Regulation of Protein Catabolism by Muscle-Specific and Cytokine-Inducible Ubiquitin Ligase E3α-II during Cancer Cachexia


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ABSTRACT

The progressive depletion of skeletal muscle is a hallmark of many types of advanced cancer and frequently is associated with debility, morbidity, and mortality. Muscle wasting is primarily mediated by the activation of the ubiquitin-proteasome system, which is responsible for degrading the bulk of intracellular proteins. E3 ubiquitin ligases control polyubiquitination, a rate-limiting step in the ubiquitin-proteasome system, but their direct involvement in muscle protein catabolism in cancer remains obscure. Here, we report the full-length cloning of E3α-II, a novel “N-end rule” ubiquitin ligase, and its functional involvement in cancer cachexia. E3α-II is highly enriched in skeletal muscle, and its expression is regulated by proinflammatory cytokines. In two different animal models of cancer cachexia, E3α-II was significantly induced at the onset and during the progression of muscle wasting. The E3α-II activation in skeletal muscle was accompanied by a sharp increase in protein ubiquitination, which could be blocked by arginine methylester, an E3α-selective inhibitor. Treatment of myotubes with tumor necrosis factor alpha or interleukin 6 elicited marked increases in E3α-II but not E3α-I expression and ubiquitin conjugation activity in parallel. E3α-II transfection markedly accelerated ubiquitin conjugation to endogenous cellular proteins in muscle cultures. These findings show that E3α-II plays an important role in muscle protein catabolism during cancer cachexia and suggest that E3α-II is a potential therapeutic target for muscle wasting.

INTRODUCTION

Mounting evidence suggests that activation of the ubiquitin-proteasome system underlies the profound skeletal muscle wasting seen in catabolic disease states, including cancer (1–3). Most intracellular proteins in skeletal muscle are degraded through the ubiquitin-proteasome system (4–5), in which proteins are marked for proteasomal degradation by the conjugation of ubiquitin molecules. Ubiquitin conjugation initially involves activation by the E1 enzyme. Activated ubiquitin is transferred to the E2 enzyme that serves as a carrier and interacts with a specific E3 enzyme (ubiquitin ligase). The ubiquitin ligase binds to the protein substrates to be degraded and catalyzes the transfer of ubiquitin from the E2 carrier enzyme to the substrate to generate an ubiquitin chain. These polyubiquitinated substrates are targeted to the 26S proteasome and rapidly degraded. Because target proteins bind to the E3 ligase before conjugation, it has been suggested that the ubiquitin ligase determines the specificity and rate of the degradative system (6). This raises the potential of E3 as a therapeutic target.

The N-end rule pathway (7) is one of the best-characterized ubiquitin ligase-dependent degradation systems, which is known to selectively degrade proteins with basic or large hydrophobic NH2-terminal residues. The UBR1 gene encoding the N-end rule ubiquitin ligase has been studied intensively in yeast (7). A mammalian counterpart of yeast UBR1, referred to as E3α, recently was reported, including a full-length mouse cDNA and a partial human cDNA (8). Biochemical studies suggest that the N-end rule pathway catalyzes the breakdown of a major fraction of soluble proteins in skeletal muscle (9–10) and is accelerated during pathologic states of muscle wasting (11). However, it remains unclear as to which ubiquitin ligase(s) is directly involved in the control of muscle protein catabolism.

Here, we have cloned the human and mouse full-length cDNAs encoding a novel N-end rule ubiquitin ligase, E3α-II, and compared its functional significance with the reported mammalian N-end rule ubiquitin ligase UBR1/E3α (ref. 8; referred to as E3α-I herein) with respect to its role in protein catabolism during cancer cachexia. We found that E3α-I and E3α-II were significantly up-regulated in skeletal muscle during cancer cachexia and that transfection of either E3α-I or E3α-II dramatically stimulates protein ubiquitination in muscle cells. However, E3α-II appeared to be more critically involved in muscle wasting because E3α-II was not only more specifically expressed in muscle tissues but also it was differentially activated by tumor necrosis factor alpha (TNF-α) or interleukin 6 (IL-6), major proinflammatory cytokines known to be involved in the development of cachexia (2, 12–14). Furthermore, E3α-II expression was significantly up-regulated at the early onset of muscle wasting when E3α-I expression was unchanged. We postulate that the novel N-end rule ubiquitin ligase E3α-II is an important downstream molecular target for muscle protein catabolism and discuss future experiments to explore the therapeutic importance of the E3α family in muscle wasting.

