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Article

The E3 Ligase MuRF1 Degrades Myosin Heavy Chain Protein in Dexamethasone-Treated Skeletal Muscle

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SUMMARY

Skeletal muscle atrophy occurs as a side effect of treatment with synthetic glucocorticoids such as dexamethasone (DEX) and is a hallmark of cachectic syndromes associated with increased cortisol levels. The E3 ubiquitin ligase *MuRF1* (muscle RING finger protein 1) is transcriptionally upregulated by DEX treatment. Differentiated myotubes treated with DEX undergo depletion of myosin heavy chain protein (MYH), which physically associates with MuRF1. This loss of MYH can be blocked by inhibition of *MuRF1* expression. When wild-type and *MuRF1*$^{-/-}$ mice are treated with DEX, the *MuRF1*$^{-/-}$ animals exhibit a relative sparing of MYH. In vitro, MuRF1 is shown to function as an E3 ubiquitin ligase for MYH. These data identify the mechanism by which MYH is depleted under atrophy conditions and demonstrate that inhibition of a single E3 ligase, MuRF1, is sufficient to maintain this important sarcomeric protein.

INTRODUCTION

The loss of skeletal muscle mass and strength are hallmarks of several cachectic syndromes, including congestive heart failure, chronic obstructive pulmonary disease (COPD), and severe burns. Notably, several cachexias are associated with increased cortisol levels (Anker et al., 1997; Hasselgren, 1999; Hong and Forsberg, 1995; Wing and Goldberg, 1993); also, muscle atrophy is a dose-limiting side effect of treatment with synthetic glucocorticoids, complicating high-dose treatment with these pharmacologic agents. Interestingly, hypercortisolemia has recently also been shown to exacerbate the atrophy-inducing effects of bed rest (Fitts et al., 2007). In a cell culture system of muscle atrophy, it was demonstrated that dexamethasone treatment induced the selective loss of myosin heavy chain protein (MYH) (Chromiak and Vandenburgh, 1992). This study indicated that glucocorticoid treatment affects muscle integrity by increasing protein breakdown of an important component of the sarcomere. It has been further shown that MYH is selectively depleted in myotube cultures treated with the atrophy-inducing cytokine TNF together with interferon (Acharyya et al., 2004) and that MYH is ubiquitinated in vivo in settings of cardiac cachexia (Schulze et al., 2005), but the mediator for these effects has not been elucidated.

A search for high-fidelity markers of the atrophy phenotype led to two genes, *MAFbx* (also called *atrogin-1*) (Bodine et al., 2001; Gomes et al., 2001) and *MuRF1* (muscle RING finger protein 1) (Bodine et al., 2001), which have been shown to be upregulated in at least 13 distinct models of atrophy, validating them as reliable markers for the atrophy phenotype (Bodine et al., 2001; Dehoux et al., 2003; Glass, 2005; Krawiec et al., 2005; Latres et al., 2005; Lee et al., 2004; Li et al., 2003, 2005; Sandri et al., 2004; Wray et al., 2003). Both *MuRF1* and *MAFbx* encode E3 ubiquitin ligases, a large family of proteins that mediate the ubiquitination of distinct protein substrates (Petroski and Deshaies, 2005; Winston et al., 1999; Yamao, 1999). MuRF1 is an enzyme of the RING-B box-coiled-coil (RBCC) or tripartite motif (TRIM) family of proteins, which exhibit intrinsic ubiquitin-conjugating activity. We previously reported the production of *MuRF1*$^{-/-}$ mice (Bodine et al., 2001). In the unperturbed state, they appear phenotypically normal; however, when examined 14 days postdenervation, significantly less muscle mass was lost in *MuRF1*$^{-/-}$ animals in comparison to wild-type littermates (Bodine et al., 2001).

There are two other MuRF family members, MuRF2 and MuRF3. MuRF proteins localize to the sarcomere (Centner et al., 2001), and it has been shown that MuRF1 associates with titin at the M band of the sarcomere, which has been proposed to maintain stability of the sarcomeric M line region (Gregorio et al., 2005; McElhinny et al., 2002). In another study, using a yeast two-hybrid method, eight myofibrillar proteins were shown to be binding partners of MuRF1: titin, nebulin, the nebulin-related protein NRAP, troponin I (TnI), troponin T (TnT), myosin light chain 2 (MLC-2), myotilin, and T-cap (Witt et al., 2005). In that study, however, none of these proteins were implicated

as substrates since there was no difference in assessed levels of interactors in *MuRF1*$^{-/-}$ animals. However, MuRF1 has been reported to function as an E3 ubiquitin ligase that catalyzes the ubiquitination of troponin I in cardiac myocytes (Kedar et al., 2004). Troponin I is therefore the only substrate previously reported for MuRF1, and it is not perturbed in skeletal muscle by the deletion of MuRF1 (Witt et al., 2005) or in myotubes by treatment with dexamethasone (B.A.C. and D.J.G., unpublished data). Therefore, it is still of interest to understand how MuRF1 modulates the atrophy phenotype.

