the i-proteasome also increased proapoptotic proteins, including Bax, as well as cleaved caspase 3, cleaved caspase 9, and cleaved poly(ADP-ribose) polymerase (PARP), suggesting that impaired differentiation is likely to occur because of significantly increased apoptosis. These results demonstrate for the first time that i-proteasomes, independent of constitutive proteasomes, are critical for skeletal muscle differentiation of mouse C2C12 cells.

The ubiquitin proteasome system (UPS) is the main intracellular protein degradation pathway that involves polyubiquitination of target proteins and subsequent proteolysis by a multicatalytic proteasome. The UPS is responsible for degrading 60 to 80% of proteins in mammalian cells and is known to be involved in many biological processes (1–3). In vertebrate cells, proteasomes can occur in different forms. The 26S proteasome, also called the standard or constitutive proteasome, is the most common form and is composed of a cylindrical catalytic core particle (20S) capped at one or both ends with 19S complexes (PA700). The 20S core can interact with one or two proteasome activators, such as the 11S activator (PA28) or PA200, to form 11S-20S, 11S-20S-11S, PA200-20S, or PA200-20S-PA200 complexes (4, 5). In addition, 20S proteasomes that are simultaneously bound to 19S, 11S, or PA200 have been observed as hybrid proteasomes (expressed as 19S-20S-11S or 19S-20S-PA200) (6–8). The proteolytic activities of the proteasomes are carried out by three β catalytic subunits in the 20S catalytic core: β1 (PSMB6), with caspase-like activity; β2 (PSMB7), with trypsin-like activity; and β5 (PSMB5), with chymotrypsin-like activity. The immune cytokine gamma interferon (IFN-γ) can induce the expression of three other catalytically active β subunits of the 20S proteasome, called β1i (PSMB9), β2i (PSMB10), and β5i (PSMB8). Each induced subunit replaces its constitutive counterpart to incorporate into the nascent 20S proteasome, referred to as the immunoproteasome (i-proteasome), and modifies peptide-bond cleavage preferences of the 20S proteasome (7, 8). Like the constitutive 26S proteasome, the i-proteasome can be composed of the inducible 20S (i20S) bound to one or two 19S complexes. Also, the activator 11S can bind to the i20S and result in more active forms of i-proteasomes capable of degrading proteins in an ATP-independent manner (6, 9). The immune-related functions of i-proteasomes have been extensively studied. The i-proteasomes play a wide spectrum of roles in regulating antigen presentation, cytokine production, T cell differentiation, and survival (10–12). More recently, the i-proteasomes have been shown to take part in non-immune-related functions, such as removing oxidized proteins, preventing protein aggregation, remodeling cardiac muscle, and regulating tumor survival (12).

Myogenesis is a complicated process controlled by the spatiotemporal expression of many myogenic regulatory factors (MRFs) and transcription factors (13–15). Under the control of these factors, the proliferating myoblasts withdraw from the cell cycle and then elongate, adhere, and fuse into multinucleated myotubes (16). After the myotubes are formed, their cellular morphology, structure, and function differ significantly from those of the myoblasts. The expression of contractile tissue-associated proteins is significantly increased in myotubes. As such, myogenic differentiation is a well-organized destruction and reconstruction process, during which new proteins are synthesized and other proteins are selectively degraded in a timely manner. The expression of specific proteins is thought to be essential for the proper progression of myogenesis, and the controlled degradation of proteins occurs throughout myogenic differentiation by cathepsins (17–19), calpains (20–22), caspases (23, 24), and UPS (25–27).

The UPS has been shown to play the most significant role among all of the intracellular degradation systems in the regulation of the myogenic process. The essential role of the UPS has been demonstrated by using proteasome inhibitors, such as MG132 and PSI, and also by knocking down specific 26S proteasome subunits. Proteasome inhibition or knockdown can block

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Address correspondence to Aldrin V. Gomes, avgomes@ucdavis.edu.

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the fusion of myoblasts and inhibit differentiation (26, 27). In 1995, Ebisui et al. showed that the 26S proteasome activity decreased and 20S proteasome activity increased after myotube formation (28). The UPS was also shown to be important in ensuring the development of muscle cells and the proper modeling of the sarcomere (3).

Although proteasomes have been implicated in myogenic differentiation, the role of i-proteasomes in this process is unknown. In this study, we report for the first time that the i-proteasomes, independent of the constitutive proteasomes, play an important role in proper skeletal muscle development. Downregulation of the i-proteasome increases oxidized proteins and increases apoptosis, resulting in impaired C2C12 skeletal muscle differentiation.

MATERIALS AND METHODS

Cell culture. C2C12 mouse myoblasts (American Type Culture Collection, Manassas, VA) were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin (growth medium [GM]) at 37°C and 5% CO_2. Confluent myoblasts (90 to 100%) were differentiated into myotubes by changing the culture medium to DMEM supplemented with 2% horse serum (differentiation medium [DM]). Cells were kept in DM until the end of day 1 (D1), D2, D4, and D6. Human skeletal muscle myoblasts were obtained from Zen-Bio Inc. (NC). Human myoblasts were grown in skeletal muscle cell growth medium (SKM-M; Zen-Bio) and differentiated using skeletal muscle differentiation medium (SKM-D; Zen-Bio). The culture medium for all experiments was changed every 2 days.

Bortezomib treatment. C2C12 myoblasts were split into 6-well plates. Confluent (90 to 100%) myoblasts were differentiated in DM supplemented with dimethyl sulfoxide (DMSO) or 10 nM bortezomib (LC Laboratories, MA). The culture medium was changed every 2 days. The final concentration of DMSO in the medium was 0.1% (vol/vol). The cells were observed and imaged under microscopy at specific days after the treatment throughout the progress of myogenesis.

Proteolytic activity measurement of 26S and 20S proteasomes, cathepsin L, and calpain. Myoblast and differentiating/differentiated cells were scraped into proteasome lysis buffer (50 mM Tris, 250 mM sucrose, 5 mM $MgCl_2$ [pH 7.4]) after three washes with ice-cold phosphate-buffered saline (PBS). The protein extraction and proteolytic assays were performed as described previously (29–31). Briefly, the cells were homogenized with a hand-held Potter-Elvehjem homogenizer. The supernatant containing the proteasomes was collected after 20 min centrifugation at 15,000 × *g*. After protein concentration determination, every sample was diluted to 1 μg/μl with proteasome lysis buffer. The caspase (β1)-, trypsin (β2)-, and chymotrypsin (β5)-like activities of proteasomes were assayed using 25 μg of protein and the fluorescence-tagged substrates Z-LLE-AMC (7-amino-4-methylcoumarin) for β1, Boc-LSTR-AMC for β2, and Suc-LLVY-AMC for β5 (Enzo Life Sciences, NY). All assays were carried out in a total volume of 100 μl per well in a black 96-well plate. The ATP-dependent 26S assays were performed in 26S buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.5 mM dithiothreitol [DTT] [pH 7.5]) after the addition of ATP at a final concentration of 100 μM. The ATP-independent 20S assay for the chymotrypsin-like proteasome activity was carried out in 25 mM HEPES (pH 7.5), 0.5 mM EDTA, and 0.03% SDS. For the caspase- and trypsin-like 20S activities, the buffer composition was 25 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.05% Nonidet P-40, and 0.001% SDS. Each assay was conducted in the absence and presence of a specific proteasome inhibitor (40 μM Z-Pro-Nle-Asp-H for caspase-like, 60 μM epoxomicin for trypsin-like, and 20 μM epoxomicin for chymotrypsin-like activity) to determine each specific proteolytic activity. The β1 inhibitor Z-Pro-Nle-Asp-H was purchased from Enzo Life Sciences, and epoxomicin was purchased from Peptides International. AMC fluorescent tags released from substrates by the specific proteasome activity were measured using a Fluoroskan Ascent fluorometer (Thermo Electron, MA) every 15 min up to 120 min at an excitation wavelength of 390 nm and an emission wavelength of 460 nm.

Calpain activity measurements were carried out in a total volume of 200 μl per well in black 96-well plates. Protein samples (25 to 50 μg) were incubated with 100 μM LLVY-AMC (Calbiochem, La Jolla, CA) in 50 mM Tris, 1 mM EDTA, 10 mM $CaCl_2$, 150 mM NaCl, 0.5 mM DTT [pH 7.5]. AMC released from substrate cleavage by calpain activity was measured using a Fluoroskan Ascent fluorometer at an excitation wavelength of 390 nm and an emission wavelength of 460 nm for up to 120 min. Each assay was conducted in the absence and presence of a specific calpain inhibitor (50 μM calpain inhibitor IV; Calbiochem, CA) to determine calpain-specific activity. This assay measures both calpain I and II activity. Cathepsin L assays were carried out in a total volume of 100 μl per well in black 96-well plates. Protein samples (25 μg) were incubated with 100 μM fluorogenic substrate (Z-Phe-Arg-AMC; Peptides International) in 100 mM sodium acetate buffer containing 1 mM EDTA and 1 mM DTT, pH 5.5. Released AMC was measured using a Fluoroskan Ascent fluorometer at an excitation wavelength of 390 nm and an emission wavelength of 460 nm for up to 120 min. Each assay was conducted in the absence and presence of a specific cathepsin L inhibitor (10 μM cathepsin L inhibitor I; Calbiochem) to determine cathepsin L-specific activity.