MATERIALS AND METHODS

Full-length cDNA Cloning. BLASTN and BLASTP searches were performed against the Amgens internal EST database (Ampelnet: Amgen, Thousand Oaks, CA) and public database (GenBank) using nucleotide and amino acid sequence of mouse UBR1 (8) as queries. Multiple sets of oligonucleotides were designed based on the identified ESTs and were used to probe commercially available skeletal muscle Marathon-Ready cDNA libraries (Clontech, Palo Alto, CA). Full-length cDNAs were generated by subcloning (15–16) using selected PCR products spanning the entire coding regions of huE3α-II, huE3α-I, and huE3α-I, verified through confirmation sequencing, and deposited into American Type Culture Collection (Manassas, VA).

Cell Culture and Transfection. C2C12 and L6 myoblasts were obtained from American Type Culture Collection. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented (Life Technologies, Rockville, MD) with 10% fetal bovine serum and 1 μg/mL insulin. Cell differentiation was induced with Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum and 1 μg/mL insulin. Myoblast differentiation was treated with 10 ng/mL TNF-α (R&D Systems, Minneapolis, MD) or with 10 ng/mL IL-6 (R&D Systems) up to 5 days. For transfection, huE3α-II and huE3α-I were subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) to generate pcDNA-E3α-II and pcDNA-E3α-I. The cultures were transiently transfected with pcDNA-E3α-II and pcDNA-E3α-I or pcDNA-E3α-II (E3α-II) or mock transfected with pcDNA3.1 only (mock) using Lipofectamine 2000 and the manufacturer’s protocols (Life Technologies).
Northern Blot Analysis. Pair-fed, non–tumor-bearing control and tumor-bearing animals (n = 6 per mouse group; n = 8 per rat group) were killed by CO2 asphyxiation at 3 and 5 days post–Yoshida ascites hepatoma (YAH) tumor implantation (rats) and at 12 and 17 days after colon-26 adenocarcinoma (C26) tumor implantation (mice). Both the medial gastrocnemius muscles were rapidly dissected and frozen immediately in liquid nitrogen. All of the gastrocnemius muscles collected from each experimental animal group were combined. RNA was isolated from each of the pooled muscle samples and from the myotube cultures by using the TRIzol reagent following manufacturer’s protocols (Life Technologies). Equal amounts of total RNA (20 μg per lane) were separated by electrophoresis through 1% agarose gels. The separated RNA was transferred to nylon membranes and cross-linked by exposure to UV light. The membranes containing mouse or rat RNA were hybridized with cDNA probes for muE3-II or muE3-I (corresponding to amino acid position 360 to 517 and 361 to 517, respectively). Human multiple tissue RNA blots (Clontech) were hybridized with CDNA probes for huE3-I or huE3-II (corresponding to amino acid position 1157 to 1388 and 1186 to 1337, respectively). Radiolabeling of cDNA probes with [32P]dCTP was performed using the Prime-It-RnT Random Primer labeling kit (Stratagene, La Jolla, CA). Membranes were prehybridized, hybridized, and washed using the method of Church and Gilbert (15) and exposed to X-ray film (Kodak, Rochester, NY) at −70°C. All of the probed blots subsequently were stripped and rehybridized with a [32P]dCTP-labeled β-actin probe (Clontech) to confirm that equal amounts of RNA were transferred to the membranes. E3 expression levels were analyzed using PhosphorImager (STORM 860; Amersham Biosciences, Piscataway, NJ) equipped with quantitation software (ImageQuant 5.0; Amersham Biosciences) and were normalized against β-actin levels.