RESULTS

Dexamethasone Induces a Loss of Myosin Heavy Chain and an Increase in MuRF1

To identify sarcomeric proteins that are perturbed under atrophy conditions, a previously described cell culture model of muscle atrophy was employed, in which C2C12 myotubes are treated with the synthetic glucocorticoid dexamethasone (DEX), resulting in phenotypic atrophy as determined by measuring myotube diameter and the loss of insoluble protein (Stitt et al., 2004). C2C12 myotubes were differentiated for 3 days and then treated for 24 hr with DEX. The cells were lysed, and the insoluble fraction, enriched for sarcomeric proteins, was homogenized. Equal amounts of insoluble material from the control versus DEX-treated cells were run on gels, which were subsequently stained with Coomassie blue. There was a single obvious difference in the protein pattern from the DEX-treated myotubes as compared to untreated myotubes (Figure 1A): the most abundantly stained protein, of nearly 250 kDa, was substantially reduced after DEX treatment. This protein was consistent in size with myosin heavy chain (MYH). To determine whether MYH was indeed the protein observed on the Coomassie-stained gel, a similar experiment was performed, and duplicate membranes were prepared and immunoblotted with antibodies recognizing isoforms of MYH. A pan-specific antibody detecting "fast" MYH isoforms (MYH1, MYH2, MYH3, MYH4, and MYH8, found in "type II" or "fast-twitch" fibers) showed a clear reduction in the amount of fast MYH isoforms after 24 or 32 hours of DEX treatment (Figure 1B). Densitometric analysis of three separate replicates revealed consistent decreases at each time point (Figure 1C). An antibody detecting the single "slow" isoform of MYH (MYH7, found in "type I" fibers) showed similar decreases after DEX treatment (Figure 1B; replicates quantified in Figure 1C). To determine whether other sarcomeric proteins were similarly lost in this model, levels of actin were determined; these were found to be unaltered during the treatment period (Figure 1B).

The E3 ubiquitin ligase *MuRF1* has been shown to be transcriptionally induced in at least 13 models of muscle atrophy (Glass, 2005). The amount of MuRF1 protein present after DEX treatment was analyzed and demonstrated to be increased, coincident with the decrease in MYH levels (Figure 1B, third panel). It was necessary to determine whether the change in MYH protein could be explained by alterations in *Myh* mRNA levels, as opposed to an increase in protein turnover. The mRNA levels of the five distinct *Myh* isoforms known to be expressed in C2C12 myotubes were analyzed (Figure 1D). Only one of the mRNAs encoding a *Myh* gene, *Myh3*, was found to be decreased; *Myh1* and *Myh4* mRNA levels were unperturbed, and *Myh7* and *Myh8* levels actually increased upon DEX treatment (Figure 1D). The data therefore indicate that the decreases observed in the protein levels are due to posttranscriptional events.

MuRF1 Physically Associates with Myosin Heavy Chain

To determine whether MuRF1 interacts with the MYH protein and might therefore have a means to perturb the turnover of MYH, C2C12 myoblast cells were infected with a retrovirus encoding human MuRF1 protein (hMuRF1) fused to the TAP epitope tag at the COOH terminus (C-TAP-hMuRF1) (Angrand et al., 2006). Infected cells were differentiated into myotubes for 3 days. As a control, uninfected C2C12 myotubes were also analyzed. Lysates were prepared and MuRF1 was immunoprecipitated using IgG Sepharose to bind to the protein A epitope on the TAP tag; the resulting immunoprecipitates were then immunoblotted for MYH using the pan-specific antibody recognizing MYH isoforms found in type II, fast fibers (Figure 1E). MuRF1 was found to be capable of immunoprecipitating MYH, whereas significantly less MYH was found when control lysates were analyzed. Total levels of MYH were determined by western blot and were found to be equivalent between control and C-TAP-hMuRF1 cells (Figure 1E). These data demonstrate that MuRF1 physically interacts with MYH.

Myosin Heavy Chain Isoform Loss Is Proteasome Dependent

Much of the protein degradation seen during skeletal muscle atrophy is due to the ATP-dependent ubiquitin proteasome system (Krawiec et al., 2005; Tawa et al., 1997). Two proteasome inhibitors, MG132 and epoxomicin (EPOX), were used to determine whether the loss of MYH observed after DEX treatment could be inhibited by blocking proteasome-mediated protein turnover. C2C12 myotubes were treated with DEX in the presence or absence of either MG132 or EPOX, and MYH protein levels were analyzed. DEX treatment resulted in the loss of both fast and slow MYH isoforms, but treatment with either MG132 or EPOX in addition to DEX rescued the decrease in MYH protein observed after DEX treatment alone (Figure 2A, panels 1 and 2, compare lane 2 with lanes 3 and 4). MuRF1 protein was increased after treatment with DEX but remained unchanged by proteasome inhibition alone (Figure 2A). Actin levels were unperturbed by any of the treatments, allowing actin to serve as a loading control (Figure 2A). These data were confirmed in three replicate plates for each experiment and quantified by densitometry (Figure 2B). These findings indicate that