Quantification of gene expression by quantitative RT-PCR (qRT-PCR). Total RNA from each myoblast or differentiation sample was extracted with an RNeasy minikit (Qiagen Inc., Germany) following the manufacturer's protocol. Total mRNA (1 μg from each sample) was used for cDNA synthesis using a mix of random hexamer primers and oligo(dT) (QuantiTect reverse transcription kit; Qiagen) according to the manufacturer's instructions. A fast SYBR green master mix (Applied Biosystems) was used to detect the accumulation of PCR product. A final volume of 10 μl of reaction mixture for every well was loaded into a 384-well qPCR plate. The reactions were performed on a AB 7900HT instrument (Applied Biosystems) using the following steps: initial enzyme activation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 15 s. All reactions were run in triplicate ($n = 3$), and the expression of all target genes was detected in three different biological samples. The results were calculated using the $2^{-\Delta\Delta CT}$ relative quantification method normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucuronidase beta (GUSB), and 18S rRNA. Primers used for RT-PCR were as follows (all 5′ to 3′, with the forward and then the reverse primer listed for each gene): 18S rRNA, TCGAACGTC TGCCCTATCAA and GCTATTGGAGCTGGAATTACCG; GAPDH, CC AGCCTCGTCCCGTAGAC and ATGGCAACAATCTCCACTTT GC; Gusb, CCGACCTCTCGAACAACCG and GCTTCCCGTTCATA CCACACC; myogenin, GCGCAGGCTCAAGAAAGTGAAT and TTTCG TCTGGGAAGGCAACA; PSMA4, AGGTCGCTTATACCAAGT GGA and ACGTTAGCATCAGATGTTATGCC; PSMB5, AGG AGCCGCGAATCGAAATG and CCAGAAGGTACGGGTTGATCTC; PSMB10, GAGGAATGCGTCCTTGGAACA and CGATGGTAGTCCCG GTCTT; PSMB3, CAGCGTCTCAAGTTCCGACTG and CGCTTCTCAT ACAGGAGGTTG; PSMB6, CTGGGAAAACCGGGAAGTCTC and GAG TCCGCTCCTAGAACCAC; PSMB7, GTGTCGGTGTTTCAGCCAC and TCCGCTTCCAAGACAGCATTC; PSMB8, ATGGCGTTACTGGATCT GTGC and GCGGAGAAACTGTAGTGTCCC; PSMB9, GGGACAACCA TCATGGCAGT and CAGCAGCGGAACCTGAGAG; PSMC5, GCA CAGAGGAATGAGCTGAAT and CGACTTCTCCAACGTAGGAGC; PSMD11, TCCATCGTGAAACGTGACATT and GGCCGTACATACTT CAGGAGTC; PSMD13, TTTGGCACCGTTTGGAAGAG and, TACCAG GGACAGAGGGTTTAC; PSME1, CCAAGGTGGATGTGTTCCGT and GAAAATAGCTCCCAAGCAGGTT; PSME3, ATGGCCTCGTTGCTG AAGG and GTGGATCTGAGTTAGGTCATGGA; PSME4, AGCGTCAA CAAGATAAGAATGCT and GCCCGATTCCTATATGCTCAAA; PSMF1, AGTGGTGACAAACGGCTACTA and ACCGGAGGGCATACAGTTCTT; PSMG3, TGACCCAGTTCGGGAAGATG and GACGGCTCTGTTTCCTG

CTT; and RPL13A, CTGTGAAGGCATCAACATTTCTG and GACCACCA TCCGCTTTTTCTT.

Western blot analysis and quantification. Cell lysates were prepared in proteasome lysis buffer containing protease/phosphatase inhibitor cocktails (Sigma). Equal amounts of protein were subjected to electrophoresis on 4 to 20% Criterion polyacrylamide gels (Bio-Rad) under reducing conditions and transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Total protein amount was detected by staining nitrocellulose membranes with Ponceau S or by imaging in stain-free gels (Bio-Rad). Membranes were then blocked for 1 h with 3% nonfat dry milk (NFM) in Tris-buffered saline (TBS) (pH 7.4) containing 0.05% (wt/vol) Tween 20 (TTBS). The membranes were washed three times in TTBS and probed for 2 h with the following primary antibodies: mouse anti-PSMC2, anti-PSMC5, anti-PSME1 (all 1:1,000; Enzo), mouse anti-PSMB8 (1:50; Enzo), rabbit anti-PSMB9 (1:1,000; Thermo Fisher Scientific), anti-PSMA6 (1:10,000; Epitomics), anti-PSMB5, -PSMB6, and -PSMB7 (1:600; all from Santa Cruz), horseradish peroxidase (HRP)-conjugated anti-GAPDH (1:10,000; Sigma), anti-myogenin (1:50), and anti-myosin heavy chain (1:200; MF-20, Developmental Studies Hybridoma Bank). All other antibodies were obtained from Cell Signaling Technologies. Following primary antibody incubation, the membranes were incubated for 1 h with horseradish peroxidase-conjugated sheep anti-mouse or -rabbit IgG (1:10,000; Sigma, St. Louis, MO). Both primary and secondary antibodies were used in TTBS containing 1% NFM, and all incubation steps were done at room temperature with gentle shaking. Immunoreaction was detected using an enhanced chemiluminescence (ECL) detection kit (Thermo Fisher Scientific). The images were obtained by ChemiDoc MP (Bio-Rad) controlled by Image Lab 4.1 (Bio-Rad). The quantification of blots was performed using Image Lab 4.1.

Native gel electrophoresis. Native gel electrophoresis was carried out on 2 to 5% gradient native acrylamide/bis separating gels with 2% stacking gels (32, 33). A 200-μg portion of total protein was loaded in each lane. Electrophoresis was carried out in TBE running buffer containing 0.5 mM DTT, 0.5 mM ATP and 2 mM $MgCl_2$. The gels were run consecutively at 30 V for 30 min, 35 V for 1 h, 40 V for 1 h, 50 V for 1 h, and then 65 V for 4 h at 4°C. The chymotrypsin-like proteolytic activity of the proteasomes was detected by incubating the gels in 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and 0.02% SDS buffer containing 50 μM proteasome substrate (Suc-LLVY-AMC) for 10 to 30 min at 37°C. Proteasome bands were identified by the free AMC that was released when the proteasomes cleaved the substrate. For immunoblotting, proteins in native gels were transferred to PVDF membranes using Bio-Rad mini-Protean 3 transfer system in Towbin's buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) for 16 h at 5 V and 4°C.

Immunoproteasome activity in D0 and D2 C2C12 cell lysates. D0 and D2 C2C12 cells were homogenized in HEPES buffer (20 mM HEPES, 20 mM KCl, 5 mM $MgCl_2$ [pH 7.5]). The homogenate was then centrifuged at 12,000 × *g* for 20 min to remove the mitochondria and nucleus. The supernatant was then centrifuged at 100,000 × *g* for 5 h to pellet the endogenous proteasomes. The 100,000 × *g* supernatants were called the cytosolic fractions, aliquoted, and used for protein degradation studies. D0 and D2 cytosolic fractions at 1.1 mg/ml (40 μl total) were incubated with HEPES buffer (control) or 1 μg human spleen 20S immunoproteasome (Enzo). After incubation for 3 or 6 h at 37°C, 4 μl of sample was removed and mixed with 98 μl of 100 mM sodium phosphate buffer (pH 6.8) and then immediately frozen at −80°C. Samples (20 μl from the 100 μl) were then mixed with 10 μl fluorescamine (0.3 mg/ml) in acetone for 1 min in a black 96-well plate and then water added to make a final volume of 200 μl. Samples were then mixed and fluorescence measured using a Fluoroskan Ascent fluorometer at an excitation wavelength of 390 nm and an emission wavelength of 460 nm.

Effect of exogenous immunoproteasome and constitutive proteasome on oxidized proteins in D2 C2C12 cell lysates. Cytosolic fractions from D2 C2C12 cells (in 50 mM Tris, 120 mM KCl, 5 mM $MgCl_2$ [pH 7.5]) were incubated with either buffer, constitutive 20S, or 20S i-proteasomes (1 μg proteasome/40 μg of total protein) and incubated at 37°C for 3 h. Digestion was stopped by boiling samples in SDS sample buffer. Oxidized proteins were detected using the Oxyblot procedure, which is described below.

***In situ* proximity ligation assay (PLA) and immunofluorescence microscopy.** The i-proteasome localization was detected using the Duolink II kit (Olink Bioscience, Uppsala, Sweden). The assays were performed according to the manufacturer's instructions for the Duolink blocking solution and detection (34). The images were taken by an LSM 510 META confocal microscope (Carl Zeiss) with 40/63× objectives. Cell images were exported using AxioVision software (Carl Zeiss) for further analysis. Immunofluorescence microscopy was carried out on C2C12 and human skeletal muscle cells fixed in PBS containing 3% formaldehyde for 20 min at room temperature. Cells were permeabilized with PBS containing 0.2% Triton X-100 for 20 min, and blocked for 1 h in 10% goat serum at room temperature. Cells were incubated overnight at 4°C with a 1:50 to 1:200 dilution of primary antibody in 1% normal goat serum and, after rinsing, were incubated for 1 h with 1 μg/ml Alexa Fluor 555-conjugated goat anti-mouse IgG in blocking buffer (1% normal goat serum). After rinsing 0.5 μg/ml of 4′6′-diamidino-2-phenylindole (DAPI) (a nuclear stain) was added to the myoblasts. For determining the in-fusion index C2C12 myoblasts were stained with myosin heavy chain and counterstained with DAPI. The fusion index was defined as the percentage of nuclei present in myosin heavy-chain (MyHC)-positive cells with three or more nuclei. Immunofluorescence images were taken with an AX10 Zeiss microscope equipped with an X-cite 120 excitation light source.

RNA interference. Suppression of the target gene PSMB9 was performed using an shRNA plasmid (pGeneClip puromycin vector) containing a puromycin resistance gene for selection (SureSilencing shRNA plasmids; Qiagen). The inserted sequences of short hairpin RNA are TCCGG AAGCTCCTACATTTAT (clone 1), TGCAAACGTGGTGAAGAACAT (clone 2), TGCTAATTCGACAGCCCTTTA (clone 3), ATGCTAATTCG ACAGCCCTTT (clone 4), and GGAATCTCATTCGATGCATAC (negative control). C2C12 cell transfection with these purified plasmids was achieved by using the Neon transfection system (Invitrogen) and following the manufacturer's standard protocol. Briefly, 70 to 90% confluent C2C12 cells were trypsinized and washed in Ca^{2+}- and Mg^{2+}-free PBS. The cell pellets were resuspended in the appropriate volume of suspension buffer R. The shRNA plasmids were added to resuspended cells at a ratio of 2 μg per 2.0×10^5 cells in a final volume of 10 μl buffer. The cells were electrically shocked using 10-μl tips on the Neon system at 1,650 V for 10 s and 3 pulses. Then, the electrically treated cell-plasmid mixture was plated in 1 ml antibiotic-free DMEM with 10% fetal bovine serum (FBS) in 12-well plates. After 12 h of incubation in 5% CO_2 at 37°C, the transfection medium was replaced with DMEM supplemented with 10% FBS and 3 μg/ml of puromycin for transfection selection. After 3 days of selection, the transfected cells were maintained in DMEM supplemented with 10% FBS and 1 μg/ml puromycin. The cells were then split into 6-well plates for proliferation or differentiation (DM with 1 μg/ml puromycin) and subjected to further experiments.

i-proteasome chemical inhibition. Confluent (90 to 100%) C2C12 or human skeletal muscle myoblasts were grown in DM supplemented with DMSO or specific i-proteasome inhibitors. The PSMB9-specific inhibitor UK-101 was used at a concentration of 1 μM, and the PSMB8-specific inhibitor LKS01 was used at 0.2 μM (35). The cells were treated with UK-101, LKS01, or the combination of UK101 and LKS01. The treated cells were collected after 2 or 4 days of differentiation induction to be used for further analysis. For time course treatment, confluent (90 to 100%) C2C12 myoblasts in six-well plates were treated for 2, 4, 8, 12, or 24 h with the combination of UK-101 and LKS01 in DM. After time course treatments, the medium containing i-proteasome inhibitors was replaced by new DM without the inhibitors, and the cells were allowed to reach 2 days of differentiation. In other experiments to determine if i-proteasome inhibition is important at the beginning of differentiation or during differ-

