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N-myristoylated ubiquitin ligase Cbl-b inhibitor prevents on glucocorticoid-induced atrophy in mouse skeletal muscle

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ABSTRACT

A DGpYMP peptide mimetic of tyrosine^{608}-phosphorylated insulin receptor substrate-1 (IRS-1), named Cblin, was previously shown to significantly inhibit Cbl-b-mediated IRS-1 ubiquitination. In the present study, we developed *N*-myristoylated Cblin and investigated whether it was effective in preventing glucocorticoid-induced muscle atrophy. Using HEK293 cells overexpressing Cbl-b, IRS-1 and ubiquitin, we showed that the 50% inhibitory concentrations of Cbl-b-mediated IRS-1 ubiquitination by *N*-myristoylated Cblin and Cblin were 30 and 120 μM, respectively. Regarding the DEX-induced atrophy of C2C12 myotubes, *N*-myristoylated Cblin was more effective than Cblin for inhibiting the DEX-induced decreases in C2C12 myotube diameter and IRS-1 degradation. The inhibitory efficacy of *N*-myristoylated Cblin on IRS-1 ubiquitination in C2C12 myotubes was approximately fourfold larger than that of Cblin. Furthermore, *N*-myristoylation increased the incorporation of Cblin into HEK293 cells approximately 10-folds. Finally, we demonstrated that *N*-myristoylated Cblin prevented the wet weight loss, IRS-1 degradation, and MAFbx/atrogin-1 and MuRF-1 expression in gastrocnemius muscle of DEX-treated mice approximately fourfold more effectively than Cblin. Taken together, these results suggest that *N*-myristoylated Cblin prevents DEX-induced skeletal muscle atrophy *in vitro* and *in vivo*, and that *N*-myristoylated Cblin more effectively prevents muscle atrophy than unmodified Cblin.

Introduction

Under unloading conditions, such as bed rest or microgravity, skeletal muscle is vulnerable to rapid atrophy. This atrophy is induced by both increased protein degradation and decreased protein synthesis [1,2]. Proteolysis is enhanced in muscle atrophy primarily due to activation of the ubiquitin–proteasome pathway [3,4]. Expression of the ubiquitin ligase Casitas b-lineage lymphoma b (Cbl-b[1]) is significantly upregulated under skeletal muscle atrophic conditions [5]. Cbl-b induces muscle atrophy, via its negative regulation of insulin-like growth factor-1 (IGF-1) signaling in skeletal muscle cells through the enhancement of ubiquitination and degradation of insulin receptor substrate 1 (IRS-1) [6,7]. Consequently, the loss of IRS-1 permits the expression of other muscle atrophy-associated ubiquitin ligase (atrogenes), including muscle atrophy F-box protein (MAFbx)/atrogin-1 and muscle RING finger protein-1 (MuRF-1) in a fork head box O (FOXO3)-dependent manner. Thus, Cbl-b is one of several atrogenes, and the inhibition of

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[1] *Abbreviations used:* atrogenes, atrophy-related ubiquitin ligase genes; Cbl-b, Casitus b-lineage lymphoma-b; DEX, dexamethasone; DMEM, Dulbecco's Modified Eagle's Medium; FoxO, fork head box O; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high performance liquid chromatography; IC_{50}, concentrations with 50% inhibitory efficacy; IGF-1, insulin-like growth factor-1; IRS-1, insulin receptor substrate-1; MAFbx, muscle atrophy F-box protein; MyHC, myosin heavy chain; mTOR, mammalian target of rapamycin; MuRF-1, muscle RING finger protein-1; NF-κB, nuclear factor-kappa B; qRT-PCR, quantitative reverse transcription polymerase chain reaction; S6K, p70 S6 kinase; TKB domain, tyrosine kinase-binding domain.

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Cbl-b-mediated ubiquitination of IRS-1 has become an attractive therapeutic target in developing treatments for muscle atrophy.

We previously developed a Cbl-b inhibitor for the treatment of unloading-mediated muscle atrophy [7]. This DGphosphrylated(p) YMP penta-peptide, named Cblin, inhibited Cbl-b-mediated IRS-1 ubiquitination and was effective against denervation-induced muscle atrophy [7]. However, a high dose of Cblin was necessary to rescue muscle atrophy because Cblin was prone to degradation by aminopeptidases and also showed low efficiency in penetrating cell membranes.

In the present study, we sought to increase the inhibitory activity of Cblin by modifying it via *N*-myristoylation. Interestingly, the *N*-terminal myristoylation of Cblin rendered the molecule highly resistant to ubiquitination. We also found that *N*-myristoylated Cblin prevented glucocorticoid-induced skeletal muscle atrophy *in vivo*. Thus, *N*-myristoylated Cblin is a novel agent that may be useful for treating muscle atrophy.

Materials and methods

Cell culture

We purchased mouse myoblastic C2C12 and human embryonic kidney HEK293 cells from DS Pharma Biomedical (Osaka, Japan). Both cells lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C under 5% CO_2 and 95% air. C2C12 myoblasts were differentiated into myotubes by replacing the medium with DMEM containing 2% horse serum.

Ubiquitination inhibitory assay

Transfection of the indicated genes into HEK293 cells was performed with 5 μg of plasmid and HilyMax transfection reagent (Dojindo, Tokyo, Japan), according to the manufacturer's instructions. The plasmids used in this study were pcDNA3-IRS-1-V5, pCEFL, pCEFL-HA-Cbl-b, and pcDNA3-FLAG-Ubiquitin. Vector alone was transfected into cells in parallel as a negative control.

Transfected HEK293 cells were treated with 10 ng/mL recombinant human long-R3 IGF-1 (Sigma, St. Louis, MO, USA) at 27 h after transfection. One hour later, cells were treated with a synthetic oligopeptide, *N*-caprylated or *N*-myristoylated peptide (Table 1), at the indicated concentrations to examine the inhibitory activity of these peptides on Cbl-b-mediated IRS-1 ubiquitination. We purchased all synthetic oligopeptides and *N*-caprylated and *N*-myristoylated peptides tested in this study from GenScript (NJ, USA).