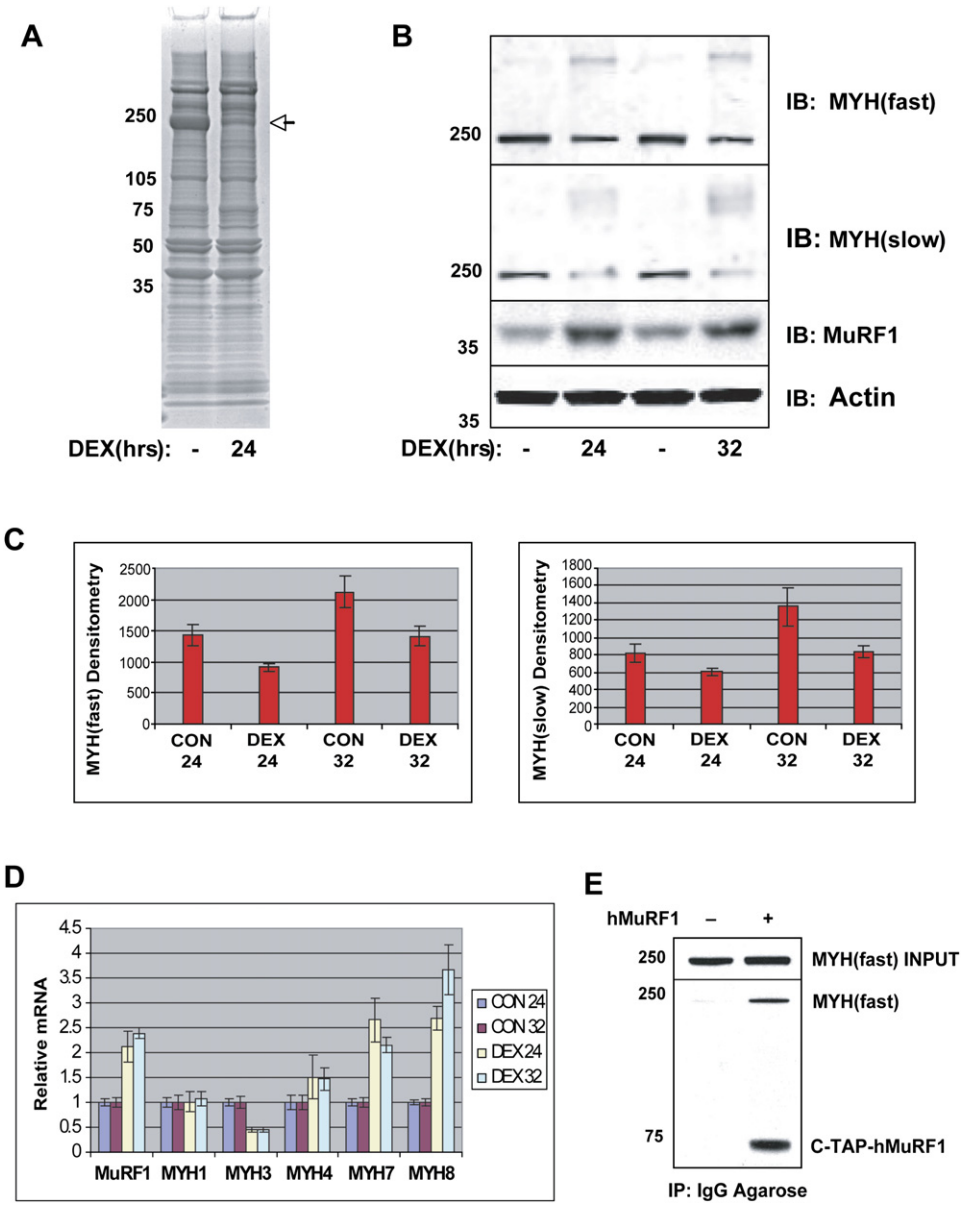

Figure 1. Dexamethasone Induces an Increase in MuRF1 and a Concomitant Decrease in MYH

Myotubes were differentiated for 3 days prior to dexamethasone (DEX) treatment.

(A) Detergent-insoluble protein samples were analyzed by SDS-PAGE and subsequent Coomassie staining. Protein lysates were from untreated (lane 1) and 24 hr DEX-treated (lane 2) myotubes. The arrow indicates the loss of a protein with a molecular weight of ~250 kDa.

(B) Analysis of myosin heavy chain (MYH) isoforms by immunoblotting (IB). Top panel: "fast," type II isoforms (MYH(fast)); second panel: "slow," type I isoforms (MYH (slow)); third panel: MuRF1; bottom panel: actin. On each panel, lysates from untreated (–) and 24 or 32 hr DEX-treated myotubes are presented. A high-molecular-weight ladder consistent with ubiquitinated material is visible in the DEX-treated MYH lanes, coincident with the loss of MYH protein at 250 kDa.

(C) Densitometric analysis of MYH isoforms in (B), shown as mean density (in arbitrary units) from three replicate plates. Error bars in (C) and (D) represent standard error of the mean (SEM).

(D) *MuRF1* and individual *Myh* mRNAs from untreated (CON 24 and CON 32) and DEX-treated (DEX 24 and DEX 32) myotubes. RNA analysis was performed by qPCR. Graphs depict the mean relative fold changes and are representative of three replicate plates.

(E) MuRF1 associates with MYH in C2C12 myotubes. IB analysis of MYH coimmunoprecipitated (IP) with C-TAP-hMuRF1 is presented. C-TAP-hMuRF1 is detected as a result of the affinity of the protein A portion of the TAP tag for IgG. Total levels of MYH from the lysates are shown in the top panel.

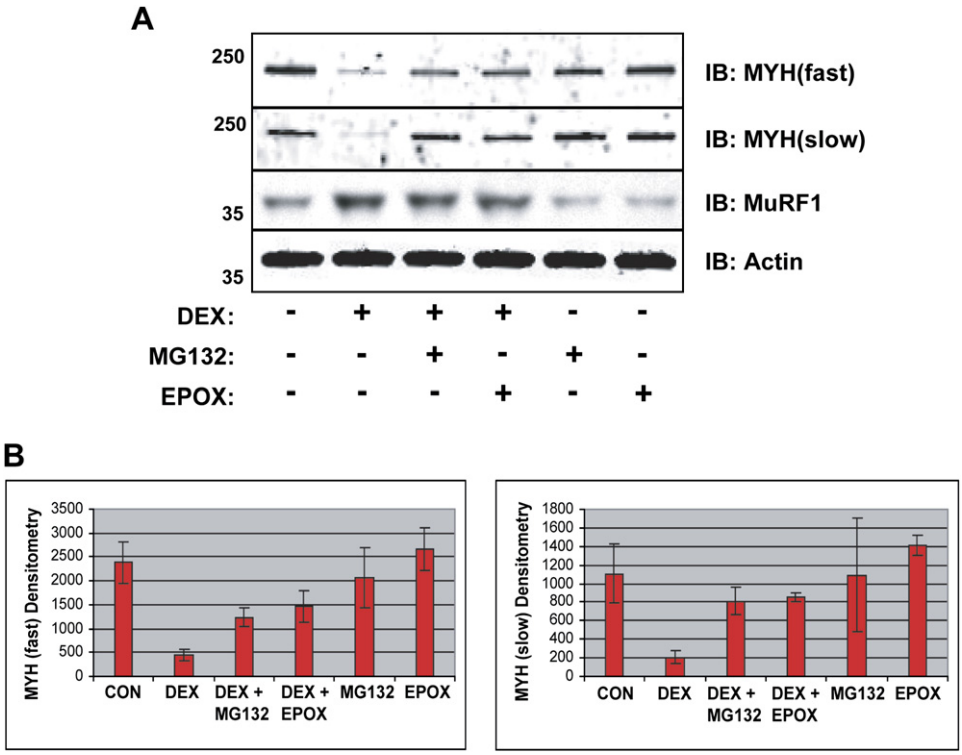

Figure 2. Dexamethasone-Induced Loss of Myosin Heavy Chain Is Blocked by Proteasome Inhibitors

(A) IB analysis of MYH isoforms, MuRF1, and actin after DEX treatment in the presence (+) or absence (−) of MG132 or epoxomicin (EPOX).