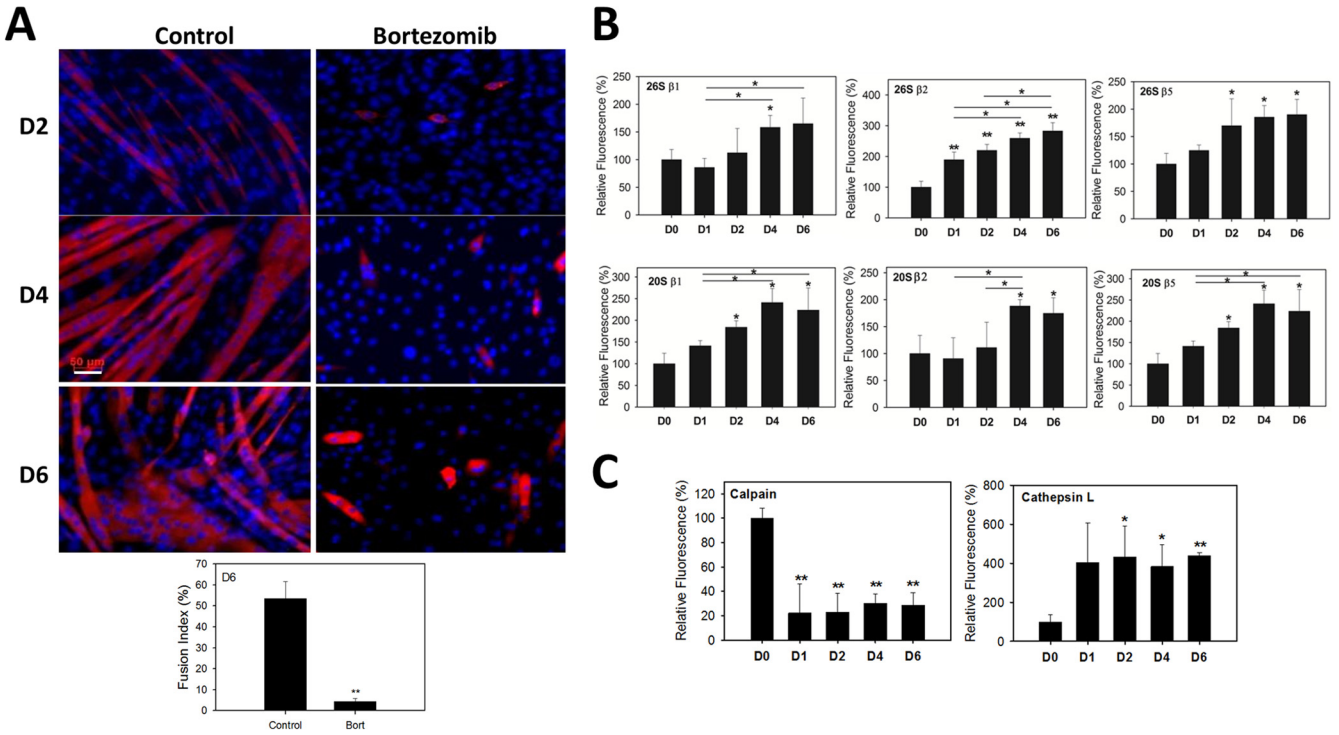

FIG 1 Dynamic changes of intracellular proteolytic activities during myogenic differentiation and the effects of proteasome inhibition on C2C12 cell differentiation. (A) Bortezomib, the specific proteasome inhibitor, inhibits myogenesis. C2C12 myoblasts (90% confluent) were induced to differentiate in the presence or absence of 10 nM bortezomib for 2 days (D2), 4 days (D4), or 6 days (D6). Images represent three independent experiments. Magnification, ×10. Scale bar, 50 μm. The graph shows the fusion index of C2C12 cells grown in differentiation medium for 6 days after the initiation of differentiation. (B) 26S and 20S proteasome activities increase during myogenic differentiation. Proteasome activities were measured in cell lysates prepared from D0 myoblasts, D1, D2, and D4 differentiating cells, and D6 differentiated cells (myotubes) using fluorescently labeled proteasome substrates. The caspase-like (Z-LLE-AMC, β1), trypsin-like (Boc-LSTR-AMC, β2), and chymotrypsin-like (Suc-LLVY-AMC, β2) proteasome activities were assayed using 25 μg of protein with or without specific proteasome inhibitors. 26S and 20S proteasome activities were determined in the presence of ATP and SDS, respectively. The released free AMC fluorescence was quantified using a 390-nm filter on a Fluoroskan Ascent fluorometer. (C) Calpain activity remains lowered and cathepsin L activity increases during myogenic differentiation. Calpain and cathepsin L activities were measured in D0 to D6 cell lysates using fluorescently labeled substrates. Each value in panels B and C is the mean of at least three measurements ($n = 3$); error bars denote standard deviations (SD) (*, $P < 0.05$; **, $P < 0.01$).

entiation or both, C2C12 cells were allowed to differentiate for 2 days, and then i-proteasome inhibition was carried out for 2 days.

Detection of oxidized proteins. Oxidized carbonyl groups on proteins were detected using the Oxyblot procedure provided by the OxiSelect protein carbonyl immunoblot kit (Cell Biolabs, Inc.). The Oxyblot procedure allows detection of carbonyl derivatives of Pro, Arg, and Lys, which were introduced by oxidative reactions with oxides of nitrogen or ozone or by metal catalyzed oxidation. After gel electrophoresis and electrotransfer to a PVDF membrane, the carbonyl groups on the protein side chains were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) using 10 mM 2,4-dinitrophenylhydrazine (DNPH; Sigma) in 2 M HCl. Then, a general Western blot protocol was followed. The DNP-derivatized proteins were detected by incubation with rabbit anti-DNP (1:200) and then anti-rabbit antibody–HRP (1:10,000; Sigma), both in 3% nonfat milk–TTBS, and subsequent chemiluminescence detection of HRP signal.

Apoptosis induction. C2C12 myoblasts were split into 24-well plates. Confluent (90 to 100%) myoblasts were differentiated in DM supplemented with DMSO or 20 μM apoptosis inhibitor II {1-[(3,4-dichlorophenyl)methyl]-1*H*-indole-2,3-dione; Enzo Life Sciences}. The final concentration of DMSO in the medium was 0.1% (vol/vol). The cells were observed and imaged under microscopy 48 h after treatment.

Measurement of intracellular ROS production. Reactive oxygen species (ROS) were measured at 488 nm with a FACSCalibur flow cytometer (BD Bioscience) using the cell-permeative compound 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) (Molecular Probes).

Statistics. Results are expressed as means ± standard deviations (SD) from at least three independent experiments. The comparisons were performed using the Student *t* test or one-way analysis of variance (ANOVA). *P* values of <0.05 were defined as statistically significant.

RESULTS

Proteasome activities increase during C2C12 cell differentiation. It has been shown that the differentiation of rat L6 myoblasts cells can be inhibited by the nonspecific proteasome inhibitors MG132 and PSI (26). However, little is known about the proteasome activities during muscle differentiation. To explore the role of the proteasomes in C2C12 cell differentiation, we used bortezomib, a specific proteasome inhibitor, to pharmacologically inhibit the proteasome activity at the time of myogenic induction. The control cells (no inhibitor) fully differentiated into myotubes after 6 days of growth in differentiation medium (DM). However, the differentiation of C2C12 cells was inhibited by 10 nM bortezomib (Fig. 1A). The caspase-, trypsin-, and chymotrypsin-like catalytic activities of 26S and 20S proteasomes were assayed by using selective cleavage of fluorescent substrates targeted by each proteolytic class. The dynamic changes of proteasome activities in C2C12 cells were measured in lysates of myoblasts (D0) and cells differentiated for 1 day (D1), D2, D4, and D6. All catalytic activities of 20S and 26S proteasomes were gradually increased

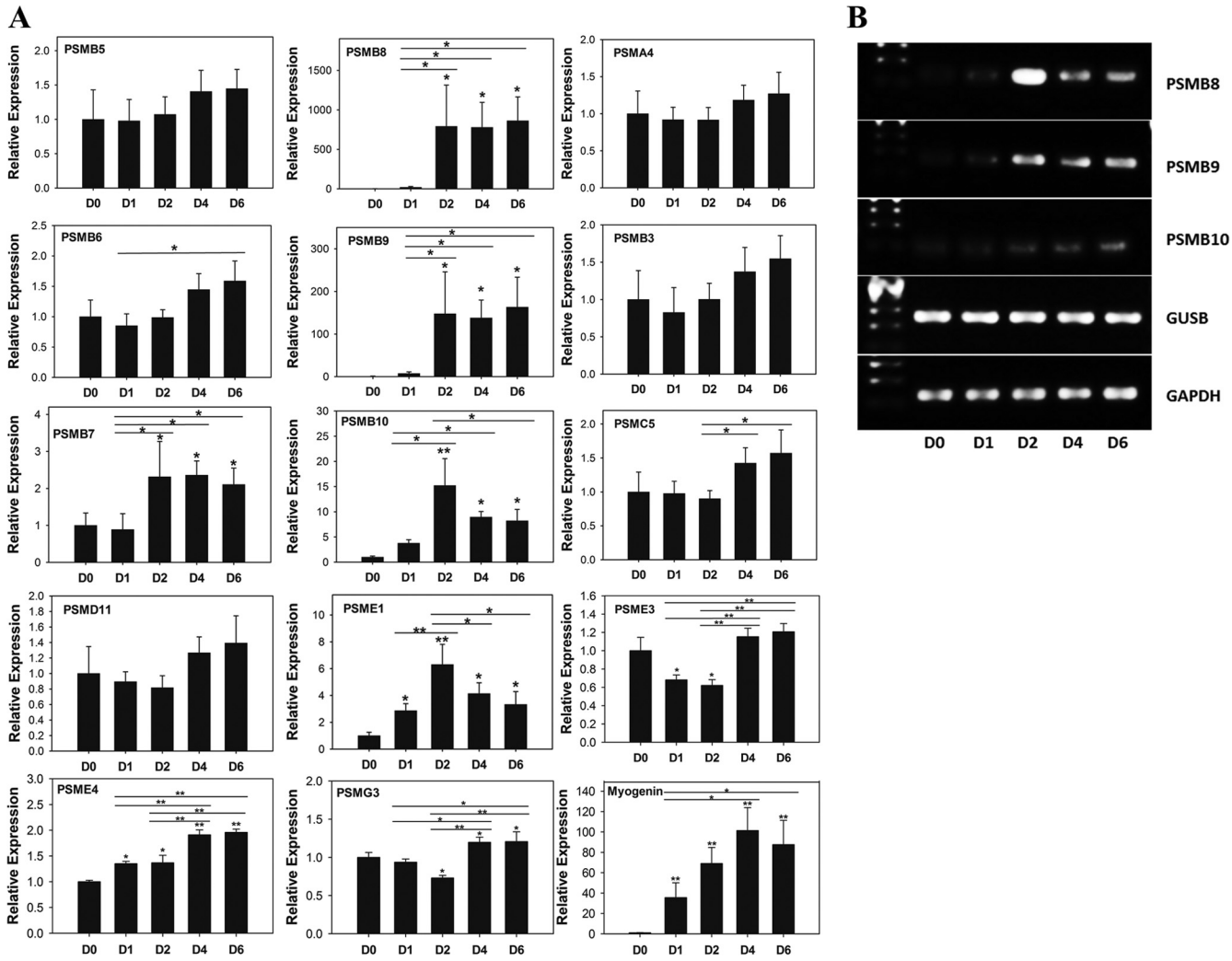

FIG 2 Proteasome subunit gene expression changes during C2C12 cell differentiation. (A) qRT-PCR was utilized to determine the gene expression of constitutive and inducible proteasome subunits as well as proteasome activators in D0 myoblasts, D1, D2, and D4 differentiating cells and D6 differentiated cells (myotubes). The inducible subunits PSMB8, PSMB9, and PSMB10 showed the largest increases in gene expression during C2C12 cell differentiation. Values are means and SD ($n = 3$) (*, $P < 0.05$; **, $P < 0.01$). (B) Agarose gel showing the fragments of RT-PCR products for the inducible subunits and two normalization controls (GUSB and GAPDH).