RESULTS

Full-length cDNA Cloning of Human E3α-II and E3α-I and Murine E3α-II. To critically evaluate the function of the N-end rule pathway in protein catabolism, we set out to clone the N-end rule ubiquitin ligases. Using the reported murine UBR1/E3α DNA sequence (8) as query, we performed extensive bioinformatic analysis against the public (GenBank) and the Amgen internal (Amgenesis) EST databases, followed by homology cloning. This led to the isolation of mouse and human full-length cDNAs encoding the entire open reading frame of a novel E3 ubiquitin ligase, E3α-II (referred to as muE3α-II and huE3α-II, respectively). We denote it as E3α-II because of its structural homology and activity resemblance to the known murine N-end rule ubiquitin ligase UBR1/E3α (referred to as muE3α-I herein; ref. 8). For structural analysis, we also isolated the full-length cDNA encoding human E3α-I (referred to as huE3α-I). Transformation with full-length E3α-II or E3α-I cDNA appeared to be toxic to Escherichia coli, and as a result, the frequencies of getting transformants containing the full-length E3α-II or E3α-I cDNA inserts were <1/500. We verified the sequences of the full-length cDNA inserts by confirmation sequencing against multiple clones and cloned the confirmed full-length cDNAs into the mammalian expression vector pcDNA3.1 for transfection purposes. To our knowledge, this is the first successful isolation of full-length E3α-II and the first successful attempt to construct full-length E3α-II and E3α-I mammalian expression vectors. To define the genomic structures of the human E3α family, we performed genomic database analysis using the full-length human E3α-II and E3α-I cDNAs as queries. The results revealed that the human E3α-I gene consists of 47 exons and is located on chromosome 6, whereas the human E3α-II gene is made up of 48 exons and is located on chromosome 15. Fig. 1 shows the sequence alignment of huE3α-II, muE3α-II, and huE3α-I with the reported mouse E3α/UBR1 (muE3α-I; ref. 8) at amino acid level. E3α-II and E3α-I exhibit ~58% overall sequence homology and greater homology within a number of highly conserved regions, including domains I through V and the basic residue-rich region originally reported for yeast UBR1 (8, 17). In addition, 41 identical cysteine residues and the residues in yeast UBR1 that were reported to be necessary for type 1 or type 2 substrate binding (7, 8, 17–18) are found to be conserved. These structural features identify E3α-II as a new N-end rule ubiquitin ligase and clearly define the existence of a mammalian E3α ubiquitin ligase family.

Muscle-specific Expression of E3α-II. Northern blot analysis using E3α-II– or E3α-I–selective probes (see Materials and Methods) revealed that the tissue expression profile of E3α-II was different from that of E3α-I, E3α-II is the more muscle-specific form of E3α family (Fig. 2A) in human tissues and was found to be most highly expressed in skeletal muscle with moderate expression in heart, minor expression in kidney, and minimal expression in other tissues examined, including brain, colon, thymus, spleen, liver, intestine, placenta, lung, and peripheral WBCs. In contrast, moderate levels of E3α-I (Fig. 2C) were found to spread through many nonmuscle tissues examined, although skeletal muscle and heart had relatively higher levels of the E3α-I message. Similar results were obtained from Northern blot analysis using mouse and rat tissues (data not shown). The E3α-II–selective probes detected two bands in blots containing human or mouse RNA isolated from muscle tissues, including a higher molecular weight band of >7.5 kb and a lower molecular weight band of ~6 kb (Fig. 2A and B). However, when rat tissue RNA was examined, only a single band of >7.5 kb was detected with the same E3α-II–selective probes (Fig. 3B). Whether the lower molecular weight band seen in human and mouse Northern blots corresponds to an alternatively spliced form of E3α-II or a homologous gene needs to be further investigated.