(B) Densitometric analysis of MYH isoforms from (A) shown as mean density (in arbitrary units) from three replicate plates. Error bars represent SEM.

proteasome-dependent activity is required for the loss of MYH induced by DEX treatment.

MuRF1 Is Required for the Loss of Myosin Heavy Chain Protein

Given that MuRF1 is responsible for a significant amount of the muscle loss observed under atrophy conditions (Bodine et al., 2001; Cai et al., 2004), our findings that MuRF1 and MYH physically interact and that inhibition of the proteasome rescues the loss of MYH during DEX treatment prompted us to determine whether MuRF1 is required for the decrease in MYH levels during DEX treatment. To answer this question, C2C12 myotubes were infected with an adenovirus expressing a short hairpin RNA (shRNA) recognizing the sequence encoding *MuRF1* (shMuRF1) or a control virus expressing a noncoding shRNA (shCON). Twenty-four hours after infection, cells were treated with DEX. After an additional 24 hr post DEX treatment, the cells were lysed and RNA was extracted. To determine the specificity of the *MuRF1* shRNA, mRNA levels of the three MuRF family members, *MuRF1*, *MuRF2*, and *MuRF3*, were analyzed by quantitative PCR (qPCR). *MuRF1* mRNA levels were significantly decreased by infection with shMuRF1 to less than 20% of that observed in control myotubes (Figure 3C). As expected, *MuRF1* mRNA levels were increased, by nearly 70%, after DEX treatment (Figure 3C). When shMuRF1 was introduced into myotubes and the cells were subsequently treated with DEX, *MuRF1* mRNA levels were reduced to below 50% of control levels (Figure 3C). Analysis of *MuRF2* and *MuRF3* mRNA levels revealed that neither one was affected by infection with shMuRF1 (Figure 3C). A duplicate experiment was performed, and the cells were lysed for immunoblot analysis. Myotubes that were treated with DEX in the presence of shCON demonstrated the same decrease in MYH protein and the same increase in MuRF1 protein observed in Figure 1 and Figure 2; myotubes treated with only shCON served as the negative control in this experiment (Figure 3A, compare lanes 1 and 2). In contrast, when myotubes were treated with shMuRF1, there was a significant decrease in MuRF1 levels in comparison to the shCON-treated cells; furthermore, while DEX slightly increased MuRF1 protein levels in these cells, the final level was less than that observed in control myotubes (Figure 3A, compare lanes 4 and 1). In these DEX-treated myotubes, in which MuRF1 levels were inhibited by shMuRF1 expression, there was almost no loss of either fast or slow MYH (Figure 3A, compare lanes 4 and 2). Actin protein levels remained unchanged, allowing actin to serve as a loading control (Figure 3A, panel 4).

The results of three replicate plates from a single experiment were quantified, confirming that MuRF1 is required for the loss of MYH protein (Figure 3B). To determine whether the changes in MYH protein levels were due to alterations in mRNA, *Myh* isoform mRNA was determined

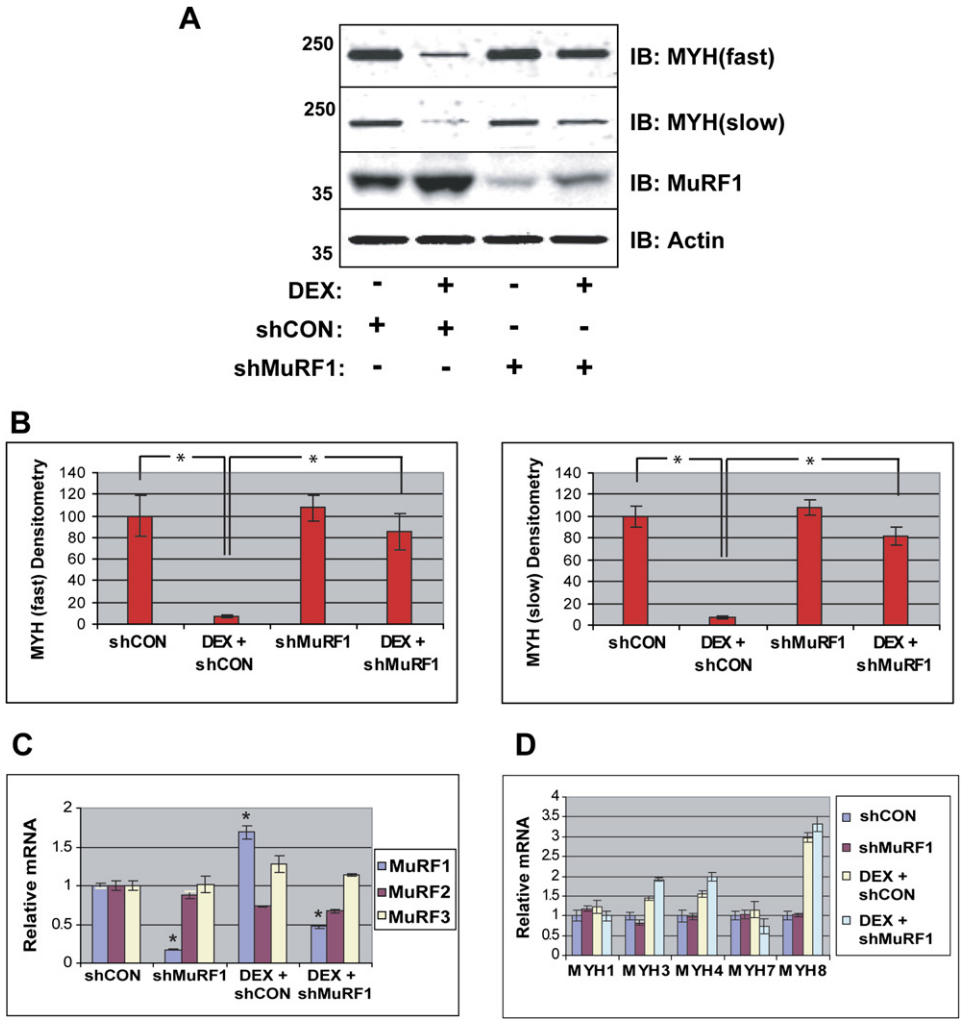

Figure 3. MuRF1 Is Necessary for the Dexamethasone-Induced Loss of Myosin Heavy Chain in C2C12 Myotubes

