Muscle wasting occurs when rates of protein degradation outstrip rates of protein synthesis. Accelerated rates of protein degradation develop in atrophying muscle largely through activation of the ubiquitin-proteasome pathway. The complexity of the ubiquitination process, however, has hampered our understanding of how this pathway is activated in atrophying muscles and which enzymes of the ubiquitin conjugation system are responsible. Recent studies demonstrate that two ubiquitin–protein ligases (E3s), atrogin-1/MAFbx and MuRF1, are critical in the development of muscle atrophy. Other experiments implicate E2 14k and E3 /H9251, of the N-end rule pathway, as important players in the process. It seems likely that multiple pathways of ubiquitin conjugation are activated in parallel in atrophying muscle, perhaps to target for degradation specific classes of muscle proteins. The emerging challenge will be to define the protein targets for, as well as to develop inhibitors of, these E3s.

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Keywords: Atrogin-1, MAFbx, MuRF1, UBR1, Ubiquitin, E3

1. Introduction

Muscle wasting and fiber atrophy are seen frequently as the result of poor nutritional states and diseases, including cancer, uncontrolled diabetes, uremia, and sepsis. Beyond its contractile function, muscle serves as a major protein reservoir that can be mobilized in stressful states as a source of amino acids for energy. The reduction in function and...
strength of muscle that results from atrophy is a major factor contributing to the morbidity characteristic of these disease states. Recent work has demonstrated that the rapid atrophy in experimental models of these various diseases is due primarily to enhanced degradation of muscle protein (Lecker, Solomon, Mitch, & Goldberg, 1999; Lecker, Solomon, Price et al., 1999). In all types of atrophying muscle, a common set of transcriptional adaptations and biochemical changes enhance the cell’s capacity for protein breakdown (Hasselgren & Fischer, 2001; Lecker, Solomon, Mitch et al., 1999; Lecker, Solomon, Price et al., 1999; Lecker et al., 2004; Mitch & Goldberg, 1996). Clearly, a better understanding of the cellular pathways of protein degradation that are activated during muscle atrophy is not only of scientific interest, but may also help direct us towards rational preventative measures and treatments. Towards this goal, a number of major advances have recently been made suggesting that multiple protein degradation enzymes are activated during muscle wasting.

In muscle as in all other tissues, the vast majority of intracellular proteolysis occurs through the ubiquitin-proteasome system (Rock et al., 1994). Moreover, it is now well-established that this pathway is of primary importance in the enhanced protein degradation that occurs in muscle as it atrophies (Lecker, Solomon, Mitch et al., 1999; Lecker, Solomon, Price et al., 1999). This system is characterized by the concerted action of enzymes that link chains of the polypeptide cofactor ubiquitin onto proteins to mark them for degradation (Glickman & Ciechanover, 2002) (Fig. 1). This tagging process leads to their recognition by the 26S proteasome, a large multisubunit, multicatalytic protease complex that degrades ubiquitinated proteins to small peptides (Baumeister, Walz, Zuhl, & Seemuller, 1998). Three enzymatic components are required to link chains of ubiquitin onto proteins destined for degradation. E1 (Ub-activating enzyme) and E2s (Ub-carrier or conjugating proteins) prepare ubiquitin for conjugation but the key enzyme in the process is the E3 (Ub-protein ligase), since it couples the activated ubiquitin to the protein substrate (Koegl et al., 1999). Clearly, a better understanding of the enzymatic conjugation cascade (Schuffner, Nuber, & Huber, 1995; Scheffner, Nuber, & Huibregtse, 1995; Rock et al., 1994) is the E3 (Ub–protein ligase), since it couples the activated ubiquitin to the protein substrate (Fig. 1). The E3, then, is the component of the conjugation apparatus that confers specificity to the system. While only a single E1 protein and a few dozen E2 proteins exist in cells, there are more than a thousand E3s. Generally, E3s fall into two broad categories: they either contain HECT (homologous to E6-AP carboxy-terminus) domains or RING fingers (Jackson et al., 2000). Like E2s, HECT-domain E3s directly bind activated ubiquitin and thus are actual components of the enzymatic conjugation cascade (Schuffner, Nuber, & Huber, 1995). RING-containing E3s catalyze ubiquitin conjugation by a less direct mechanism, probably by creating an appropriate microenvironment for transfer of activated ubiquitin (on the E2) to a lysine residue on the protein target (Lorick et al., 1999; Seol et al., 1999). The HECT-domain family is the smaller of the two groups, first identified in E6-AP (Huibregtse, Schuffner, Beaudenon, & Howley, 1995) that targets the viral E6 protein for degradation. More recent work also has found HECT domains in Nedd4 that ubiquititates the epithelial sodium channel (Staub et al., 1996) and KIAA10 (You & Pickart, 2001) (see below).