Role of E3α Family in Muscle Protein Catabolism during Cancer Cachexia. To examine the role of the E3α gene family in muscle protein catabolism during disease states, we analyzed the expression levels of E3α-II and E3α-I in skeletal muscle using two tumor-implantation models of cancer cachexia: YAH-130 tumor-bearing rats (19, 22) and C26 tumor-bearing mice (20, 21). These tumor-bearing models mimic human cancer cachexia with a pronounced loss of muscle mass at low tumor burden. The tumor-bearing animals also show a reduction in food intake (19–22). However, the rapid muscle
wasting could not be accounted for by reduced food intake because pairing feeding that simulated the semifasting condition in the tumor-bearing state had no significant effect on muscle mass. It has been shown that in the YAH model, the rate of muscle protein catabolism increases rapidly in a manner that is independent of food intake by 63% to 90% at 5 days of tumor implantation (19, 22). Detailed descriptions of food intake, progressive weight loss, and loss of muscle protein mass and selective activation of ubiquitin-proteasome protein catabolism are provided elsewhere (19, 22). Under pairing conditions, a progressive loss of skeletal muscle weight was observed ranging from 4% to 9% decrease at the early stage (12 days post-C26 implantation and 3 days post–YAH-130 implantation) to 25% to 26% decrease at the late stage (17 days post-C26 tumor implantation and 5 days post–YAH-130 tumor implantation) relative to the non–tumor-bearing paired-fed control animals (Fig. 3A). We examined in parallel the expression levels of E3/H9251-I and E3/H9251-II and the protein catabolism are provided elsewhere (19, 22). Under pairing conditions, a progressive loss of skeletal muscle weight was observed ranging from 4% to 9% decrease at the early stage (12 days post-C26 implantation and 3 days post–YAH-130 implantation) to 25% to 26% decrease at the late stage (17 days post-C26 tumor implantation and 5 days post–YAH-130 tumor implantation) relative to the non–tumor-bearing paired-fed control animals (Fig. 3A). We examined in parallel the expression levels of E3-I and E3α-II and the
ubiquitin-protein conjugation activities in skeletal muscle at the early and late stages of muscle wasting in these models. Changes in E3/H9251-II occurred concordantly with the early onset and the continued progression of muscle atrophy in both models examined. As shown in Fig. 3, at the early stage of muscle wasting, there was a significant (approximately twofold) increase in E3/H9251-II expression in the gastrocnemius muscle of the tumor-bearing animals as compared with that in pair-fed control animals, whereas the level of E3α-I appeared to be unaltered (Fig. 3B). At the later stage of muscle wasting, increased expression of E3α-II (more than threefold) and E3α-I (twofold to threefold) was detected in the gastrocnemius muscle of the tumor-bearing animals as compared with their expression levels in pair-fed, non-tumor-bearing control animals (Fig. 3B). Moderate levels of E3α-I and E3α-II were expressed in heart; however, Northern blot analysis revealed no significant alteration in either E3α-I or E3α-II expression in heart at the early and late stages of tumor implantation of mice bearing C26 (data not shown). To determine the rates of ubiquitin conjugation in skeletal muscle from the animal models, muscle lysates (fraction II) of gastrocnemius muscles from tumor-bearing and pair-fed control animals were subjected to ubiquitination reactions in the presence of 125I-ubiquitin (see Materials and Methods). In parallel with the increase in E3α-II expression at the early stage, as well as with the increases in E3α-II and E3α-I expression at the late stage, there was a significant increase in ubiquitin conjugation activities in the gastrocnemius muscle of the YAH-130 tumor-bearing rats (Fig. 3C) and C26 tumor-bearing mice (data not shown). We also examined whether the activation of the N-end rule pathway mediated the accelerated muscle protein ubiquitination. Ubiquitin conjugation assays revealed that the gastrocnemius muscle lysates from the YAH-130 tumor-bearing rats (Fig. 3C) and the C26 tumor-bearing mice (data not shown) catalyzed the ubiquitination of exogenously added 125I-κ-lactalbumin, a bona fide N-end rule substrate, significantly faster than those from pair-fed control animals. Moreover, the addition of the E3α-selective inhibitor ArgME (7, 10, 18) to the muscle lysates virtually abolished the accelerated ubiquitination activity seen in the cachectic muscle lysates (Fig. 3C). In a separate study using recombinantly produced E3α-II, we confirmed that E3α-II was capable of catalyzing ubiquitin conjuga-
Fig. 4. Differential induction of E3α-II expression by TNF-α and IL-6 and the effect of E3α transfection on protein ubiquitination in C2C12 myoblast cultures. A, Northern blot analysis of the effects of TNF-α or IL-6 treatment on E3α-II and E3α-I expression in differentiated C2C12 myoblast cultures. The same Northern blots were stripped and rehybridized with a [32P]dCTP-labeled β-actin probe, and no significant difference in β-actin levels was detected (data not shown). B, TNF-α or IL-6 treatment elicited marked increase in ubiquitination activity in C2C12 cultures as revealed by ubiquitin conjugation assays. Ubiquitin conjugation to endogenous cellular proteins (left) and to exogenously added 125I-α-lactalbumin (right). C, Transfection with E3α-II or E3α-I significantly stimulates ubiquitination activity in C2C12 cultures. Transient transfection was performed using human E3α-II or E3α-I (E3α-II and E3α-I). Mock transfection with the pDNA vector (MoxE) was performed as control. Cell lysates were prepared from the cultures at 48 hours after transfection and subjected to ubiquitin conjugation reactions (see Materials and Methods). Left, ubiquitin conjugation to endogenous cellular proteins; right, ubiquitin conjugation to exogenously added α-lactalbumin, which was inhibited by ArgME ubiquitin conjugation activities, were quantified by measuring the radioactivity incorporated into the ubiquitin conjugates using Phosphor-Imager.