Effect of N-myristoylated Cblin on dexamethasone (DEX)-treated C2C12 myotubes

Differentiated C2C12 myotubes were treated with the oligopeptide of interest at the indicated concentrations 1 h before treatment with 10 μM DEX (Sigma). Myotubes were photographed via phase contrast microscopy at ×20 magnification. Myotube diameters were measured for a total of 300 myotubes from at least 15 fields with BZ Analyzer (Keyence, Osaka, Japan). Measurements were conducted in a blinded fashion; the myotube images were coded, and the investigator was unaware of the treatment group (control, DEX, or DEX plus peptide). Results were expressed as the percentage of the mean diameter of myotubes in the control group.

Table 1
Peptides used in these studies.

Cblin	H-Asp-Gly-Tyr(PO_3H_2)-Met-Pro-OH
Control peptide	H-Val-Gly-Tyr(PO_3H_2)-Leu-Arg-OH
C14-Cblin	Myr-Asp-Gly-Tyr(PO_3H_2)-Met-Pro-OH
C14-control-peptide	Myr-Val-Gly-Tyr(PO_3H_2)-Leu-Arg-OH
C8-Cblin	Cap-Asp-Gly-Tyr(PO_3H_2)-Met-Pro-OH

Myr, myristoyl group; Cap, capryl group.

Effects of N-myristoylated Cblin on DEX-induced muscle atrophy in vivo

Female C57BL/6 mice (11-weeks-old) from Japan SLC (Shizuoka, Japan) were injected intraperitoneally with 3 mg/kg DEX daily for 10 days, as described previously [8]. Cblin and *N*-myristoylated Cblin at concentrations of 6 mg (9.1 μmol) and 2 mg (2.3 μmol), respectively, in phosphate-buffered saline were intramuscularly injected into the lateral gastrocnemius muscle of DEX-treated mice every other day. Contralateral gastrocnemius muscles of DEX-treated mice were injected with the same volume of phosphate-buffer saline as control mice. Isolated gastrocnemius muscles were immediately frozen in chilled isopentane and liquid nitrogen and stored at −80 °C until analysis. The experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of Tokushima University.

Measurement of intracellular incorporation of oligopeptides

HEK293 cells at 90% confluence were treated with Cblin or *N*-myristoylated Cblin for 2 h. Next, cells were washed with 0.1 M glycine–HCl buffer (pH 3.0), deproteinized, and lysed. The lysate was separated via high performance liquid chromatography (HPLC) on a TSK gel ODS-80Ts column (4.6 mm × 250 mm, Tosoh, Tokyo, Japan). Buffer A was 0.1% trifluoroacetic acid in water; buffer B was 0.1% trifluoroacetic acid in acetonitrile. The column was equilibrated in 10% buffer B, then eluted with a linear gradient of 20–100% buffer B over 10 min at a flow rate of 1 mL/min.

Western blotting and immunoprecipitation

Western blotting and immunoprecipitation analyses were performed as described previously [9]. We used the following antibodies: anti-HA.11 (BabCo, Richmond, CA, USA), anti-V5 (Invitrogen, Carlsbad, CA, USA), anti-FLAG M2, anti-fast/slow-twitch-type myosin heavy chain (MyHC), anti-α-tubulin, anti-ubiquitin (Sigma), anti-IRS-1 (Calbiochem, La Jolla, CA, USA), anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Cbl-b (Cell Signaling, Boston, MA, USA).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was performed with SYBR™ Green dye and an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) as described previously [10]. Oligonucleotide primers for amplification are listed in Table 2.

Histochemical analysis

Sections were counterstained with hematoxylin and eosin. The cross-sectional area of myofibers was measured using BZ Analyzer (Keyence).

Other assays

Cell-free ubiquitination assays were performed as described previously [7]. Protein concentration was determined by the method of Lowry et al. as described previously [9].

Table 2
Primer sequences for qRT-PCR.

Target gene		Sequence	Length (bp)
Cbl-b	S	5′-GAGCCTCGCAGGACTATGAC-3′	241
	AS	5′-CTGGCCACTTCCACGTTATT-3′	
GAPDH	S	5′-ACCCAGAAGACTGTGGATGG-3′	125
	AS	5′-TTCAGCTCTGGGATGACCTT-3′	
MAFbx/atrogin-1	S	5′-GGCGGACGGCTGGAA-3′	100
	AS	5′-CAGATTCTCCTTACTGTATACCTCCTTGT-3′	
MuRF-1	S	5′-ACGAGAAGAAGAGCGAGCTG-3′	179
	AS	5′-CTTGGCACTTGAGAGGAAGG-3′	

AS, antisense primer; S, sense primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

All data are expressed as the mean ± standard deviation of 3–5 individual samples per group. Differences between groups were analyzed via one-way analysis of variance with SPSS (release 6.1, SPSS Japan, Tokyo, Japan). Differences between two groups were tested with Scheffé's test. $P < 0.05$ was considered statistically significant.

Results

Enhanced efficacy of N-myristoylated Cblin as a Cbl-b inhibitor

We performed the cell-free ubiquitination assay and the ubiquitination assay in the cells to clarify whether the myristoylation could contribute to the increased inhibitory activity of Cblin or its upregulated incorporation into the cells. In the cell-free ubiquitination assay (Fig. 1A), both Cblin and C14-Cblin similarly effectively inhibit IRS-1 ubiquitination by Cbl-b, indicating that myristoylation did not affect the inhibitory efficacy of Cblin. In another ubiquitination inhibitory assay using Cbl-b- and IRS-1-transfected HEK293 cells, *N*-myristoylated Cblin more effectively inhibited Cbl-b-mediated IRS-1 ubiquitination than Cblin (Fig. 1B). The concentrations with 50% inhibitory efficacy (IC_{50}) of Cblin and *N*-myristoylated Cblin were 120 and 30 μM, respectively. Myristoylated control peptide exerted no inhibitory activity on Cbl-b-mediated IRS-1 ubiquitination. Thus, *N*-myristoylation possibly causes peptides to increase their permeability into cells in addition to the resistance against aminopeptidase.