The vast majority of E3s, however, contain RING finger domains. These 40–60 residue zinc-binding motifs contain core cysteine and histidine amino acids arranged in a C3HC4 (“RING”) or C3HSC3 (“RING-H2”) pattern (Deshaiæs, 1999). RING fingers are typically found in a small protein that forms one subunit of a larger complex that together bears ubiquitin ligase activity. The prototype of such an E3 is the SCF (Skp1-Cullin-Fbox) complex (see below, Fig. 2) (Cardozo & Pagano, 2004; Deshaïæs, 1999). RING fingers have also been found, however, in many single subunit E3s such as E3x/UBR1, MuRF1 (Muscle-specific RING Finger protein 1) and Mdm2 (see below, Fig. 2) (Ellenbææs, Dobbelstein, Roth, Shenk, & Levine, 1996; Kwon et al., 1998; Spencer, Eliazer, Hara, Richardson & Olson, 2000). Recently, a novel group of enzymes with ubiquitin ligase activity have been identified. These U-box domain proteins, such as UFD2 and CHIP, contain atypical RING fingers which lack the canonical cysteine residue usually required for Zn2+ coordination (Hatakeyma & Nakayama, 2003). These proteins are sometimes referred to as E4s, and may in some cases act in association with other E3s to enhance the number and rate of ubiquitin residues attached to substrates (Koegl et al., 1999).

2. **E246, E3α and the N-end rule pathway**

The key role of the Ub-proteasome pathway in muscular wasting has helped shift research in the field to-
Fig. 1. Ubiquitin conjugation to protein substrates. Ubiquitin chains are conjugated to substrate proteins destined for destruction by a multistep process. First, the carboxyl terminal glycine residue of ubiquitin (Gly76) is activated by thiolester linkage to E1, in a reaction that consumes ATP, and releases AMP and pyrophosphate. The activated ubiquitin is then transferred to a sulfhydryl group on an E2 (ubiquitin carrier or conjugating protein). The activated ubiquitin is then conjugated to the ε-amino group of a lysine in the substrate protein, in a reaction catalyzed by an E3. Subsequent ubiquitin moieties are then conjugated to the prior ubiquitin in a similar cascade, usually at the ε-amino group of ubiquitin Lys48.
Fig. 2. Structure of E3s involved in muscle atrophy. E3 components are indicated by dark grey shading. Note that the SCF Atrogin-1 E3 complex is made up of four components. Atrogin-1, the F-box component, presumably also binds to the protein substrate ubiquitinated by the complex. The RING finger motifs within each of these E3s are noted in black. E2: ubiquitin carrier protein.

Towards identifying, from the very large number of enzymes involved in ubiquitin conjugation and specifically Ub-protein ligases (E3s) in muscle, the specific ones responsible for the activation of proteolysis during atrophy. Three candidates show promise as important players. E3α (also called UBR1) was the first to be implicated in the atrophy process (Solomon & Goldberg, 1996; Solomon, Baracos, Sarraf, & Goldberg, 1998; Solomon, Lecker, & Goldberg, 1998). This large, 200 kDa, single subunit ubiquitin-protein ligase contains a modified RING-H2 domain and acts together with the ubiquitin carrier protein, E214k. It is believed to recognize substrate proteins that begin with unblocked hydrophobic or basic amino acids (called the N-end rule pathway) (Kwon, Xia, Davydov, Lecker, & Varshavsky, 2001; Varshavsky, Turner, Du, & Xie, 2000; Xie & Varshavsky, 1999). The S. cerevisiae homolog of UBR1 also has been demonstrated to degrade coherins in a step required for chromosome segregation (Rao, Uhmann, Nasmyth, & Varshavsky, 2001). To complicate matters, in addition to recognizing proteins at their amino termini, S. cerevisiae UBR1 can also recognize substrates based on internal motifs, and can be allosterically activated by binding hydrophobic and basic amino acids (Du, Navarro-Garcia, Xia, Tasaki, & Varshavsky, 2002; Turner, Du, & Varshavsky, 2000).