Myotubes were infected 2 days postdifferentiation with adenovirus expressing a short hairpin RNA directed against MuRF1 (shMuRF1) or a control virus (shCON) targeting no known mammalian sequence. Twenty-four hours after infection, the myotubes were treated with DEX for an additional 24 hr.

(A) IB analysis of MYH isoforms, MuRF1, and actin after shRNA knockdown of MuRF1 and/or DEX treatment. The myotubes treated with shCON alone (lane 1) serve as the negative control. DEX (lane 2) induces the loss of fast (MYH(fast)) and slow (MYH(slow)) myosin heavy chain isoforms; this loss of MYH isoforms is blocked by shMURF1 (lane 4). Treatment with shMURF1 does not affect MYH levels but does reduce MuRF1 to levels below that seen in control (compare lane 3 to lane 1).

(B) Densitometric analysis of MYH isoforms from (A) shown as mean density (in arbitrary units) from three replicate plates. *$p < 0.01$ versus shCON + DEX by ANOVA and Tukey's post hoc test. Error bars in (B)–(D) represent SEM.

(C) mRNA levels of the three MuRF family members, *MuRF1*, *MuRF2*, and *MuRF3*, analyzed by qPCR after MuRF1 knockdown and DEX treatment. Graphs depict mean relative fold changes and are representative of three replicate plates. Each mRNA expression level was determined relative to the mean expression level of that mRNA in control cells. *$p < 0.01$ versus shCON by ANOVA and Tukey's post hoc test.

(D) Individual *Myh* mRNAs from MuRF1 knockdown and DEX-treated myotubes. Graphs depict mean relative fold changes and are representative of three replicate plates.

by quantitative TaqMan PCR. The mRNA levels of *Myh1* and the single slow isoform, *Myh7*, were unaltered by DEX or by shRNA knockdown of MuRF1; the isoforms *Myh3*, *Myh4*, and *Myh8* were all increased between 1.5- and 3-fold after DEX treatment alone, a change that was not affected by MuRF1 knockdown and therefore does not account for the sparing effect of MuRF1 inhibition (Figure 3D). These data indicate that the DEX-induced MYH loss in C2C12 myotubes is posttranscriptional and that MuRF1 does not influence the mRNA levels of the *Myh* isoforms analyzed. Therefore, inhibition of *MuRF1* expression is sufficient to block the posttranscriptional loss of MYH observed after DEX treatment.

Dexamethasone-Induced MYH Loss Is Attenuated in *MuRF1*$^{-/-}$ Mice

To determine whether the sparing of MYH loss seen in DEX-treated C2C12 myotubes after MuRF1 knockdown

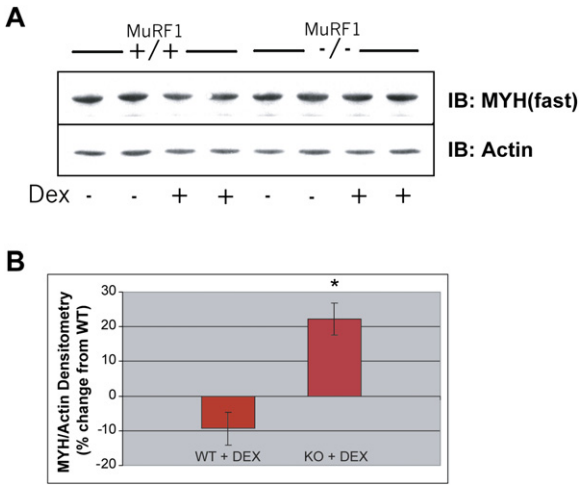

Figure 4. Myosin Heavy Chain Is Relatively Spared in *MuRF1* Null Animals Undergoing DEX-Induced Atrophy

Homozygous *MuRF1* knockout (KO) mice and wild-type (WT) control mice were treated for 14 days with 1 mg/kg DEX or saline.

(A) IB analysis of MYH and sarcomeric actin from gastrocnemius muscle was performed for all animals in the study. A representative blot is shown.

(B) The immunoreactive bands were subjected to densitometric analysis, and the ratio of MYH to actin was determined. The mean MYH:actin ratio was plotted for WT and KO mice treated with DEX as the percent change from WT mice treated with saline. *$p < 0.01$ versus WT saline-treated mice by ANOVA and Tukey's post hoc test. Error bars represent SEM.

is recapitulated in vivo, we utilized the previously described *MuRF1* null (*MuRF1*$^{-/-}$) mouse model (Bodine et al., 2001). Wild-type (WT) mice were treated with DEX or saline (n = 9 and n = 8, respectively) for 14 days, as were *MuRF1*$^{-/-}$ mice (n = 6 and n = 7, respectively). Gastrocnemius muscle weights were taken and normalized to the animals' starting body weight. Equal amounts of protein homogenates were analyzed by immunoblot for fast MYH isoforms, as well as for sarcomeric actin. MYH levels were decreased when comparing WT saline- and DEX-treated samples (Figure 4A). Furthermore, there was a 15% loss of mass in DEX-treated WT gastrocnemius muscles in comparison to controls (data not shown). In contrast, the levels of actin did not appear to be consistently altered between groups (Figure 4A).