Enhanced inhibition of N-myristoylated Cblin on DEX-induced myotube atrophy

Next, we examined the inhibitory effects of *N*-myristoylated Cblin on DEX-induced atrophy in C2C12 myotubes. As reported in our previous study [7], DEX treatment for 3 days and 5 days decreased the thickness of C2C12 myotubes by 20% and 35%, respectively, compared to vehicle-treated myotubes, and Cblin restored this DEX-induced decrease in C2C12 myotubes (Fig. 2A). Interestingly, *N*-myristoylated Cblin significantly facilitated the effect of Cblin on DEX treatment in C2C12 myotubes, while myristoylated control penta-peptides had no effect. In contrast, *N*-caprylated Cblin did not change the inhibitory effects of Cblin on the DEX-mediated decrease in C2C12 myotube diameter (Fig. S1A). Consistently, DEX treatment reduced the amounts of slow- and fast-twitch-type-MyHC (Fig. 2B, lane 2). The decrease in amounts of these MyHC proteins was restored by treatment of Cblin and *N*-myristoylated Cblin (Fig. 2B, lanes 4 and 5), but not by *N*-myristoylated control peptide (Fig. 2B, lane 3).

Differentiated C2C12 myotubes contained low levels of Cbl-b protein (Fig. 2C). Compared to vehicle-treated C2C12 myotubes, DEX treatment stimulated Cbl-b expression at the mRNA and protein levels (Fig. 2C). In parallel with the DEX-mediated increase in Cbl-b protein expression, IRS-1 levels were significantly decreased in C2C12 myotubes. Although both Cblin and *N*-myristoylated Cblin significantly prevented the DEX-induced degradation of IRS-1, the preventive efficacy of *N*-myristoylated Cblin was greater than that of Cblin (Fig. 2C). The vehicle or *N*-myristoylated control peptide had no effect on the Cbl-b protein expression and DEX-induced degradation of IRS-1(Fig. 2C). Consistent with greater inhibition by *N*-myristoylated Cblin on IRS-1 degradation, *N*-myristoylated Cblin inhibited DEX-mediated IRS-1 ubiquitination at a lower concentration than did Cblin (Fig. 2D).

We also investigated whether *N*-myristoylated Cblin suppressed the DEX-mediated expression of atrogenes such as MAFbx/atrogin-1and MuRF-1 in C2C12 myotubes (Fig. 2E). Compared to vehicle treatment, 6-h DEX-treatment of C2C12 myotubes upregulated atrogene expression at the mRNA level. *N*-Myristoylated Cblin significantly prevented the upregulated expression of MAFbx/atrogin-1 and MuRF-1. In contrast, neither Cblin nor *N*-myristoylated Cblin affected DEX-mediated Cbl-b expression (Fig. 2E). Since the expression of MAFbx/atrogin-1 and MuRF-1 (but not Cbl-b) is regulated through IGF-1 signaling, our observation that neither Cblin nor *N*-myristoylated Cblin affected DEX-mediated Cbl-b expression makes sense. *N*-Caprylated Cblin did not affect MAFbx/atrogin-1 expression in DEX-treated C2C12 myotubes (Fig. S1B).

Efficiency of N-myristoylated Cblin incorporation into HEK293 cells

To confirm the finding that *N*-myristoylation increase the permeability of Cblin into cells (Fig. 1), we measured the amount of Cblin incorporated into HEK293 cells using our HPLC system. The retention times of synthetic Cblin and *N*-myristoylated Cblin on HPLC were 20.1 and 33.6 min, respectively (Fig. 3A and B). The absorbance values of 50 nmol of Cblin and *N*-myristoylated Cblin were 493,000 and 315,000, respectively, on our HPLC system. Untreated HEK293 cells did not contain any Cblin and *N*-myristoylated Cblin (Fig. 3C). When the lysate of Cblin-treated cells were subjected to the HPLC system, the result indicates that only small amounts of non-myristoylated Cblin were taken up by cell (Fig. 3D). The amount of incorporated Cblin was estimated as 0.35 nmol, since the absorbance of the peak I on Fig. 3D was 3451. In contrast, the amount of incorporated *N*-myristoylated Cblin, which was estimated as 3.65 nmol, was approximately 10-folds higher than the amount of Cblin (Fig. 3E). Interestingly, we detected a small amount of Cblin in the lysates of cells treated with *N*-myristoylated Cblin (Fig. 3D), indicating that subset of the pool of *N*-myristoylated Cblin was demyristoylated in cells. When we simultaneously applied Cblin and *N*-myristoylated Cblin to HEK293 cells at the same concentration (50 nmol/dish), the amounts of incorporated Cblin and *N*-myristoylated Cblin was 0.30 and 3.6 nmol, respectively, and were similar to these in the cells only treated with *N*-myristoylated Cblin (Fig. S2).

Effect of N-myristoylated Cblin on DEX-induced skeletal muscle atrophy in vivo

Finally, we confirmed whether *N*-myristoylated Cblin was available for DEX-induced skeletal muscle atrophy *in vivo*. Since the inhibitory efficacy of *N*-myristoylated Cblin on IRS-1 degradation was 4-fold larger than that of Cblin in C2C12 myotubes (Fig. 2C), we intramuscularly injected the approximately one-fourth amount of *N*-myristoylated Cblin compared with Cblin into DEX-treated mice.