throughout the differentiation process; in D6 myotubes, all proteasome activities were upregulated by 50 to 200% (Fig. 1B). In particular, the 26S trypsin-like activity increased just after 24 h of differentiation induction, while most other catalytic activities increased after 48 h of induction.

Calpain activity and cathepsin L activity. In this study, we measured the activity of calpains and cathepsin L in C2C12 myoblasts and myotubes to determine how they were changed during myogenic differentiation. Compared to D0 myoblasts, calpain activity was lowered by 60 to 80% and cathepsin L activity was upregulated by 400% throughout the 6 days of differentiation (D1 to D6) (Fig. 1C).

Differential expression of proteasome subunits. To determine whether the increase in proteasome activity was due to an increase in the relative amount of proteasomes, the expression of specific subunits of the 19S, 20S, and 11S particles was investigated at both the mRNA (Fig. 2) and protein (Fig. 3) levels. First, the mRNA expression of the catalytic subunits of the constitutive 26S proteasome and the inducible subunits was measured by qPCR. In this study, the mRNA expression of myogenin (skeletal muscle differentiation marker) increased 30-fold by D1 and further increased 100-fold by D4 (Fig. 2A). There were significant increases in the mRNA expression of several proteasome subunits, including the PSMB7 catalytic subunit by D2. By D6, the mRNA level of PSMB7 was upregulated by a factor of 2. There were no significant increases in the mRNA expression of PSMA4, PSMB3, PSMB5, PSMB6, PSMC5 (19S regulator ATPase subunit Rpt 6), or PSMD11 (19S regulator non-ATPase subunit Rpn5) during differentiation relative to myoblasts (Fig. 2).

In contrast, the mRNA levels of all three catalytic subunits of the i-proteasome were increased dramatically by D2. PSMB9 showed a dramatic upregulation of mRNA expression (150-fold), so C2C12 cells with PSMB9 knockdown were used in subsequent experiments to further investigate the role of i-proteasomes in myogenesis. PSMB8 and PSMB10 mRNA levels were increased 700-fold and 15-fold, respectively. The agarose gels of some RT-

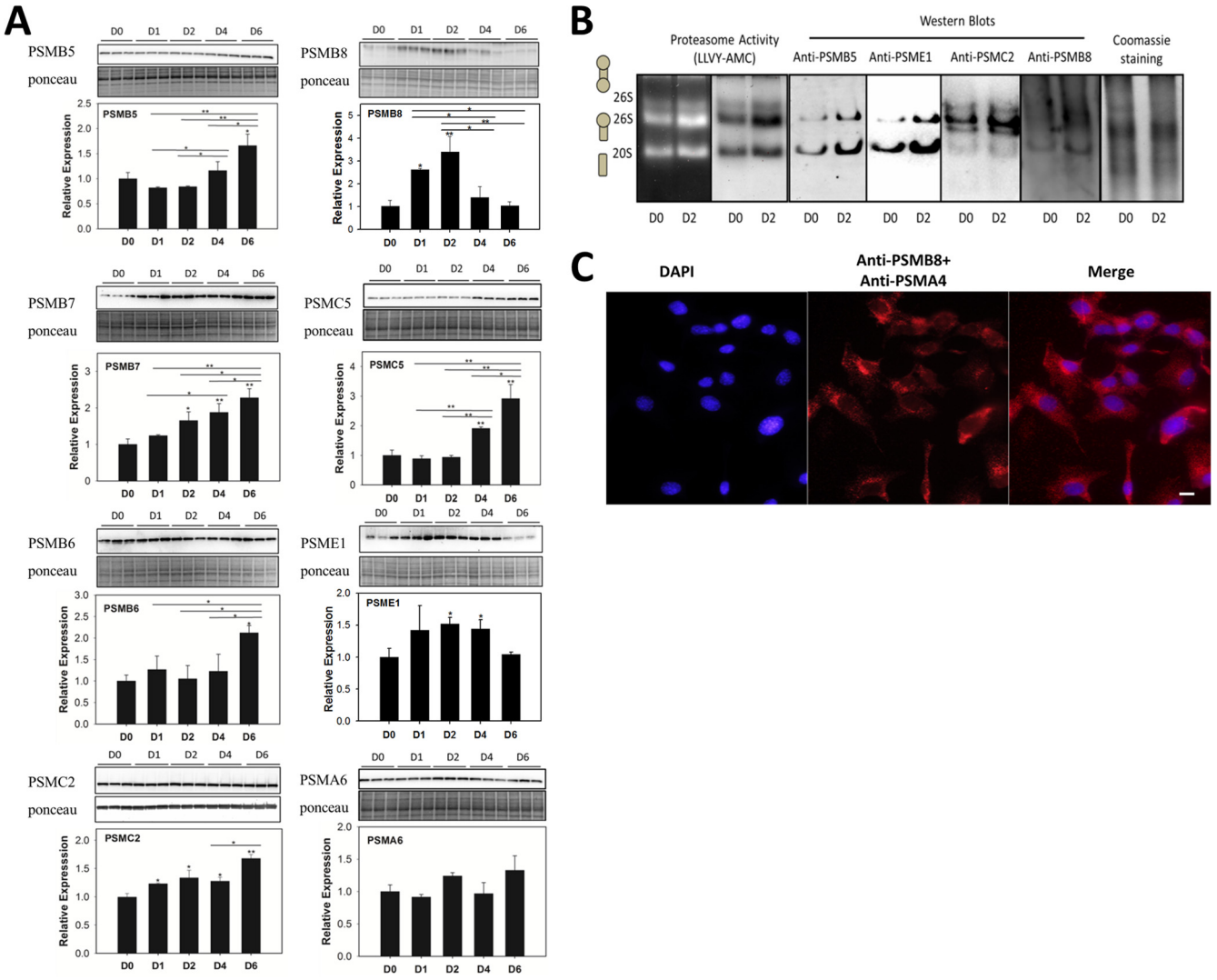

FIG 3 Localization and dynamic changes of proteasome subunit expression during C2C12 cell differentiation. (A) Protein expression of the constitutive catalytic proteasome subunits PSMB5, PSMB6, and PSMB7, the i-proteasome subunit PSME1, the inducible subunit PSMB8, the 19S subunits PSMC2 and PSMC5, and the 20S subunit PSMA6. Each lane represents a different sample, and three different samples for each experimental condition are included to show the variation between samples at the same time points. Relative expression values are means ± SD (*, $P < 0.05$; **, $P < 0.01$). (B) Native gel electrophoresis of D0 and D2 cell lysates. Each lane contains 200 μg of lysate protein which was run on 2 to 5% acrylamide gels and incubated with 50 μM Suc-LLVY-AMC to detect proteasome activity. The clear region on the gel (first image on the left) corresponds to cleaved LLVY-AMC, indicating proteasome activity. The second image on the left is an inverted image of in-gel proteasome activity showing the upregulation of proteasome activity in D2 lysates compared to D0 lysates. After detecting proteasome activity, the gels were immunoblotted with different antibodies to proteasome subunits or stained for total protein with Coomassie blue R-250. (C) The cultured cells (D0) in the chamber slide were immunostained by the rabbit-derived antibody to the inducible subunit PSMB8 and the mouse-derived antibody to the 20S α-type subunit PSMA4. The slide was subsequently incubated with two PLA probes (Duolink II anti-mouse Minus and Duolink II anti-rabbit Plus) followed by the DNA amplification procedure. The panels show cells with DAPI-stained nuclei, immunostained cells, and a merged image. The images were taken using an Zeiss LSM 510 META confocal microscope with 40×/63 objectives. Scale bar, 20 μm.

PCR products showed significant increases in the inducible subunits by D2 (Fig. 2B).

The mRNA expression of PSME1 (11S subunit α) was also upregulated after just 1 day of differentiation with maximum expression after 2 days of myogenic induction (Fig. 2A). Previously, the upregulation of PSME1 has sometimes been shown to occur together with an increase in the inducible proteasome subunits (6, 36). On the other hand, PSME3 and PSME4 (components of proteasome activator complexes) showed different expression patterns. The mRNA expression of PSME4 increased gradually, reaching 2-fold upregulation by D6. But PSME3, the nucleus-localized subunit, decreased after differentiation induction and then returned to the expression level comparable to that of myoblasts by D4. These major activators and regulators of the proteasome exhibited dynamic mRNA expression throughout the differentiation process and should be investigated in future studies regarding their individual roles in myogenesis. In addition, because the amount of proteasomes is determined by not only the expression level of every subunit but also the assembly of the proteasomes, we determined the mRNA expression of the proteasome assembly regulator PSMG3, which was found to be unregulated at D4 and D6.