Differential Induction of E3α-II by TNF-α and IL-6. Using differentiated myoblast cultures, we examined whether TNF-α and IL-6 were capable of activating the E3α family in muscle cells because these two major proinflammatory cytokines were known to be the key humoral mediators of muscle wasting and cachexia in the C26 and YAH-130 tumor-implantation models used in our studies. IL-6 was reported as a cachetic factor in the development of cancer cachexia in the C26 model (20, 21), whereas TNF-α was shown to mediate the activation the ubiquitin-dependent proteolytic system in the YAH model (23). Fig. 4A shows the results of Northern blot analysis of E3α-II and E3α-I levels in differentiated C2C12 myoblast cultures that had been incubated with or without treatment with TNF-α or IL-6. Remarkably, treatment with either TNF-α or IL-6 resulted in a twofold to fourfold induction in E3α-II expression without detectable alteration in E3α-I expression. Parallel examination of the ubiquitination activities in lysates of TNF-α- or IL-6–treated cultures revealed that TNF-α or IL-6 treatment led to a significant increase in the ubiquitin conjugation to endogenous cellular proteins (Fig. 4B, left) and to exogenously added α-lactalbumin (Fig. 4B, right). These data show that TNF-α and IL-6 stimulate protein ubiquitination in muscle cells via, at least in part, the induction of E3α-II. Many proinflammatory cytokines, including TNF-α and IL-6, have been shown to be involved in human cachectic disease states, such as cancer cachexia, AIDS, inflammatory cachexia, renal cachexia, burns, and sepsis (1–3, 23–28). E3α-II activation in muscle may be an important molecular mechanism by which proinflammatory cytokines and possibly other cachetic factors induce protein catabolism and muscle wasting.

Effect of Transfection E3α-I and E3α-II on Protein Ubiquitination in Muscle Cell Cultures. To show conclusively that increased E3α gene expression leads to accelerated ubiquitination in muscle cells, we transfected mouse C2C12 and rat L6 myoblast cultures with huE3α-II and huE3α-I and performed ubiquitin conjugation assays using freshly prepared cell lysates. Transfection of C2C12 cultures with either huE3α-II or huE3α-I dramatically stimulated the ubiquitination of the endogenous cellular proteins (Fig. 4C, left). Similar results were obtained from E3α-II– or E3α-I–transfected L6 myoblast cultures (data not shown). Lysates from E3α-II– or E3α-I–transfected cultures catalyzed ubiquitin conjugation to exogenously added 125I-α-lactalbumin significantly faster than those from control cultures, whereas addition of ArgME virtually completed blocked this accelerated ubiquitin conjugation activity (Fig. 4C, right). Therefore, an increase in the expression of either E3α-II or E3α-I is sufficient to cause accelerated protein ubiquitination in muscle cells. Ubiquitin conjugation is the prerequisite and rate-limiting step in proteasome-mediated proteolysis. Our results are consistent with the report that the N-end rule pathway activity mediates the breakdown of a major fraction of muscle proteins (9, 10) and further pinpoint a pivotal role of E3α-II and E3α-I, rate-limiting enzymes of the pathway, in the regulation of muscle protein catabolism.