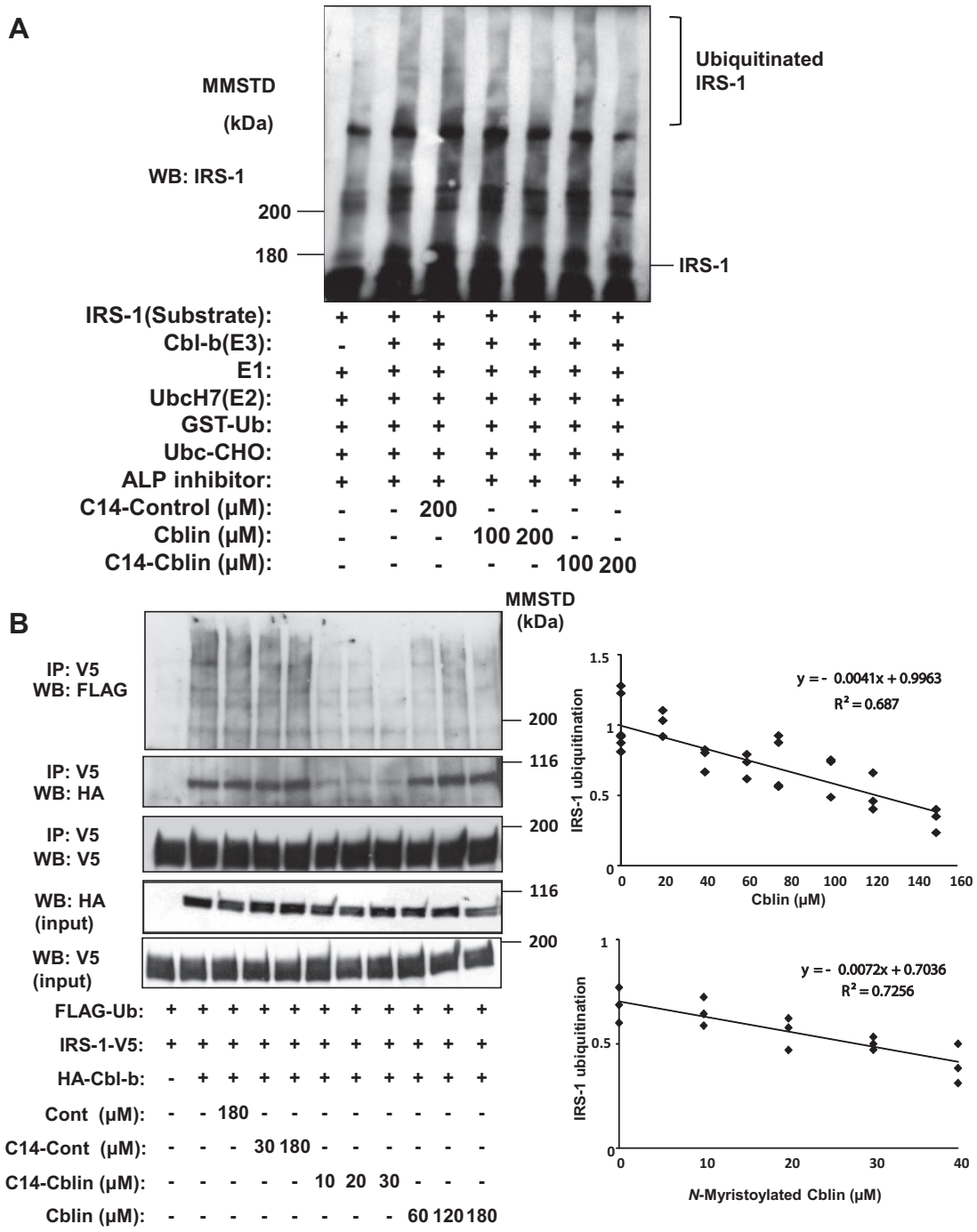

Fig. 1. *N*-myristoylated Cblin inhibits IRS-1 ubiquitination. (A) A cell-free ubiquitination assay reveals Cbl-b-mediated IRS-1 ubiquitination in the absence and presence of *N*-myristoylated Cblin or Cblin. Data are representative of three experiments. (B) Left, western blotting (WB) as a dose–response assay to compare peptide IC50. HEK293 cells were transfected with combinations of ubiquitin (FLAG-Ub), IRS-1 (IRS-1-V5), and Cbl-b (HA-Cbl-b) for 24 h, followed by treatment with 100 nM epoxomicin and Cblin, *N*-myristoylated Cblin (C14-Cblin), Cblin, or control peptide (Cont). Then, whole cell lysates were immunoprecipitated (IP) to isolate IRS-1 (V5), and IP-V5-associated proteins were separated by WB. The blot was probed for ubiquitin (FLAG), Cbl-b (HA), IRS-1 (V5), and input protein of Cbl-b (HA) and IRS-1 (V5) in turn from the top. Data are representative of three experiments. Right, inhibition of IRS-1 ubiquitination at various Cblin and *N*-myristoylated Cblin concentrations. For three experiments, the total area of IRS-1 ubiquitination was quantified with image-analysis software (ImageJ), and IC50 was determined from a correlation analysis (line).

Intraperitoneal injection of DEX for 10 days resulted in significantly decreased gastrocnemius muscle wet weight and cross-sectional area in mice by 20% and 40%, respectively (Fig. 4A and B). Intramuscular injection of Cblin and *N*-myristoylated Cblin significantly protected muscle from DEX-induced decreases in gastrocnemius muscle wet weight and cross-sectional area. Interestingly, 2 mg (2.3 μmol) of *N*-myristoylated Cblin injection prevented DEX-induced muscle atrophy, whereas in the case of Cblin injection, 6 mg (9.1 μmol) was necessary in this case and our previous report [7]. Consistent with the observed cross-sectional area change in whole muscle fibers, treatment with Cblin and *N*-myristoylated Cblin shifted the distribution pattern of cross-sectional area to the right, compared with the distribution of DEX-treated gastrocnemius muscle (Fig. 4B). The shift of the