Furthermore, the total protein expression of proteasome subunits was examined via Western blotting for PSMB5, PSMB6, PSMB7, PSMB8, PSMB9, PSMA6, PSMC2, PSMC5, and PSME1 (Fig. 3A). The protein expression for PSMB8 and PSME1 showed a bell-shaped curve with the maximal level at D2, which was consistent with their respective mRNA expression patterns. PSMB9 showed increased protein expression with maximum expression at D6 (data not shown). Although mRNA expression levels for PSMB5 and PSMB6 were not increased, their corresponding protein expression levels were increased by D6. Moreover, PSMB7 protein expression was increased by D2, which was reflective of the increase in mRNA expression. The ATP-dependent 19S regulator subunits PSMC2 and PSMC5 both showed a gradual increase in protein expression from D0 to D6 (Fig. 3A). Because native gel electrophoresis allows the detection of native proteasome amount and activity in cells, chymotrypsin-like proteasome activity and Western blotting of PSMB5, PSMB8, PSMC2, and PSME1 were performed after separating cell lysates on the native gel. The native gel showed that the 20S and 26S proteasome activities in C2C12 cells increased after 2 days of differentiation in comparison to those in myoblasts (Fig. 3B). The expression levels of these subunits were also clearly increased by D2 compared to D0 myoblasts (Fig. 3B). The amount of PSMB5, PSMB8, and PSME1 was increased in both the 20S and 26S complexes by D2 relative to D0. The upregulation ratio of PSMB8 was drastically higher than that of PSMB5 during C2C12 cell differentiation. The top three bands in the PSMC2 (19S subunit) immunoblot represent doubly capped 26S, singly capped 26S, and free 19S, in order of molecular weight. The free 19S had no proteolytic activity and was not observed in the gel activity determination.

i-proteasomes are mainly localized in the cytosol of C2C12 cells. The localization of i-proteasomes in C2C12 cells was determined by rabbit anti-inducible PSMB8 (β5i) and mouse anti-20S α-type subunit PSMA4 (α3) by using an *in situ* proximity ligation assay (PLA). After the primary and secondary antibody reactions were completed, the images were visualized with a fluorescence microscope. The images revealed that the i-proteasomes were localized mainly in the cytosol of C2C12 myoblasts (Fig. 3C). The i-proteasomes remained predominantly in the cytosol after differentiation (data not shown). In addition, the PSME1 and PSMA4 containing proteasomes were also found to be mainly localized in the cytosol of C2C12 cells (data not shown).

Suppression of PSMB9 expression by shRNA causes myogenic defects. Due to the striking increase in the mRNA expression of the inducible i-proteasome subunits, PSMB9 mRNA expression was knocked down by shRNA to determine the importance of the i-proteasome in C2C12 myogenesis. After shRNA transfection, C2C12 cells were treated with 3 μg/ml puromycin. Three days later, untransfected cells died due to lack of puromycin resistance, and the remaining cells were maintained in 1 μg/ml puromycin. The confluent shRNA-transfected cells were induced to differentiate in 6-well plates. C2C12 cells transfected with negative control (N) or clone 4 (C4) differentiated normally in DM. However, myotube formation of C2C12 cells transfected with clones 2 and 3 (C2 and C3) was significantly impaired (Fig. 4A). Immunohistochemistry of myosin heavy chain (MyHC), a skeletal muscle differentiation marker, at D2 and D4 in control and C3-transfected myoblasts was utilized to determine the infusion index for differentiation (Fig. 4A). C3-transfected myoblasts showed significantly lower in-fusion indexes than the control plasmid-transfected myoblasts. Western blots showed that the protein expression of MyHC and myogenin (another skeletal muscle differentiation marker) was significantly less in cells with C2 and C3 than in the negative control (N) (Fig. 4B). MyoD expression was also significantly lower in C3 than in N (Fig. 4C).

To evaluate the effects of shRNA on the target gene expression, PSMB5 to -10 were measured by qRT-PCR (Fig. 4D). PSMB9 expression was found to be decreased by about 65 to 75% at the gene level in the cells transfected with C2 or C3 compared to N. PSMB8 expression was also decreased when PSMB9 was knocked down. However, PSMB5, -6, and -7 were not affected at the gene level (Fig. 4D) in the cells with C2 or C3 compared to control cells. In addition, the 26S proteasome activities in D0 myoblasts and D2 differentiating cells were measured after PSMB9 suppression by shRNAs. Caspase- and chymotrypsin-like activities of the 26S proteasome were decreased in D0 myoblasts transfected with C3 or C2 shRNA, and caspase- and trypsin-like activities were further decreased by D2 (Fig. 4E and F). To determine if the proteasome activity decrease was due to the decreased protein expression of the constitutive proteolytic proteasome subunits, Western blotting was performed for PSMB5, PSMB6, and PSMB7 subunits. The protein expression of the constitutive proteolytic proteasome subunits was not affected by any of the shRNA clones (Fig. 4G).

Reduction in i-proteasome activity by specific i-proteasome inhibitors causes myogenic defects. Two of the best established i-proteasome inhibitors, UK-101 (PSMB9 inhibitor) and LKS01 (PSMB8 inhibitor), were used individually or in combination to investigate the involvement of i-proteasomes in C2C12 cell differentiation (Fig. 5A and B). Both UK-101 and LKS01 reduced myotube formation, but UK-101 exhibited a greater degree of myogenic inhibition. The combination of both inhibitors prevented the upregulation of myogenin and significantly reduced the expression of MyHC after 2 days, 4 days and 6 days of differentiation (Fig. 5B). The use of both inhibitors also reduced MyHC expression in a time-dependent manner; longer exposure to the inhibitors caused greater inhibition of MyHC throughout 24 h of differentiation induction (Fig. 5C). C2C12 myoblasts which were allowed to differentiate for 2 days and then allowed to differentiate for a further 2 days with i-proteasome inhibitors showed impaired differentiation (Fig. 5D and E). Western blots of differentiating myoblasts exposed to i-proteasome inhibitors for 2 days showed that i-proteasome inhibition reduced MyHC and myogenin, demonstrating that the i-proteasome is not just important at the start of differentiation but during differentiation as well. To determine if i-proteasome inhibition also impaired differentiation in primary skeletal muscle cells, human skeletal muscle myoblasts were treated to induce differentiation in the presence or absence of bortezomib or i-proteasome inhibitors, UK-101 and LKS01, for 4 days. Human skeletal muscle myoblasts showed a significantly lower fusion index ($P < 0.001$) when exposed to i-proteasome inhibitors and compared to control (Fig. 5F). Human skeletal muscle myoblasts also showed significant impairment in differentiation with the proteasome inhibitor bortezomib. These results demonstrate that the impairment of skeletal muscle differentiation by i-proteasome inhibition also occurs in human skeletal muscle.

Reduction in i-proteasome activity increases the levels of oxidized proteins. It is known that ROS are increased during differentiation, which would be expected to increase the levels of oxidized proteins in differentiating cells (37). However, protein

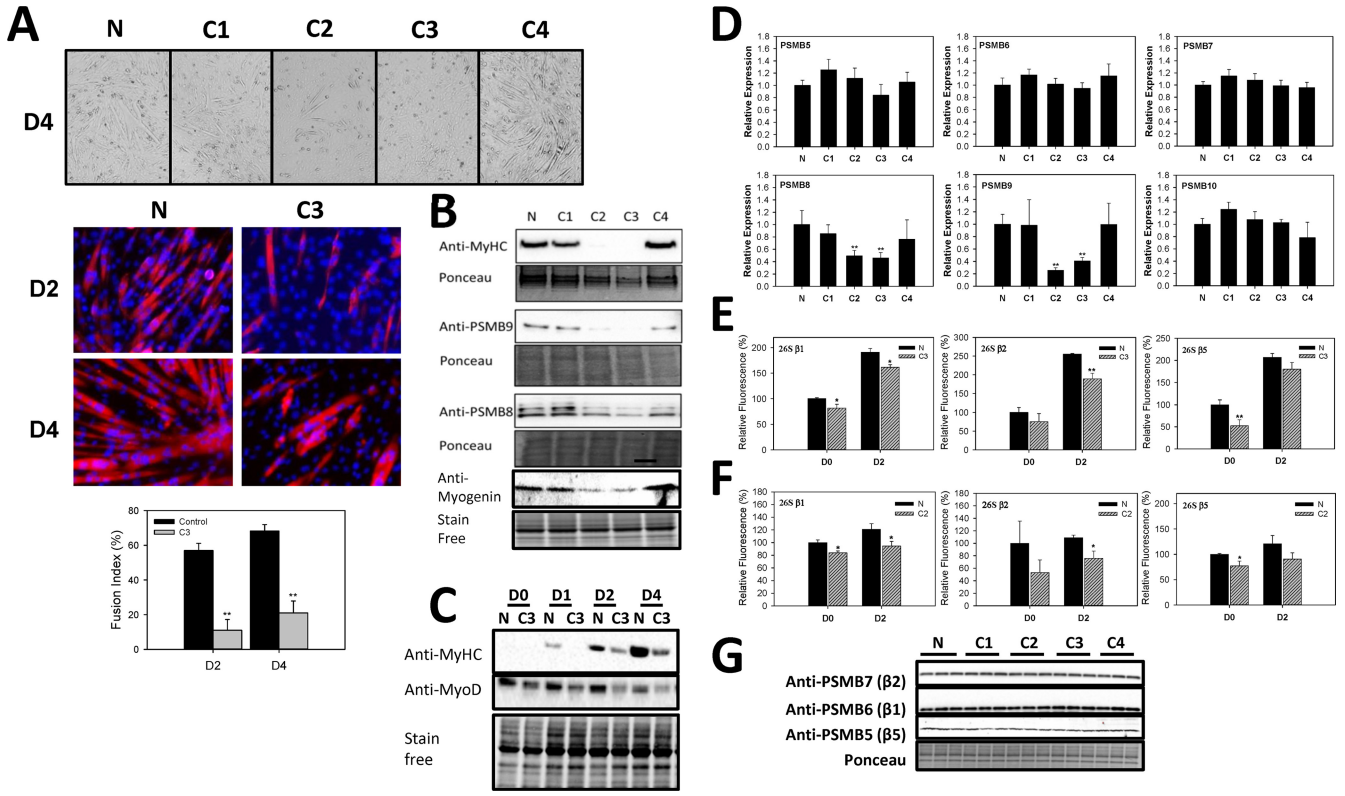

FIG 4 Effects of PSMB9 suppression on C2C12 cell myogenic differentiation. (A) (Top) C2C12 cells were transfected with a negative control and four different shRNAs to PSMB9 and treated to induce differentiation after puromycin selection. The images were taken at 4 days after the initiation of differentiation. (Bottom) Immunofluorescence analysis of skeletal myosin heavy chain (MyHC) in C2C12 cells grown in differentiation medium for 2 or 4 days in the presence or in the absence of C3 shRNA. The graph shows the fusion indexes of C2C12 cells grown in differentiation medium for 2 days and 4 days after the initiation of differentiation. (B) Expression of MyHC, PSMB9, PSMB8, and myogenin at D2 in C2C12 cells containing the control shRNA (N) or the four PSMB9 shRNAs (C1, C2, C3, and C4). C2 and C3 shRNAs showed the best suppression of PSMB9, PSMB8, and MyHC. (C) Effect of C3 shRNA on MyHC and MyoD protein expression at D0, D1, D2, and D4. (D) qRT-PCR of constitutive and inducible proteolytic proteasome subunits in D2 differentiated cells. Only the RNA expression of the inducible subunits PSMB8 and PSMB9 was suppressed. Values are means and SD ($n = 3$) (**, $P < 0.01$). (E) Proteolytic activities (caspase [βi], trypsin [β2], and chymotrypsin [β5] like) of the proteasomes in C2C12 cell lysates from cells containing different shRNAs at D0 and D2. Values are means and SD ($n = 3$) (*, $P < 0.05$; **, $P < 0.01$). (F) Proteasome activity of PSMB9 knockdown cells with C2. (G) Protein expression of constitutive proteolytic proteasome subunits in PSMB9 knockdown cells with different shRNA clones after 2 days of differentiation. The blot shows results for three replicates for each clone.