MYH loss was compared relative to actin by densitometric analysis of the immunoreactive bands, revealing a nearly 10% loss of MYH relative to actin in DEX-treated WT mice (Figure 4B). In contrast, the MYH:actin ratio in DEX-treated *MuRF1*$^{-/-}$ animals increased significantly as compared to either WT or *MuRF1*$^{-/-}$ mice treated with saline or to WT mice treated with DEX (1.23 ± 0.06 versus 1.01 ± 0.04, 0.91 ± 0.05, and 1.02 ± 0.03, respectively; $p < 0.01$) (Figure 4B). The increase in MYH relative to actin in *MuRF1*$^{-/-}$ mice treated with DEX was greater than 29% when compared to WT animals treated with DEX (Figure 4B; 9% loss in WT + DEX versus 20% increase in *MuRF1*$^{-/-}$ + DEX). Despite the sparing of MYH, MuRF1-deficient muscle mass was spared by only 3% relative to WT muscle after DEX treatment (data not shown).

These findings highlight a preferential sparing of MYH in comparison to the sarcomeric protein actin in DEX-treated mice deficient for MuRF1, indicating the specific role of MuRF1 in the regulation of MYH.

Myosin Heavy Chain Protein Is Ubiquitinated by MuRF1 In Vitro

To determine whether MYH protein is a substrate for MuRF1 ubiquitin ligase activity, in vitro ubiquitination assays were performed utilizing purified recombinant GST-His-hMuRF1 protein, purified myosin II protein from rabbit skeletal muscle, yeast E1, recombinant hUbcH5c, and His_6-biotin-hUB. Reactions were conducted with or without myosin present, and the results were analyzed by immunoblotting for ubiquitin using streptavidin-conjugated HRP (Figure 5). When increasing concentrations of GST-His-hMuRF1 were used in the assay, a corresponding increase in the amount of ubiquitinated product was observed above the molecular weight for myosin (Figure 5A). In control lanes, where no myosin was included, there was significantly less ubiquitinated material visible above the molecular weight for myosin, indicating that the increase in signal after addition of MYH is caused not by auto-ubiquitinated GST-His-hMuRF1 but by ubiquitination of myosin (Figure 5A). We next performed a time course of myosin ubiquitination using a single concentration of 50 nM GST-His-hMuRF1 (Figure 5B). As the reaction proceeded, we observed an increased accumulation of ubiquitinated products above the molecular weight of myosin that was not observed in lanes that did not contain MYH (Figure 5B). These results indicate that MuRF1 can directly ubiquitinate myosin in vitro.

DISCUSSION

MuRF1 was previously demonstrated to be transcriptionally upregulated in multiple models of skeletal muscle atrophy (Glass, 2005). When *MuRF1*$^{-/-}$ animals are subjected to atrophy conditions—via either denervation or the coexpression of a constitutively active form of the NF-κB activator IKK—significantly less muscle is lost than in *MuRF1*$^{+/+}$ control mice, indicating that MuRF1 is more than a marker of the atrophy process, it is in part responsible for the increased protein turnover that occurs during atrophy (Bodine et al., 2001; Cai et al., 2004).

The question that remained was clear: How does MuRF1 mediate skeletal muscle atrophy? Several important studies have demonstrated that MuRF1 can interact with key sarcomeric proteins, including titin at the M band of the sarcomere, which has been proposed to maintain stability of the sarcomeric M line region (Gregorio et al., 2005; McElhinny et al., 2002), as well as nebulin, NRAP, TnI, TnT, MLC-2, myotilin, and T-cap, but no difference was seen in these proteins when *MuRF1*$^{-/-}$ and control animals were compared (Witt et al., 2005), nor have there been reports of these proteins being perturbed in models of atrophy. Of all the previously identified

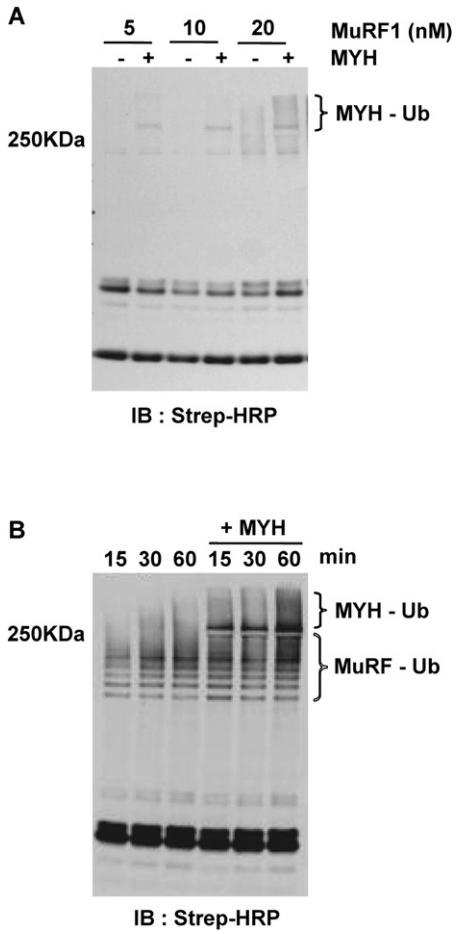

Figure 5. MuRF1 Ubiquitinates Myosin Heavy Chain Protein In Vitro

MYH (1 μM) was incubated with increasing concentrations of GST-His-hMuRF1 for 1 hr or with 50 nM GST-His-hMuRF1 over a time course. (A) IB analysis of in vitro myosin ubiquitination with increasing amounts of GST-His-hMuRF1. Reactions were carried out for 1 hr at room temperature.

(B) Time course of in vitro myosin ubiquitination by 50 nM GST-hMuRF1. All reactions were performed at room temperature. MYH-Ub indicates ubiquitinated MYH, which is only in evidence in the presence of MYH; MuRF-Ub indicates auto-ubiquitinated MuRF1, which is apparent in the lanes with or without MYH.

interacting proteins, only one, TnI, has been shown to be a ubiquitination substrate of MuRF1 in cardiac myocytes (Kedar et al., 2004). However, in our studies, TnI levels in skeletal myotubes were not altered by DEX treatment or by inhibition of MuRF1 (data not shown), consistent with the previous report comparing troponin levels in WT and *MuRF1* null mice (Witt et al., 2005).