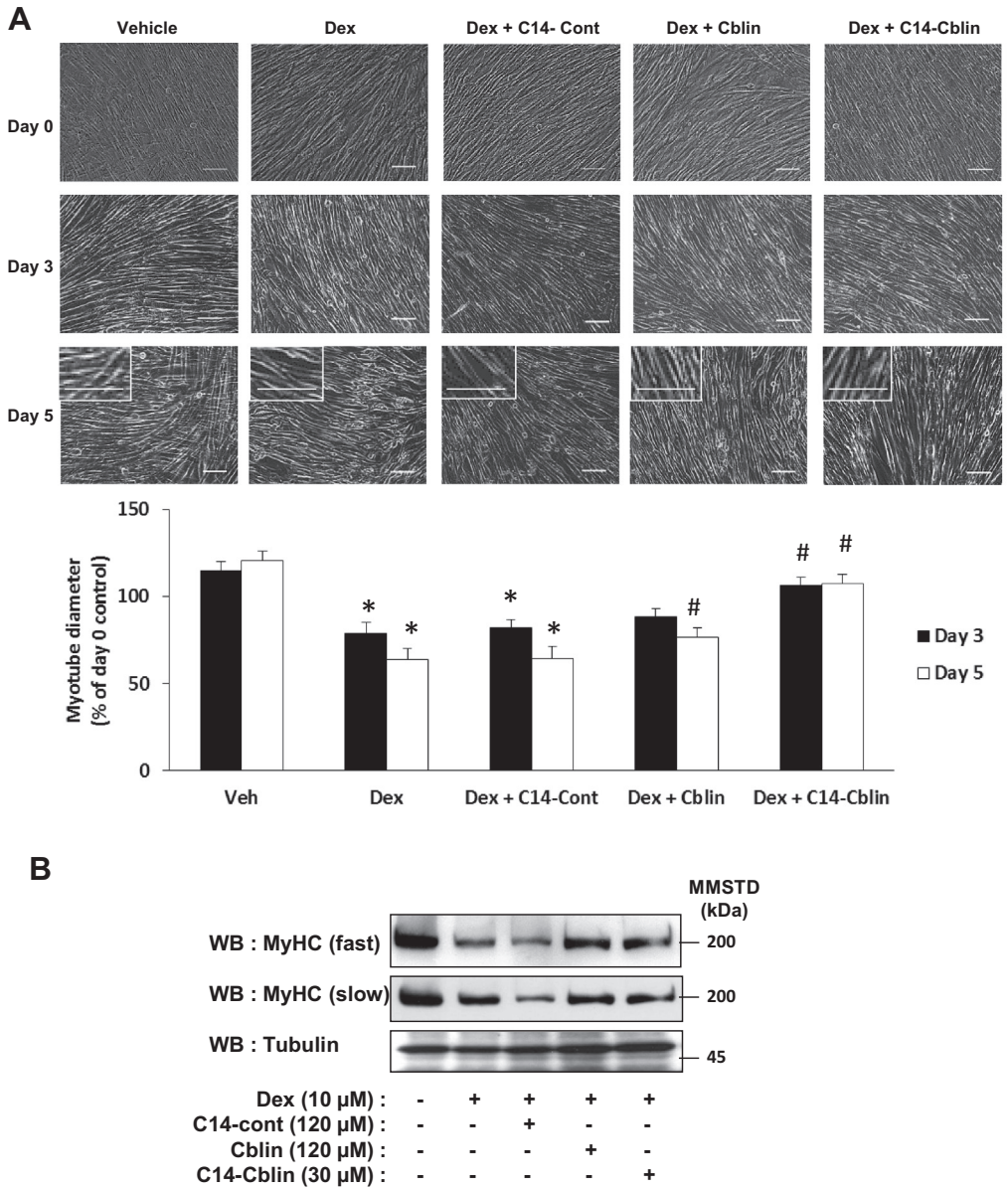

Fig. 2. *N*-myristoylated Cblin prevents dexamethasone (DEX)-induced myotube atrophy and expression of atrogenes in C2C12 myotubes. Differentiated myotubes were treated with vehicle (ethanol) or 10 μM DEX each day for 5 days. 120 μM of Cblin, 30 μM of *N*-myristoylated Cblin (C14-Cblin), or 120 μM of *N*-myristoylated control peptide (C14-Cont) were added every 12 h. (A) Top myotube diameters from randomly selected fields were quantified (Materials and methods). Scale bar, 100 μm. Bottom, diameters were expressed as a percent of the mean control diameter. Results are mean ± standard error of the mean; $n = 300$ per group. $^{*}P < 0.05$ compared to vehicle treatment; $^{\#}P < 0.05$ compared to DEX treatment. (B) Myotubes were treated with the indicated peptides for 1 h before treatment with vehicle or 10 μM DEX for 5 days. Whole cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), followed by western blotting (WB) to visualize the indicated proteins. (C) Left, myotubes were treated with the indicated peptides for 1 h before treatment with vehicle or 10 μM DEX for 4–6 h. Right, the ratio of Cbl-b or IRS-1 to tubulin (internal control) was evaluated with densitometric analysis. Data are mean ± standard deviation ($n = 3$). $^{*}P < 0.05$ compared to vehicle treatment; $^{\#}P < 0.05$ compared to DEX treatment. (D) Differentiated C2C12 myotubes were treated with each peptide for 1 h; then, DEX and 100 nM epoxomicin were added for 4 h. Whole-cell lysates were immunoprecipitated (IP) with an anti-IRS-1 antibody and subjected to WB. Multi-UB, antibody to ubiquitin. The input Cbl-b and IRS-1 protein levels were also examined by WB. (E) qRT-PCR of the expression levels of atrogenes (MAFbx/atrogin-1, MuRF-1, and Cbl-b) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) under the indicated conditions. Data are mean ± standard deviation ($n = 3$). $^{*}P < 0.05$ compared to vehicle treatment; $^{\#}P < 0.05$ compared to DEX treatment. C14-Cblin, *N*-myristoylated Cblin; C14-Cont, *N*-myristoylated control peptide.

distribution pattern by *N*-myristoylated Cblin was larger than that by Cblin (Fig. 4B). DEX treatment decreased the amount of IRS-1 protein (Fig. 4C, lanes 8–10 and 12). While both Cblin and *N*-myristoylated Cblin significantly prevented DEX-mediated IRS-1 degradation, the inhibition efficacy of *N*-myristoylated Cblin was larger than that of Cblin (Fig. 4C, lanes 7, 8, 11, and 12). DEX treatment upregulated the expression of MAFbx/atrogin-1, MuRF-1, and Cbl-b at the mRNA level (Fig. 4D). Cblin (9.1 μmol) and *N*-myristoylated Cblin (2.3 μmol) significantly prevented the upregulated expression of MAFbx/atrogin-1 and MuRF-1 to a similar extent. In contrast, Cblin nor *N*-myristoylated Cblin changed Cbl-b expression.