**DISCUSSION**

We have identified a novel mammalian N-end rule ubiquitin ligase E3α-II and elucidated its critical role in mediating muscle protein...
ubiquitination and in particular its regulation during cancer cachexia and by proinflammatory cytokines. Our data show that E3α-II plays a key role in muscle protein catabolism in the two experimental models of cancer cachexia examined. At the early onset of muscle wasting in the C26 and YAH-130 tumor-implantation models, a differential induction of E3α-II, but not E3α-I, occurred concomitantly with a significant increase in ubiquitin conjugation to endogenous muscle proteins and to N-end rule model substrate α-lactalbumin. At this early stage of muscle wasting, addition of E3α-selective inhibitor ArgME completely abolished the increased ubiquitination activity, indicating that the activation of E3α-II was responsible for the accelerated ubiquitin conjugation activity. That overexpression of E3α-II leads to accelerated protein ubiquitination has been unequivocally shown with our E3α-II transfection experiments. Similar to what was observed in cancer cachexia models, transfected muscle cultures show a sharp increase in ubiquitin conjugation to endogenous proteins and to exogenously added N-end rule model substrate. It is noteworthy that E3α-II is not only muscle specific but also the proinflammatory cytokine-inducible form of the E3α family. TNF-α and IL-6 treatment differentially induced the expression of E3α-II and a parallel increase in ubiquitin conjugation activity in cultured myotubes without affecting E3α-I expression levels. We also examined MAPb/Atrogmin-1 expression in myotube cultures treated with TNF-α or IL-6 but observed no significant change in its expression (data not shown). Our in vitro results corroborate our in vivo findings in YAH and C26 cachexia models, in which TNF-α and IL-6 were known to play a key role in protein catabolism and the development of muscle wasting. Two other ubiquitin ligases, including MuRF1 (29), a RING finger protein, and MAVb or Atrogmin-1 (29, 30) of the SCF family, recently also have been reported to play a role in muscle atrophy. Conceivably, multiple ubiquitin ligases may operate in muscle atrophy by different mechanisms, with each playing a nonredundant role. Additional experiments will be needed to clarify the relative contribution of different ubiquitin ligases to muscle wasting under different disease conditions.

Further studies are required to better understand the importance of the E3α ubiquitin ligase family in catabolic disease states. These include identifying the physiologic substrates for E3α-II and E3α-I in skeletal muscle, elucidating various signaling events that regulate the activity of the E3α family, and analyzing the effects of E3α blockade through gene ablation and/or the design of selective small molecule inhibitors on animal’s tolerance to cachectic challenges. Ubiquitin ligases are attractive molecular targets for manipulation of proteolysis because they are muscle-specific isoforms, and their activation may be specific to different forms of muscle wasting, such as disuse atrophy or that associated with cancer and inflammation. These features may potentially allow for local suppression of muscle catabolism without affecting the basal proteolytic processes in nonmuscle tissues or associated with essential functions, such as antigen processing in antigen-presenting cells. Activation of the ubiquitin-proteasome system is common to many models of cancer cachexia regardless of whether one or another hormone, cytokine, or other factors appear to be the humoral signal for the system’s activation (31). The position of E3 in the span of the pathway of muscle protein catabolism that is common to multiple hormones, cytokines, and other factors would allow for a simplification of antitumoral therapies directed at this step rather than attempts to individually monitor and manipulate humoral mediators of diverse types in cancer patients.

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REFERENCES

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