The connection between E3/H9251 and muscle atrophy began with the finding that mRNA for E214k and UBR1 are increased in atrophying muscles (Fischer, Sun, Gang, Pritts, & Hasselgren, 2000; Lecker, Solomon, Mitch, et al., 1999; Lecker, Solomon, Price, et al., 1999; Wing & Banville, 1994). In addition, experiments in cellular extracts prepared from atrophying muscles show increased rates of ubiquitin conjugation via this pathway, and inhibitors E3/H9251 reduce accelerated ubiquitin conjugation rates back to baseline levels (Lecker, Solomon, Mitch, et al., 1999; Lecker, Solomon, Price, et al., 1999; Solomon, Baracos, et al., 1998; Solomon, Lecker, et al., 1999). Because the vast majority of cellular proteins begin with methionine and are acetylated, the specific proteins ubiquitinated by E3/H9251 in muscle, and how they may be generated are quite unclear. The finding of an important role of this enzyme in the accelerated ubiquitin conjugation that occurs during atrophy suggests that there might be an undefined proteolytic enzyme that clips muscle proteins and generates free amino-terminal residues that can be recognized by this E3. Both calpains (Williams et al., 1999) and caspases are obvious candidates for such endoproteases and there may be emerging evidence that caspases are able to cleave muscle proteins and generate such fragments. dIAP1, a drosophila inhibitor of apoptosis, is cleaved by caspases leading to a short form of dIAP1 that is rapidly degraded via the N-end rule pathway (Ditzel et al., 2003). Furthermore, caspase 3 is known to be able to cleave myosin light chain (Moretti et al., 2002), and Mitch and colleagues (Du et al., 2004) recently demonstrated that caspase 3 can also cleave purified actomyosin complex in vitro to yield a 14 kDa actin fragment. This actin fragment was also found in “atrophy” cultured muscle cells treated with staurosporine (to activate cas-
pase 3) or deprived of serum (to mimic atrophy induced by food deprivation), as well as in atrophying muscles from diabetic and uremic rats. Interestingly, although this fragment seems to be further degraded by the Ub–proteasome pathway, the identity of its amino-terminal residue and whether E3s/UBR1 and the N-end rule pathway is involved in its ubiquitination is not known. These data have, however, led Mitch and coworkers to suggest that caspase 3 cleavage of actin might be an initial step in the enhanced proteolysis in muscle wasting conditions.

Most recently, a homologue of E3α, E3α-II, was identified by Han and coworkers (Kwak et al., 2004). This protein shares 60% overall sequence homology with E3α and was previously found by Varshavsky and his investigators, who named it UBR2 (Kwon et al., 1998, 2001). Han and colleagues found a dramatic up-regulation of E3α-II in atrophying muscles of tumor-bearing rodents. Interestingly, transfection of E3α-II into muscle cells in culture markedly increases rates of N-end rule ubiquitination (Kwak et al., 2004). Their data suggest that E3α-II or the combination of E3α and E3α-II may be responsible for the increased rates of ubiquitination by the N-end rule pathway found in atrophying muscle.

The findings in cell extracts from atrophying muscle, that rates of ubiquitin conjugation by E214k and E3α/UBR1 are increased, have led to experiments in mice lacking these proteins to test whether such animals are resistant to the development of muscle atrophy. Both the E214k (HR6B−/−) and E3α (UBR1−/−) knockout mice, however, develop muscle atrophy at the same rate as controls in response to food deprivation (Adegoke, Bedard, Roest, & Wing, 2002; Kwon et al., 1998, 2001; Roset et al., 1996). Furthermore, UBR2 null mice have normal muscles, though no studies have been performed to date to measure their response to catabolic stimuli (Kwon et al., 2003). These negative results are complicated by the fact that another isoform of E214k/HR6B exists (HR6A) (Roset et al., 1996) and at least one other protein with homology to UBR1 and UBR2 (i.e. UBR3) is present in mammalian muscle tissue (Kwon et al., 1998, 2001). The redundancy of enzymes in this pathway, then, makes it difficult to draw definitive conclusions regarding the role of the N-end rule pathway in muscle wasting. Clearly, animals with these multiple isoforms deleted will need to be generated to be able to definitively test the importance of the N-end rule pathway. This may be difficult or impossible to perform as it is already known that HR6A/B (Roset et al., 1996) and UBR1/2 (Kwon et al., 2001) double knockouts are not viable.