Discussion

Glucocorticoids are used as therapeutic agents due to their potent anti-inflammatory and immunosuppressive functions [12]; however, their side effects include skeletal muscle atrophy. It was previously reported that activation of the IGF-1/PI3K/AKT

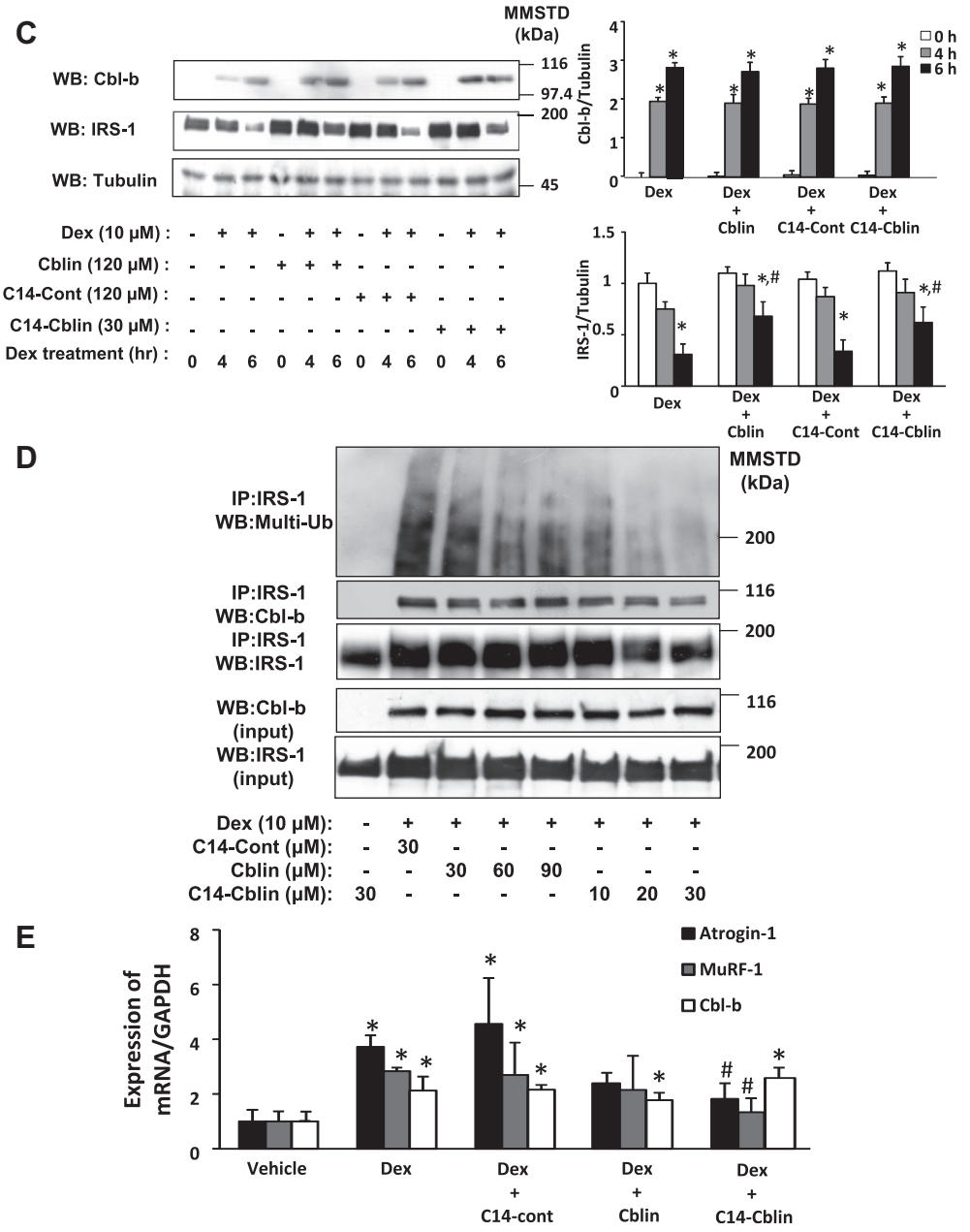

Fig. 2 (*continued*)

pathway rescued DEX-mediated atrophy through phosphorylation and inactivation of FOXO1/FOXO3 [13]. That observation implied that inhibition of the FOXO1/FOXO3 pathway was a primary mechanism underlying DEX-induced atrophy. Thus, we hypothesized that Cblin may inhibit DEX-induced skeletal muscle atrophy through activation of the IGF-1 signaling pathway.

We previously reported that Cblin peptide prevented skeletal muscle atrophy induced by denervation *in vivo* [7]. We also solved the structure of Cblin and the TKB domain of Cbl-b via X-ray crystallography (in submitted). These results revealed that the phosphotyrosine of Cblin is bound to the positive charged pocket of the Cbl-b TKB domain, resulting in competitive inhibition against the Cbl-b-IRS-1 interaction. However, high doses of Cblin were necessary to prevent denervation-mediated muscle atrophy *in vivo*, due to its low permeation into cells and its instability *in vivo*. To address these issues, we examined the effects of *N*-myristoylated Cblin on DEX-induced myotube atrophy in mouse C2C12 myotubes and in mice, since *N*-myristoylation of peptides has been reported to increase cell membrane permeation and thermostability [11,14]. In fact, lower concentrations of our *N*-myristoylated Cblin (IC_{50} = 30 μM) than Cblin (IC_{50} = 120 μM) were required for inhibition *in vitro* (Fig. 1B). *N*-myristoylated Cblin displayed a 10-fold larger accumulation in the intracellular fractions of HEK293 cells than did Cblin (Fig. 3). Consequently, the present results indicate that *N*-myristoylated Cblin facilitates the inhibition of DEX-mediated myotube atrophy by approximately 4-fold *in vitro* and *in vivo*, compared with Cblin (Figs. 2 and 4).