oxidation has not been examined previously in differentiating skeletal muscle cells. Detection of oxidized proteins showed that oxidized protein levels are increased during C2C12 cell differentiation, with maximal levels being observed after 2 days (D2) of myogenic induction (Fig. 6A). C3 cells showed higher levels of oxidized proteins than control (N) cells in both myoblasts and differentiating cell, suggesting that i-proteasome suppression may increase oxidized protein levels (Fig. 6B and F). However, it was possible that ROS would be higher in C3 cells, which would also increase the levels of oxidized proteins. While a small increase in ROS was detected in C3 myoblasts relative to N myoblasts, the increase was not statistically significant (Fig. 6C). After 12 h of differentiation, no difference in ROS levels was observed between C3 and N cells. Therefore, it is not likely that ROS was responsible for the increase in oxidized proteins observed with i-proteasome suppression. Chemical inhibition of the i-proteasome resulted in the accumulation of oxidized proteins (Fig. 6D). The degradation of C2C12 cytosolic lysates from myoblasts and 2-day-old differentiating cells were measured by incubating cytosolic lysates (which were centrifuged to remove the endogenous proteasomes) with purified 20S immunoproteasome. Fluorescamine forms a fluorescent adduct with the N termini of peptides generated by proteasomal cleavage and is readily measured using a fluorometer. The immunoproteasome showed very little activity in cytosolic fractions from myoblasts but significantly more activity in cytosolic fractions from two-day-old differentiating cells (Fig. 6E). These results demonstrate that the two-day-old differentiating cells contain more immunoproteasome substrates than the myoblasts. Homogenates from C2C12 cells that had differentiated for 2 days were incubated with constitutive proteasome (c20S), i-proteasome (i20S), or buffer (control) for 3 h at 37°C and then boiled with sample buffer. The amount of oxidized proteins in these samples suggests that i-proteasomes degrade oxidized proteins in D2 myoblast homogenates to a significantly greater degree than constitutive proteasomes (Fig. 6F). Interestingly, although the amount of protein in sample was the same, samples incubated with c20S and i20S showed decreased total protein staining, suggesting that some of the proteins were being degraded and possibly ran at the dye front.

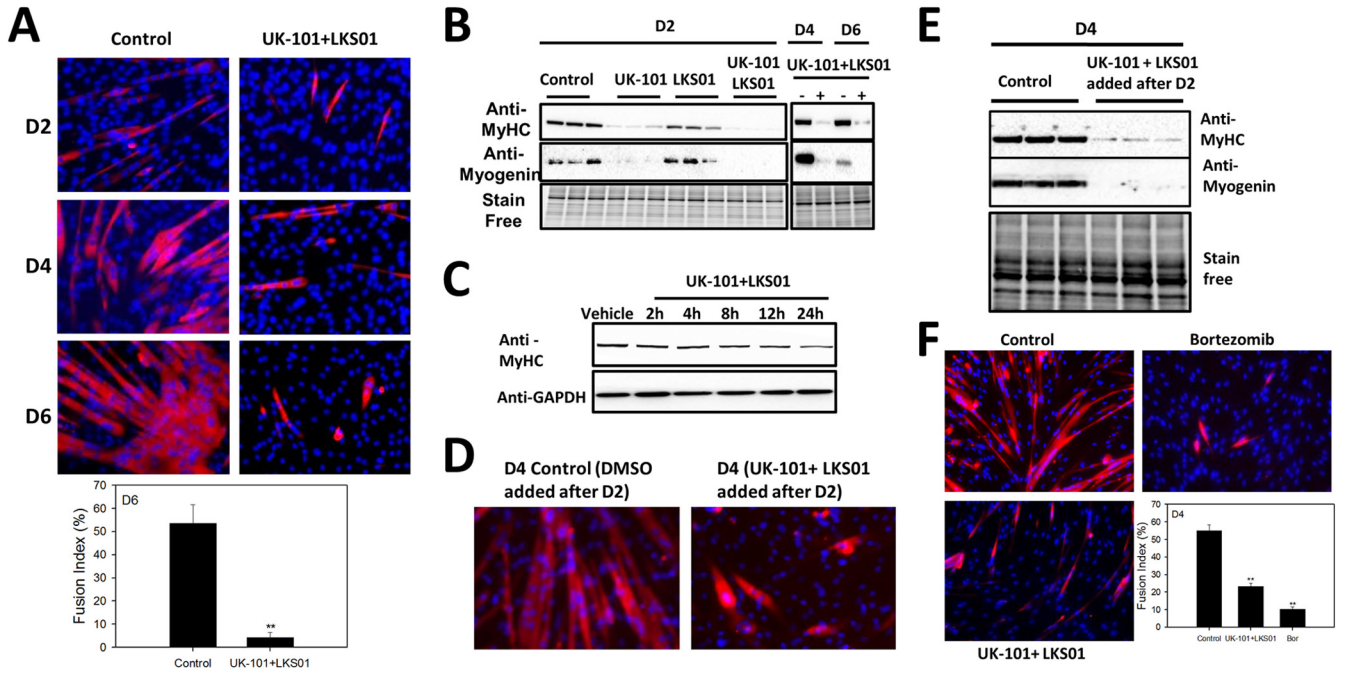

FIG 5 Effects of inducible subunit inhibitors on C2C12 cell and human skeletal muscle myogenic differentiation. (A) C2C12 myoblasts (90% confluent) were treated to induce differentiation in the presence or absence of i-proteasome inhibitors, UK-101 and LKS01, for up to 6 days (D6). Images show immunofluorescence of MyHC (red) and DAPI (blue). Each image is representative of three independent experiments. The graph shows the fusion index in the presence and absence of i-proteasome inhibitors at D6. (B) Western blots of MyHC and myogenin levels 2 days (D2), 4 days (D4), and 6 days (D6) after differentiation induction in the presence and absence of i-proteasome inhibitors. (C) Western blots of MyHC and GAPDH after two i-proteasome inhibitors (UK-101 and LKS01) were incubated with C2C12 cells for different lengths of time ranging from 2 h to 24 h. Then the medium with inhibitors was replaced with the medium lacking inhibitors, and the cells were left to differentiate for a total of 48 h. (D) Immunofluorescence images of MyHC (red) and DAPI (blue) in myoblasts differentiated for 2 days, after which DMSO or UK-101 and LKS01 were added and the myoblasts allowed to further differentiate for 2 more days. (E) Western blots of MyHC and myogenin to determine the effects of i-proteasome inhibition on differentiation after 2 days of differentiation has already occurred. (F) Human skeletal muscle myoblasts were treated to induce differentiation in the presence or absence of bortezomib (Bor) or i-proteasome inhibitors, UK-101 and LKS01, for 4 days (D4). Images show immunofluorescence of MyHC (red) and DAPI (blue). The graph shows the fusion index of C2C12 myoblasts grown in differentiation medium for 4 days after the initiation of differentiation in the presence of proteasome (bortezomib) or i-proteasome inhibitors compared to control myoblasts. Each image is representative of three independent experiments.

Upregulation of apoptosis in i-proteasome-suppressed C2C12 myoblasts. Protein expression of proapoptotic proteins in control (N) and PSMB9 knockdown (C3) myoblasts and myotubes after 2 to 6 days of differentiation showed increased levels of Bax at D0, D2, and D4, while Bad was elevated at D4 and D6 (Fig. 7A). The active enzymes involved in apoptosis (cleaved caspase 9 and cleaved caspase 3) were both increased at D0, D2, and D4 in PSMB9 knockdown cells. The well-established apoptotic cleavage product, cleaved poly(ADP-ribose) polymerase (PARP), was also elevated at D2 and D4 in the PSMB9 knockdown cells. These results all suggest that apoptosis is significantly increased in the PSMB9 knockdown myoblasts relative to the control myoblasts. Induction of apoptosis using apoptosis activator 2 in differentiating C2C12 myoblasts caused prevented differentiation (Fig. 7B). Apoptosis activator 2 is known to increase procaspase 9 processing and subsequent caspase 3 activation (38). Other key enzymes known to be involved in C21C2 differentiation (glycogen synthase kinase 3β [GSK3β] and p38) were also investigated by immunoblotting. Both GSK3β and p38 were found to be unaffected by immunoproteasome suppression (Fig. 7C). Total protein staining (Ponceau stain) and GAPDH were used as normalization controls (Fig. 7C).

DISCUSSION

The complex process of myogenic differentiation, which begins with cell cycle arrest and ends with the fusion of individual myoblasts into multinucleated myotubes, was previously associated with the UPS (25–27). However, the results of these initial studies were limited and contradictory. The specific proteasome inhibitor bortezomib (10 nM) suppressed the myogenic differentiation of C2C12 cells, consistent with previous studies, suggesting that proteasome inhibition has a very strong relationship with proper myogenic differentiation. Differentiation of human skeletal muscle myoblasts was also found to be suppressed with bortezomib. Initial studies by us showed significantly increased levels of i-proteasome-specific subunits, suggesting a possible involvement of the i-proteasome in muscle differentiation. In the present study, we explored the role of the i-proteasomes in the differentiation of C2C12 cells to understand the unknown relationship between i-proteasome function and myogenic differentiation.