To identify an atrophy-relevant MuRF1 substrate, this study initially focused on the synthetic glucocorticoid DEX as a method to induce myotube atrophy in cell culture; it has previously been shown that DEX treatment can induce atrophy and the upregulation of *MuRF1* and *MAFbx* in myotubes (Latres et al., 2005; Sacheck et al., 2004) and the selective loss of MYH by increasing protein turnover (Chromiak and Vandenburgh, 1992). In the present study, a Coomassie-stained gel comparing lysates from control and DEX-treated myotubes demonstrated that a single protein, consistent in molecular weight with MYH, was highly abundant in control cells but much less evident in lysates from DEX-treated myotubes. An immunoblot confirmed that the protein was MYH, and antibodies that discriminate between the slow and fast isoforms of the protein showed that both classes were significantly affected by the treatment. The decrease in MYH was found to be coincident with an increase in MuRF1 protein levels in these myotubes; this last point is noteworthy because most prior studies focused on *MuRF1* mRNA levels alone. An indication that MuRF1 and MYH might be in a common pathway is that the two proteins directly interact—epitope-tagged MuRF1 protein coimmunoprecipitated with MYH protein. Also suggestive is the fact that MYH levels can be maintained when the ubiquitin-proteasome system is inhibited by the use of either of two proteasome inhibitors, indicating that MYH is being degraded via the proteasome. The demonstration that selective inhibition of MuRF1, using MuRF1-specific shRNA that does not inhibit other MuRF family members, is sufficient to maintain MYH levels, even in the presence of DEX treatment, implicates MuRF1 directly as the key modulator of MYH levels.

Mice that are null for *MuRF1* have been described previously (Bodine et al., 2001). These animals were used to assess whether the absence of MuRF1 was sufficient to spare MYH levels under atrophy conditions in vivo. Both WT and *MuRF1*$^{-/-}$ mice were treated with DEX for 2 weeks. In this experimental setting, MYH was decreased in WT muscle relative to total protein, but not in the lysates from *MuRF1*$^{-/-}$ muscle. When the relative changes in MYH were assessed, the results were more striking: in WT animals, MYH was lost to a 10% greater degree than actin under conditions of DEX treatment, whereas in the *MuRF1* null animals, there was an increase in MYH relative to actin, indicating that myosin is preferentially spared under atrophy conditions in the absence of MuRF1. Interestingly, in contrast to denervation or the upregulation of constitutively active IKK, in which *MuRF1* deletion has been previously shown to block atrophy (Bodine et al., 2001; Cai et al., 2004), DEX treatment of *MuRF1* null animals did not result in a statistically significant decrease in atrophy in comparison to DEX treatment of WT animals. This indicates that sparing of MYH is not sufficient to avoid DEX-induced atrophy, that there are apparently distinct atrophy mechanisms engaged upon DEX treatment in comparison to denervation and IKK upregulation, and that loss of MuRF1 is not sufficient to stop these. The in vivo data are reminiscent of studies on humans treated with high doses of steroids. A corticosteroid-induced myopathy has been reported in which there is a preferential loss of thick filaments, containing myosin, and a relative preservation of thin, actin-containing filaments (Hanson et al., 1997).

Direct biochemical evidence as to the mechanism by which MuRF1 causes the turnover of MYH is provided

by the demonstration that MuRF1 functions as an E3 ubiquitin ligase for MYH in an in vitro ubiquitination reaction (Figure 5). This study therefore provides evidence that the critical sarcomeric protein MYH is a substrate for MuRF1 and that loss of MuRF1 is sufficient to maintain MYH levels during muscle atrophy.

These findings suggest a model as to the order of sarcomeric protein breakdown during muscle atrophy. Since MYH is lost out of proportion to other contractile proteins during atrophy in WT animals and since other contractile apparatus components seem to be maintained in *MuRF1* null animals, the data suggest that MYH degradation occurs quite early in the sarcomere breakdown process, such that blocking this step protects sarcomeric integrity. The fact that MYH is lost to a greater degree than other proteins also suggests a particular model of atrophy in which a single key protein is removed, disrupting the integrity of the system without necessarily having to remove each individual protein to the same extent. Also, the data indicate that inhibition of other proteases, such as the calpains, may not be required to maintain MYH levels, though it is still possible that MuRF1 perturbs calpain or other proteolysis pathways by some additional mechanism. The finding of a single MuRF1 substrate that is perturbed during skeletal muscle atrophy does not eliminate the possibility that MuRF1 acts on additional important substrates—and the prior report that a MuRF family member can perturb serum response factor (SRF) (Lange et al., 2005) makes one wonder whether MuRF1 also functions away from the sarcomere during atrophy. However, the demonstration in the present study that one of the main components of the sarcomere, MYH, can be rescued solely by eliminating MuRF1 increases focus on the contractile apparatus as the target for MuRF1 activity during skeletal muscle atrophy and provides the basis for future studies to determine the order and mechanism of protein breakdown of the sarcomere.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment

C2C12 myoblasts (ATCC) were cultured and differentiated as described previously (Rommel et al., 1999). For shRNA experiments, cells were infected with adenovirus on day 2 postdifferentiation and treated with DEX for 24 hr starting on day 3. When proteasome inhibitors were used, they were added for the last 8 hr before harvesting cells on day 4.

For the MuRF1/MYH coimmunoprecipitation experiment, C-terminal TAP-tagged MuRF1 (C-TAP-hMURF1) was stably expressed in C2C12 myoblasts by retrovirally mediated gene transfer. Cells were differentiated into myotubes for 3 days. TAP-tagged MuRF1 and associated proteins were coimmunoprecipitated as described previously (Angrand et al., 2006).