We also compared the inhibitory efficacies of Cblin peptides with myristoylation and caprylation. Myristoylated (14-carbon) Cblin suppressed the expression of MAFbx/atrogin-1 and prevented DEX-induced myotube atrophy more effectively than did caprylated (8-carbon) Cblin (Fig. S1). Myristoylation and caprylation

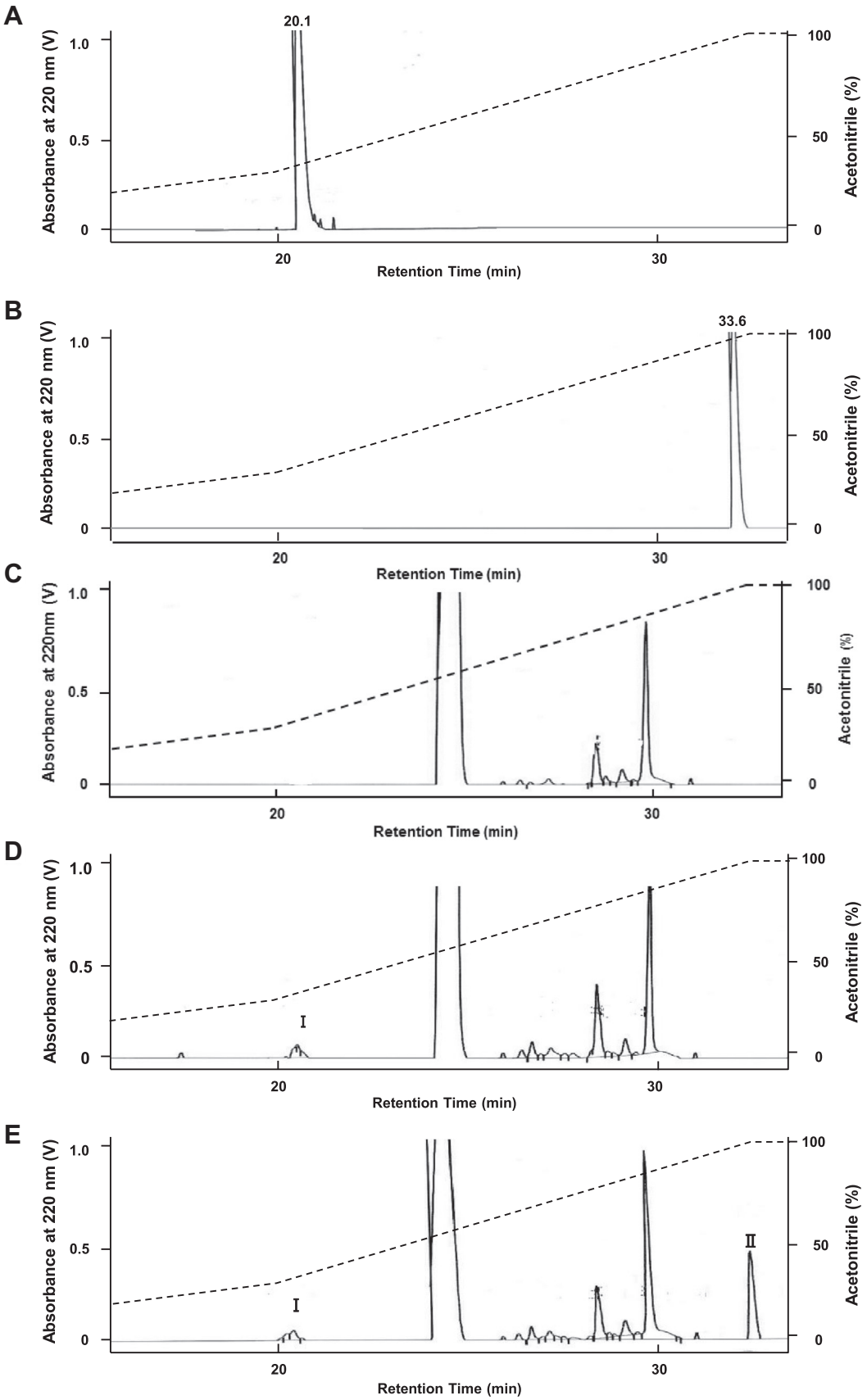

Fig. 3. *N*-myristoylation enhances the intracellular delivery of Cblin. HPLC elution profiles for Cblin (20.1 min) (A) and *N*-myristoylated Cblin (33.6 min) (B). HEK293 cells were treated with vehicle (PBS) (C), 50 nmol Cblin (D) or *N*-myristoylated Cblin (E) for 2 h. After washing with 0.1 M glycine–HCl buffer (pH 3.0) and deproteinization, lysates were subjected to the HPLC. I and II indicate the elution point of Cblin and *N*-myristoylated Cblin, respectively. (—): Absorbance at 220 nm; (—): gradient of elution buffer.