All three proteolytic activities of the proteasomes were measured at five time points, at day 0 (D0; myoblasts), day 1 (D1), day 2 (D2), day 4 (D4), and day 6 (D6; fully differentiated myotubes). The caspase-, trypsin-, and chymotrypsin-like proteasome activities of 20S and 26S proteasomes increased during the differentiation process. This suggests that the proteasome activity has a pos-

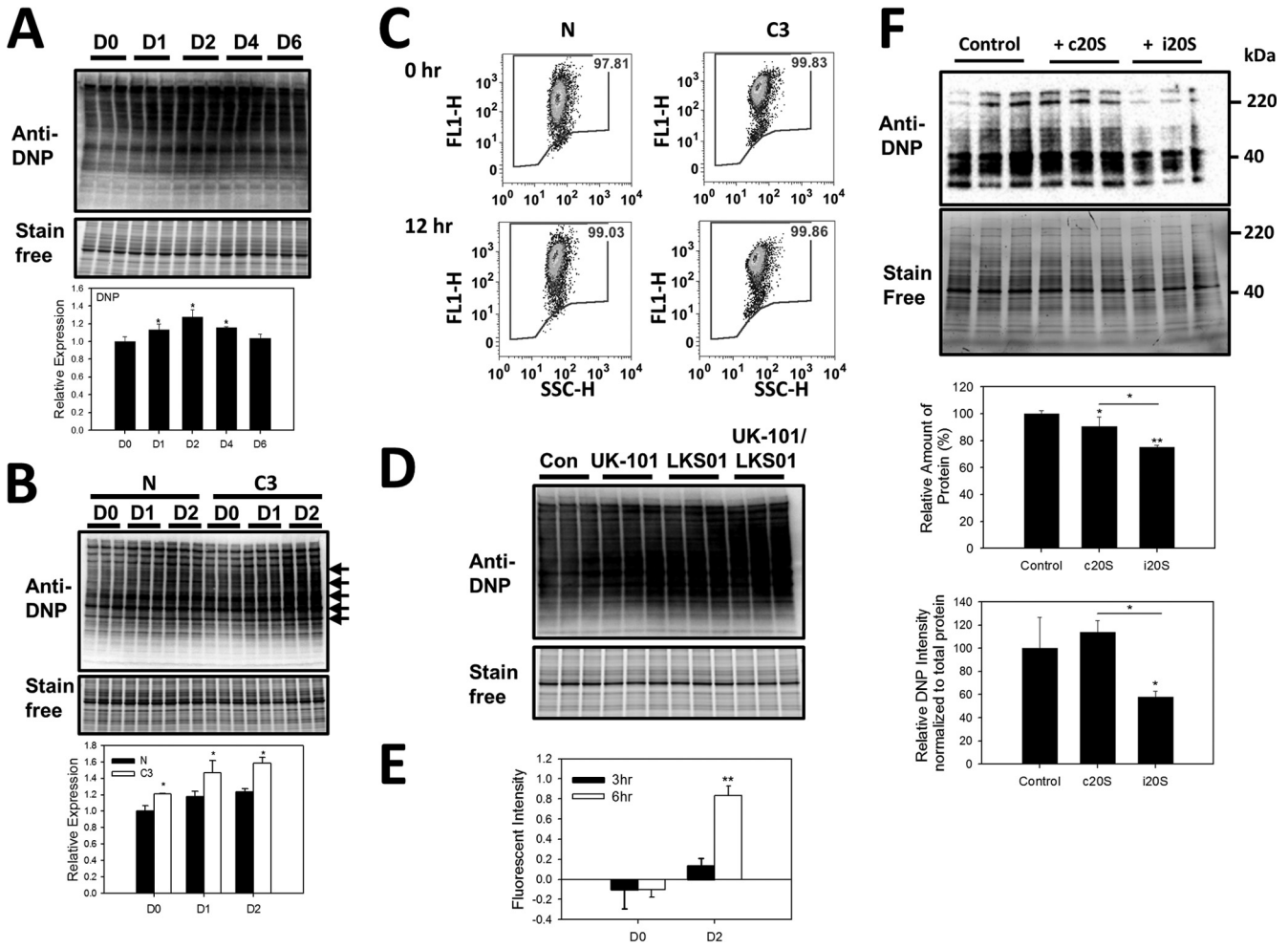

FIG 6 Effects of i-proteasome suppression on oxidized proteins. (A) Oxyblot of C212 cell lysates from D0 myoblasts, D1, D2, and D4 differentiating cells and D6 differentiated cells (myotubes). Carbonyl groups on proteins were first conjugated to DNP; the DNP-bound proteins were then detected using an anti-DNP antibody. Oxidized protein levels increase to a maximum at D2 during C2C12 cell differentiation. (B) Oxyblot of C212 cell lysates from D0 myoblasts and D1 and D2 differentiating C3 and control cells. With C3 shRNA knockdown of PSMB9, protein oxidation further increased in myoblasts and differentiating cells up to D2. Arrows indicate bands which increased in intensity in C3 cells relative to N cells. (C) ROS level in C3 and control cells as measured by flow cytometry. Samples were either myoblasts (D0) or 12-h differentiating C3 and control (N) cells which were harvested, and ROS were measured with the fluorescence probe 2′,7′-dichlorodihydrofluorescein (DCFH). The image is representative of three different experiments. SSC-H, side-scattered light; FL1-H, fluorescent light. (D) Oxyblot of C212 cell lysates from D2 differentiating cells treated with different i-proteasome inhibitors. Carbonyl groups on proteins were first conjugated to DNP; the DNP-bound proteins were then detected using an anti-DNP antibody. The PSMB9-specific inhibitor UK-101 was used at a concentration of 1 μM, and the PSMB8-specific inhibitor LKS01 was used at 0.2 μM. (E) Cytosolic fractions lacking proteasomes from D0 and D2 C2C12 cells were incubated with exogenous 20S i-proteasomes at 37°C for 3 to 6 h. Increases in cleaved proteins were determined by the fluorescent compound fluorescamine, which forms a fluorescent adduct with the N termini of peptides generated by proteasomal cleavage. Results shown are fluorescamine adducts in the cytosolic fractions in the presence of i-proteasomes subtracted from fluorescamine adducts in the absence of exogenous i-proteasomes incubated under the same conditions ($n = 4$). Higher levels of fluorescent adducts represent greater levels of protein degradation. **, $P < 0.001$. (F) C2C12 cell D2 homogenates were incubated with constitutive proteasome (c20S), i-proteasome (i20S), or buffer (control) for 3 h at 37°C and then boiled with sample buffer. Western blotting was subsequently carried out to detect oxidized proteins (5 μg protein/lane). Each blot shows results for three replicates for each condition. Although the amount of protein in each lane was 5 μg, samples incubated with c20S and i20S showed decreased total protein staining, suggesting that some of the proteins were being degraded and possibly ran at the dye front (top graph). Quantification of the results showed that i-proteasomes significantly degraded oxidized proteins in D2 myoblasts homogenates relative to constitutive proteasomes (lower graph). $n = 3$; *, $P < 0.05$; ** $P < 0.001$.

itive correlation with myogenic differentiation. Ebisui et al. showed that the 26S proteasome activity decreased and the 20S proteasome activity increased significantly after myotube formation (28). However, the experiments of those authors are difficult to reproduce, since they partially purified the proteasomes with a linear glycerol gradient and also measured the 20S and 26S chymotrypsin-like proteasome activities without using specific proteasome inhibitors. In our study, we did not fractionate the samples, and each type of proteasome activity was obtained by comparing the proteasome activity in the presence and absence of a specific proteasome inhibitor. We also utilized native gel electrophoresis of C2C12 cell lysates and observed increases in both 20S and 26S chymotrypsin-like activities. Both our independent methods show that both 20S and 26S proteasome activities were increased during C2C12 cell differentiation.

Calpains are calcium-dependent cytosolic cysteine proteases

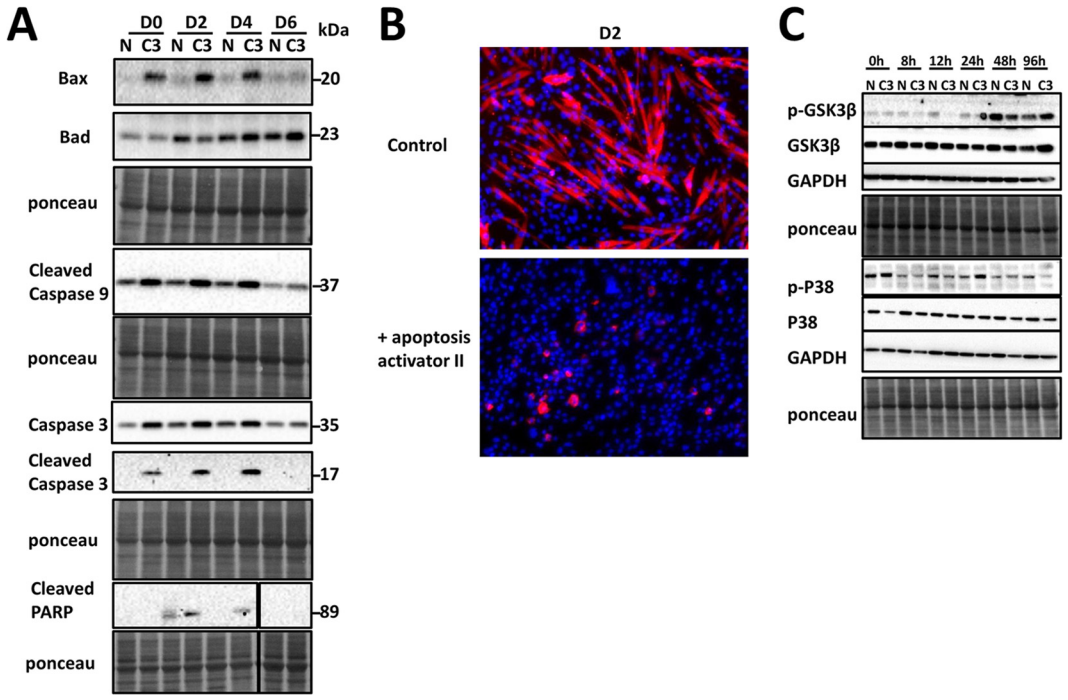

FIG 7 Upregulation of apoptosis in i-proteasome-suppressed C2C12 myoblasts. (A) Protein expression of proapoptotic proteins in control (N) and PSMB9 knockdown (C3) myoblasts and myotubes after 0 to 6 days of differentiation. (B) Immunofluorescence analysis of MyHC (red) and DAPI (blue) in C2C12 cells grown in differentiation medium in the presence or in the absence of 20 μM apoptosis activator II for 2 days. (C) Western blots of C2C12 cells for enzymes known to be involved in C2C12 cell differentiation (GSK3β and p38). Total protein staining (Ponceau stain) and GAPDH were used as normalization controls.

known to be involved in selective cellular functions to maintain homeostasis in skeletal muscle cells (39, 40). The upregulation of calpain activity is associated with muscular disorders, such as contractile dysfunction in cardiac muscle cells, and inhibiting calpain can ameliorate some aggravating effects (41, 42). Calpains have been suggested to play a significant role in myogenesis. Ueda et al. demonstrated that the mRNA level of m-calpain increased 2-fold during the first 24 h of inducing rat skeletal myoblast differentiation and then decreased to the normal level (normalized to the 0-h calpain mRNA expression level) within the following 72 h (21). We found that the calpain activity levels were significantly lower after the first 24 h (D0) of inducing differentiation. Our assay measured both calpain I and calpain II activities, and it is possible that these calpains have different expression and proteolytic activities during skeletal muscle differentiation. However, the suppressed calpain activity after D0 suggests that lower levels of calpain activity may be important for differentiating C2C12 cells.