Biochemicals and Antibodies

Myotubes were treated with 100 μM DEX (Sigma-Aldrich) dissolved in ethanol. MG132 and EPOX (Calbiochem) were dissolved in DMSO and used at 10 μM and 100 nM, respectively. Mouse monoclonal antibodies against fast MYH isoforms (MY32), the slow MYH isoform (NOQ7.5.4D), and actin (catalog #A2103) were from Sigma-Aldrich. Hybridoma cell supernatant containing anti-MuRF1 monoclonal antibodies (Regeneron Pharmaceuticals), donkey anti-mouse HRP (Rockland Biochemicals), IRDye 800CW goat anti-mouse IgG and IRDye 680 goat anti-rabbit IgG (Li-Cor Biosciences), and benzonase (EMD Biosciences) were used.

Western Blotting

Myotubes were scraped into PBS containing 1% NP40, 1 mM EDTA, 10 mM NaF, and protease inhibitors (lysis buffer). Myotube homogenates were rocked at 4 degrees for 15 min and centrifuged for 10 min at 13,000 rpm. The supernatant was collected, and the insoluble material was resuspended in 10% glycerol, 30 mM KCl, 20 mM Tris (pH 7.6), 2 mM $MgCl_2$, 2 mM DTT, 2 mM NaF in H_2O containing benzonase at 2.5 kU/ml. Samples were homogenized and incubated on ice for 15 min. Protein concentrations of insoluble suspensions were assayed by BCA Protein Assay Kit (Pierce). Samples were solubilized in SDS-PAGE sample buffer, and equal amounts of protein were loaded per lane of 4%–20% SDS polyacrylamide gels and transferred onto PVDF membranes. Membranes were blocked in TBS with 5% (w/v) milk powder. Primary and secondary antibodies were incubated in TBS with 0.1% Tween 20 and 2.5% milk. Immunoreactivity was detected by ECL and exposed to film or imaged with a Li-Cor Odyssey imaging system.

For MuRF1 coimmunoprecipitation experiments, C2C12 myotubes transduced retrovirally with an expression cassette encoding C-TAP-hMURF1 were scraped into PBS containing 1% NP40, 1 mM EDTA, 10 mM NaF, and protease inhibitors. Human IgG Sepharose was added to 2 ml of lysate (1 mg protein/ml). Uninfected C2C12 myotube lysates at the same protein concentration were used as a negative control. Membranes were probed with anti-MYH (fast) antibody (Sigma) to corroborate coimmunoprecipitation with c-TAP-hMuRF1.

RNA Analysis

RNA was isolated using TRI reagent (Molecular Research Center, Inc.) per the manufacturer's protocol. cDNA synthesis was performed using a QIAGEN QuantiTect kit. qPCR was performed using an Applied Biosystems 7900HT Real-Time PCR machine. The TaqMan probe sets MuRF1 (Mm01185221_m1), MuRF2 (Mm01292963_g1), MuRF3 (Mm00491305_m1), MYH1 (Mm01332500_gH), MYH2 (Mm00454991_m1), MYH3 (Mm01332469_g1), MYH4 (Mm01332541_m1), MYH7 (Mm00600555_m1), and MYH8 (Mm00838799_g1) were purchased from Applied Biosystems.

Adenovirus and RNAi

A shRNA corresponding to the sequence 5′-GAACATGGACTACTT TACT-3′ within mouse MuRF1 was designed according to Invitrogen's BLOCK-iT adenoviral expression system. A control viral construct targeting no known RNA sequence was similarly assembled. The adenoviral expression constructs were sent to Welgen, Inc. (Worcester, MA, USA) for production and purification. The purified viruses were used at a final concentration of 2.4×10^7 pfu/ml.

In Vitro Ubiquitination

For the in vitro ubiquitination assay, yeast E1, human recombinant UbcH5c, and human N-terminal-hexahistidine-tagged biotinylated ubiquitin (His_6-biotin-hUB) were purchased form Boston Biochem, Inc.; GST-His-hMuRF1 was bacterially expressed and purified by Blue Sky Biotech, Inc.; and MYH type II protein, purified from rabbit skeletal muscle, was purchased from Cytoskeleton, Inc. The reaction was carried out by addition of 125 nM E1; 4 μM UbcH5c; 5 μM His_6-biotin-hUB; 5, 10, 20, or 50 nM GST-His-hMuRF1; and 1 μM myosin to the reaction buffer (50 mM HEPES [pH 7.5] containing 5 mM MgATP solution [Boston Biochem, Inc] and 0.6 mM DTT). GST-His-hMuRF1 was preincubated for 30 min on Reacti-Bind anti-GST coated plates (Pierce); reactions were carried out for 1 hr at room temperature or in a time course and ended by transfer of the reaction mix to a tube, addition of SDS-PAGE sample buffer, and heating. Reactions were run in 4%–12% Bis-Tris gels with 1× MES running buffer (Invitrogen) and transferred to PVDF membranes for immunoblotting with streptavidin-conjugated horseradish peroxidase (Pierce).

Animal Studies

MuRF1 knockout (*MuRF1*$^{-/-}$) mice were generated as described previously (Bodine et al., 2001). *MuRF1*$^{-/-}$ mice and WT littermates (3 months old, 25–30 g) were maintained on standard chow at a constant temperature (21°C) under a 12 hr/12 hr artificial light/dark cycle with unlimited access to water. DEX or saline vehicle was continuously infused via dorsally implanted osmotic minipumps (Alzet, model 2002) at 1 mg/kg for 14 days. At the completion of the experiment, the mice were sacrificed with CO_2 followed by cervical dislocation. Gastrocnemius muscles were removed, weighed, and frozen in liquid nitrogen. Homogenates were prepared in lysis buffer using a QIAGEN bead homogenizer.

Statistical Analyses

Differences between groups were analyzed using one-way ANOVA for all experiments. Significance was determined by Tukey's post hoc test. Values were considered significant at $p < 0.01$.

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