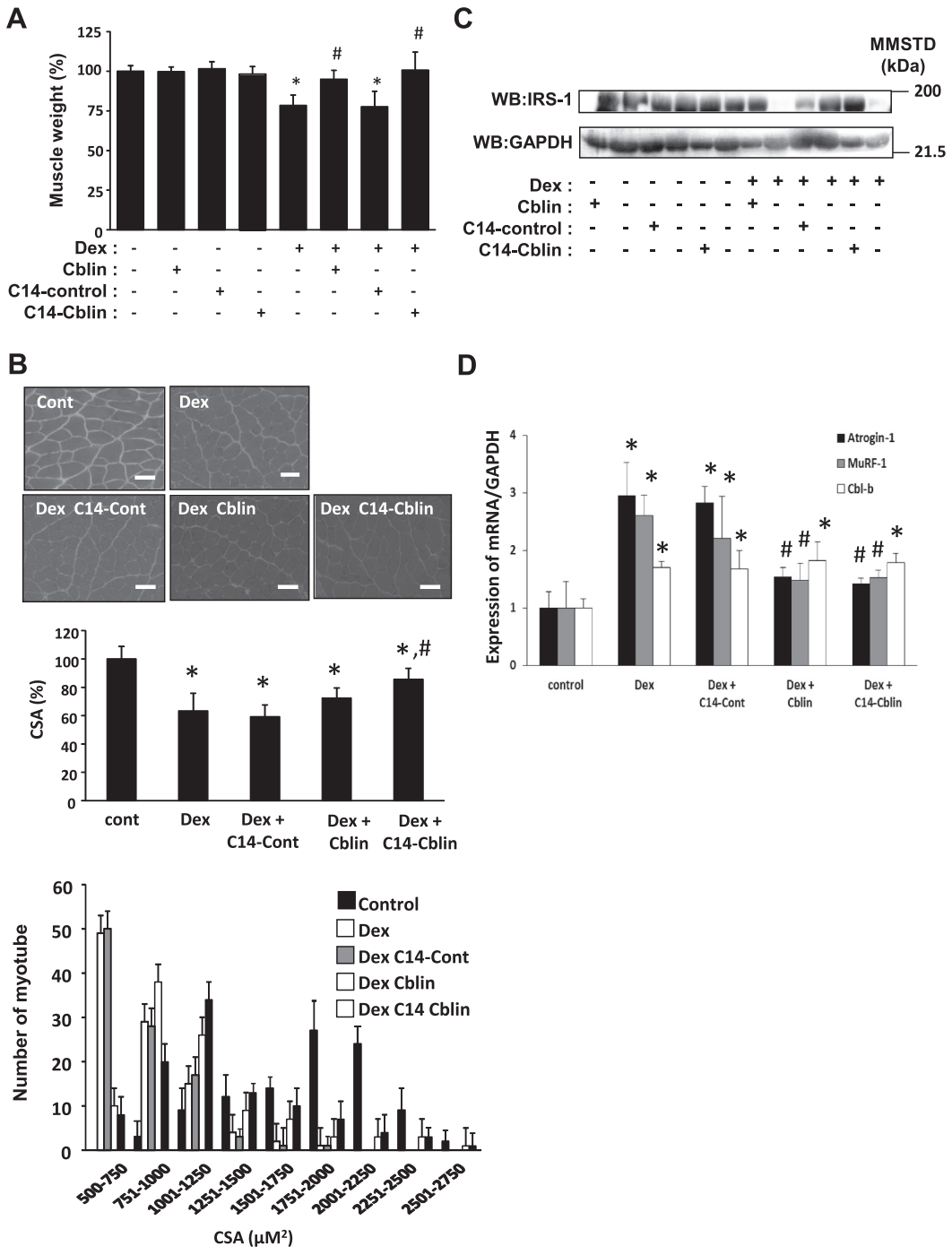

Fig. 4. *N*-myristoylated Cblin inhibits dexamethasone (DEX)-induced skeletal muscle atrophy. C57BL/6 mice received daily intraperitoneal injection with 0.1 mL of vehicle (saline, control) or DEX in saline (3 mg/kg) for 10 days. Peptide (Cblin, *N*-myristoylated control, or *N*-myristoylated Cblin) was injected into the right limb, and vehicle (dimethyl sulfoxide and saline) was injected into the left limb. (A) Wet weight of gastrocnemius muscle (Ga) was measured. Percent of muscle weight was defined as the ratio to control muscle wet weight in the mice. Data are mean ± standard deviation ($n = 5$). $^{*}P < 0.05$ compared to control; $^{\#}P < 0.05$ compared to DEX treatment. (B) Top, sections of Ga muscle were stained with hematoxylin and eosin. Middle, cross-sectional areas (CSA) of myofibers were measured. Bottom, distributions. Scale bar, 100 μm. Data are mean ± standard deviation ($n = 5$). $^{*}P < 0.05$ compared to control; $^{\#}P < 0.05$ compared to DEX treatment. (C) Homogenates from gastrocnemius muscle were subjected to SDS–PAGE and western blotting (WB) to visualize the indicated proteins. Data are mean ± standard deviation ($n = 3$). (D) qRT-PCR of the expression levels of atrogenes (MAFbx/atrogin-1, MuRF-1, and Cbl-b) and GAPDH under the indicated conditions. Data are means ± standard deviation ($n = 5$). $^{*}P < 0.05$ compared to control; $^{\#}P < 0.05$ compared to DEX treatment.

conferred equivalent resistance of Cblin peptide to aminopeptidase (data not shown). Myristoylation is more hydrophobic than caprylation, enabling myristoylated Cblin to permeate cells more readily than caprylated Cblin. Interestingly, many *N*-myristoyltransferases have been identified that catalyze covalent attachment of myristate to the *N*-terminal glycine residue of several proteins [14,15]. In contrast, to date, no enzyme has been identified that catalyzes caprylation. Thus, the myristoylation of peptides may naturally occur as a form of post-translational modification *in vivo*.

Myristate is a saturated acid. It has been shown that saturated fatty acids cause insulin resistance and type 2 diabetes [16,17]. Thus, we were concerned that myristoylation may also result in a side effect of insulin resistance. However, the adverse effects of fatty acids arise at high concentrations ($\geqslant$500 μM). In this study,

N-myristoylated Cblin caused inhibition at low concentrations (IC_{50} = 30 μM), suggesting that myristoylated Cblin is unlikely to cause insulin resistance.

Cblin permeation and inhibition efficacies were weaker than those of *N*-myristoylated Cblin (Fig. 3B). However, Cblin, a penta-peptide Cbl-b inhibitor, was taken up into HEK293 cells and significantly inhibited DEX-induced skeletal muscle atrophy (Fig. 4). Recent investigations reported that sodium-coupled oligopeptide transporters 1 and 2 transport peptides that consist of at least five amino acids into neuronal cells [18]. Based on those findings, we speculate that skeletal muscle may express novel transporters, similar to the neural transporters, which may facilitate Cblin uptake into the cytosol of myotubes. Future studies will seek to identify transporters for penta-peptides.

In our previous study, we found that Cbl-b is a negative regulator of IGF-1 signaling on unloading stress [7]. Some signals, such as myostatin/Smad2/3/Akt and kruppel-like factor 15/mTOR, are reported to be involved in glucocorticoid-induced skeletal muscle atrophy [19]. In this study, Cbl-b expression was also stimulated by DEX, leading to IRS-1 degradation (Figs. 2 and 4). This observation showed that Cbl-b expression participates in glucocorticoid-induced skeletal muscle atrophy. Treatment with Cblin or *N*-myristoylated Cblin significantly protected skeletal muscle from DEX-induced atrophy by suppressing the degradation of IRS-1 (Figs. 2 and 4). Thus, treatment with *N*-myristoylated Cblin targeting Cbl-b may suppress the side effects of glucocorticoids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.abb.2015.02.006.

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