Cathepsin L is one of the major lysosomal cysteine proteases that degrades misfolded proteins and produces essential peptide hormones for cellular homeostasis (43). Although cathepsins are abundant in fetal muscle cells, they are poorly expressed in adult skeletal muscle, with a lower protein turnover rate (30). We found that cathepsin L activity was upregulated 4-fold during differentiation (D1 to D6) in comparison to myoblasts (D0) (Fig. 1C). This increase in cathepsin L activity level is likely to complement the increased protein turnover rate demonstrated by the increases in the catalytic activities of 26S and 20S proteasomes. This is the first report that demonstrates the changes in activity levels of calpain and cathepsin L during the skeletal muscle differentiation of mouse C2C12 cells.

Since the proteasome activity was altered so dramatically during the myogenic process, the expression of several proteasome subunits, including 20S, 19S and 11S subunits, was determined at the mRNA and/or protein level. The expression of the 20S α-subunit PSMA4 and the 19S non-ATPase subunits PSMD11 and PSMD13 did not show significant changes at either the mRNA or protein level. Surprisingly, all of the constitutive proteolytic subunits PSMB5, PSMB6, and PSMB7 showed an increase in protein expression, but only PSMB7 showed increased expression at the mRNA level. This suggests that degradation of proteasome subunits may be slower in differentiating cells than in myoblasts. Because the initiation of myogenic differentiation is triggered by starvation, we investigated whether the inducible subunits of the i-proteasome were altered under this condition. Expression of all three inducible subunits of i-proteasomes was increased strikingly at both the mRNA and protein levels. The mRNA expression of PSMB8, PSMB9, and PSMB10 was upregulated 700-, 150-, and 15-fold after 2 days of differentiation in comparison to the myoblasts.

The upregulation of 11S activators PSME1 and PSME2, which can be induced by IFN-γ, has often been linked to the i-proteasomes (6, 9, 36). In this study, the mRNA and protein levels of PSME1 also increased in a manner similar to that of the inducible proteasome subunits, which suggests that the 11S-i20S proteasome complex is upregulated during differentiation. Proteasome activity depends on the assembly and composition of this large complex. To confirm that i-proteasomes were actually increased, native gel electrophoresis and Western blotting (PSMB5, PSMB8, PSMC2, and PSME1) were utilized. Western blots of the intact proteasomes showed higher levels of i20S as well as 19S-i20S-11S

complexes in two-day-old differentiating cells relative to myoblasts. The protein levels of both PSMB5 and PSMB8 were also higher in D2 cells than in D0 cells, showing that there were also increased mature forms of constitutive proteasomes (20S and 26S) after differentiation induction. However, the upregulation ratio of the inducible proteasome subunits was drastically higher than that of the constitutive proteasome subunits during C2C12 cell differentiation.

The binding of PSME1 and PSME2 to the i20S has recently been shown to increase the capacity of i-proteasomes to selectively degrade oxidized proteins (44) and accelerate the generation of a subset of MHC class I-presented antigenic peptides (9, 45, 46). In addition, *in situ* PLA showed that both PSME1 associated proteasomes and i-proteasomes were mainly localized in the cytosol of C2C12 cells. Therefore, it is likely that the newly synthesized 11S protein interacts with the i20S to promote myogenic differentiation. To confirm the contribution of i-proteasomes in the myogenic differentiation process, one of the inducible subunits, PSMB9, was selected as the target for RNA interference using shRNA. Four commercial shRNA fragments in the pGeneClip plasmid were used in this study. The C3 (clone 3) shRNA worked well in suppressing PSMB9; the average inhibition of PSMB9 mRNA by C3 was 70%. The qPCR results showed that the knockdown of PSMB9 suppressed not only PSMB9 but also PSMB8 expression. PSMB9 knockdown did not affect the expression of PSMB10 or the constitutive proteasome catalytic subunits. The suppression of PSMB8 is likely due to the fact that the PSMB8 and PSMB9 genes are linked to the MHC I gene. A previous report suggests that knockdown of PSMB8 also reduced PSMB9 gene expression (47).

After RNA interference, the number of elongated or fused cells indicative of differentiation was drastically less for C3 samples than the control. The significant suppression of MyHC protein induction in C3 cells provided further evidence that the PSMB9 knockdown prevents differentiation of C2C12 cells. To determine the alteration of proteasome activities after PSMB9 knockdown, we examined the caspase-, trypsin-, and chymotrypsin-like activities from the C3 shRNA-containing cells. Caspase- and chymotrypsin-like activities of the 26S proteasomes decreased in C3-containing D0 and D2 cells compared to the control (N), consistent with the suppression of PSMB9 and PSMB8 gene expression. In most mammalian cells, the three proteolytic activities of the proteasome are mainly due to the constitutive subunits, PSMB5, PSMB6, and PSMB7. The suppression of PSMB9 decreased total caspase- and chymotrypsin-like activity by 10 to 15%, which suggests that at least 10% of the proteolytic activity is due to the i-proteasomes. i-proteasomes have been suggested to be more active than constitutive proteasomes under certain stress stimulations, and the enhanced activity of the i-proteasomes could degrade some proteins more efficiently under stress conditions (48–50). After the induction of myogenic differentiation, the rapid upregulation of the i-proteasomes is likely required to adapt to the drastic environment change for the cells under the shock from nutrient starvation. Therefore, although the i-proteasome activities account for a small proportion of the total proteasome activity, they are important for maintaining intracellular homeostasis during myogenic differentiation.

UK-101 and LKS01 have been shown to selectively target i-proteasome subunits PSMB9 and PSMB8, respectively, without affecting the constitutive proteasome subunits PSMB6 and PSMB5 (35). Differentiation of human skeletal muscle myoblasts were also found to be suppressed with UK-101 and LKS01. The use of these i-proteasome inhibitors confirmed that the i-proteasomes, independent of the constitutive proteasome, were directly involved in C2C12 cell and human skeletal muscle differentiation.

Since ROS has been shown to have controversial roles and to be increased during myogenic differentiation (37, 51), the levels of oxidized proteins at different stages of differentiation were investigated. The levels of oxidized proteins were found to be increased during differentiation, with the highest levels of oxidized proteins seen by D2. C3 cells showed higher levels of oxidized proteins than control cells during the first 2 days of differentiation and even before differentiation was induced. This increase in protein oxidation was not due to an increase in ROS as flow cytometry showed similar ROS levels between C3 and control cells. Therefore, these results strongly suggest that the increase in total oxidized proteins in C3 cells was due to i-proteasome suppression and that a major role of i-proteasomes is to degrade oxidized proteins. Incubation of proteasome-free cytosolic fractions from myoblasts and D2 cells showed that the immunoproteasome had a strong preference for degrading substrates in D2 cells, which contain higher levels of oxidized proteins. Because protein oxidation naturally increases during myogenesis, the upregulation of oxidized protein degradation by the i-proteasomes is needed to maintain relative intracellular protein equilibrium after the initiation of skeletal muscle differentiation by radical nutrient starvation. H_2O_2 increases the levels of oxidized proteins in cells, and at concentrations of 200 μM or higher, H_2O_2 inhibits C2C12 cell differentiation (52–54), suggesting that high levels of oxidized proteins suppress muscle differentiation.

Cells which contain high levels of oxidized proteins typically show increased apoptosis (55, 56). It has recently been shown that some oxidized proteins play an active role in inducing apoptosis (57), suggesting that high levels of oxidized proteins could induce apoptosis. Apoptosis is a well-documented process that involves biochemical events that lead to cell death. Bax is major component of induced cellular apoptosis through mitochondrial stress (58). Upon apoptotic stimulation, Bax increases the mitochondrial membrane permeability, resulting in the release of cytochrome *c*, activation of caspase 9 and initiation of the caspase apoptotic pathway (59). Bad is a proapoptotic member of the Bcl-2 family that acts by displacing Bax from binding to the antiapoptotic proteins Bcl-2 and Bcl-x_L (60). Bax was significantly upregulated in immunoproteasome-suppressed cells during days 0 to 4 of differentiation while Bad was increased during later stages of differentiation. These results together with the increases in cleaved caspase 9, cleaved caspase 3, and apoptotic protein product (cleaved PARP) all suggest an upregulation of the apoptotic pathway when the immunoproteasome is inhibited. To determine if increased apoptosis could prevent C2C12 cell differentiation, C2C12 cells were exposed to a well-established apoptosis inducer (apoptosis inducer II) (38). Induction of apoptosis during differentiation prevented C2C12 cell differentiation, demonstrating that regulating apoptosis is important for skeletal muscle development.

In this study, we show another example of an alternative physiological function of the i-proteasomes apart from antigen processing, that is, being an important regulator of myogenic differentiation. Our results demonstrate that i-proteasomes, independent of constitutive proteasomes, are required for myogenic differentiation in part because of their roles in regulating apopto-

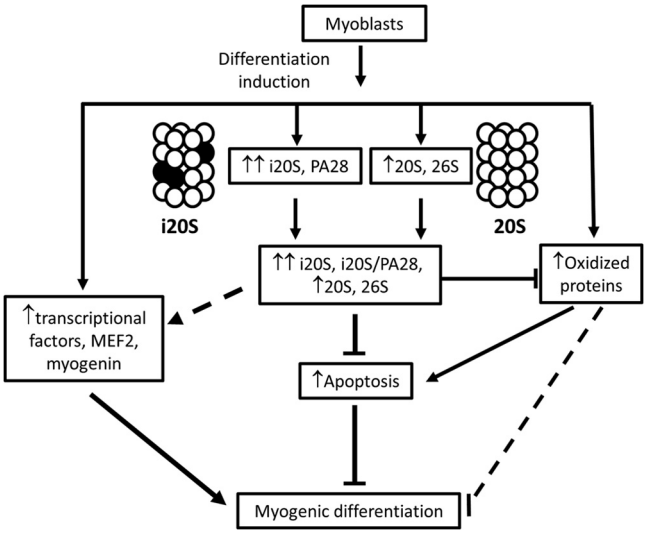

FIG 8 Schematic representation of the role of the i-proteasome in C2C12 skeletal muscle cell differentiation showing a possible role of the i-proteasome in C2C12 skeletal muscle cell differentiation.

sis and reducing oxidized proteins (Fig. 8). When myoblasts undergo differentiation, proteasome activities are upregulated by the increased expression of the proteolytic subunits PSMB6/PSMB9, PSMB7/PSMB10, and PSMB5/PSMB8 and 11S/19S activators. Protein oxidation increases as well, but the upregulated proteasome activities, including the i-proteasome activities, maintain manageable oxidized protein levels and maintain low levels of proapoptotic proteins important for differentiation. Immunoproteasome inhibition increases apoptosis and oxidized proteins, and together, they may directly or indirectly affect key transcriptional factors or protein turnover for myogenic differentiation (Fig. 8). It is currently unknown what factors regulate the transcription and translation of the inducible proteasome subunits and which proteins or pathways are specifically controlled by the i-proteasomes during differentiation. More studies are required to elucidate other possible functions of the i-proteasome involvement in the myogenic process.

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