3. Atrogin-1/MAFbx

As an independent means to identify the components of the ubiquitin-proteasome pathway activated during muscle wasting, we have used cDNA microarrays to compare the transcriptional profile in atrophying muscles from fasted mice (Jagoe, Lecker, Gomes, & Goldberg, 2002), and more recently in rats with chronic renal failure, cancer cachexia and uncontrolled diabetes (Lecker et al., 2004). These analyses led to our identification of a transcript that is induced more than eight-fold in muscle from fasted mice (Gomes, Lecker, Jagoe, Navon, & Goldberg, 2001). The transcript is also strongly induced in muscles from the diabetic, uremic and tumor-bearing animals, as well as in atrophying human muscles (Jones et al., 2004). We cloned the gene, which we named atrogin-1. It contains an F-box domain that characterizes the class of Ub–protein ligases (E3s) called SCF complexes (Gomes et al., 2001). SCF complexes are modular E3s made up of Skp1, a cullin (Cul) protein, a RING finger protein and an F-box protein (Fig. 2) (Deshaies, 1999). The F-box protein, of which atrogin-1 is an example, is the component that links the protein substrate to be ubiquitinated with the rest of the E3 and ubiquitination machinery (Cardozo & Pagano, 2004). F-box proteins, in addition to the F-box domain that binds to Skp1, also contain a substrate recognition domain. While no clear motifs are present in atrogin-1 that may suggest which proteins in muscle it recognizes (e.g. leucine rich repeats and WD40 domains that typically recognize phosphorylated proteins), our initial characterization of atrogin-1 demonstrated that the 42 kDa protein does contain two other motifs which might suggest functional roles. The protein contains both a PDZ-binding domain, implicated in protein–protein interactions (Doyle et al., 1996), and a nuclear localization sequence. Indeed, data suggest that atrogin-1 can be found in the nuclei of muscle cells (Sandri et al., 2004, and our unpublished observations). The activity of SCF complexes can be further regulated by other protein–protein interactions. Nedd8, a ubiquitin-like protein can be conjugated to the Cul1
protein, enhancing its activity and helping to recruit the E2 to the SCF complex (Pan, Kentis, Dias, Yanoah, & Wu, 2004). In addition, CAND1 (Cullin-associated Neddylation-associated) can also bind to cullin proteins and interrupt the Neddylation modification and the formation of an active SCF complex (Liu, Furukawa, Matsumoto, & Xiong, 2002; Zheng et al., 2002). It is as yet unknown whether these modifications or interactions regulate the atrogin-1 SCF complex.

Our early studies indicated that expression of atrogin-1 is restricted to striated and cardiac muscle with induction at least 12 h before significant gastrocnemius muscle weight loss occurs (Gomes et al., 2001), strongly suggesting a role for atrogin-1 in the early phases of the atrophy process. Concomitant with our studies, Glass and colleagues at Regeneron Pharmaceuticals found the same gene (which they named MAFbx, for Muscle Atrophy F-Box protein) to be induced in muscles atrophying due to denervation, immobilization and hindlimb suspension (Bodine et al., 2001). Since these initial reports, increased atrogin-1 expression has also demonstrated in the muscle from the cellular ligation and puncture model of sepsis in rats (Wray, Mammen, Hershko, & Hasselgren, 2003) and by others studying muscle during fasting and diabetes (Dehoux et al., 2004). However, perhaps the most powerful data linking atrogin-1 with the generation of muscle wasting was obtained by the Regeneron group who demonstrated that mice lacking the MAFbx gene are resistant to denervation atrophy, with a 56% sparing of muscle weight loss after 14 days of denervation (Bodine et al., 2001). Conceptually, one could imagine atrogin-1 targeting for degradation key nuclear regulatory proteins or transcription factors that in turn might lead to a decrease in protein synthesis in atrophying muscle, or conversely, it could enhance bulk muscle protein degradation directly by ubiquitinating myofibrillar components. Clearly, identification of atrogin-1 targets is of primary importance in clarifying these issues. On the other hand, recent work in the oncology field found atrogin-1 induced in gastrointestinal tumors undergoing involution after Gleevec treatment (Frolov et al., 2001), raising some question as to the true tissue specificity of atrogin-1. In addition, a recent report showed that in cardiac muscle, atrogin-1 is able to ubiquitinate and target for degradation calcineurin A (Li et al., 2004). In the heart, transgenic overexpression of atrogin-1 was able to prevent the calcineurin-mediated cardiac hypertrophy that usually occurs following aortic banding (Li et al., 2004). It is not yet known if calcineurin A is a target of atrogin-1 in skeletal muscle, though calcineurin signaling pathways seem to affect fiber type more than fiber size in that tissue (Serrano et al., 2001), leading one to predict that other atrogin-1 substrates may be more critical in striated muscle. Finally, atrogin-1 has been reported to interact with and degrade myoD, a transcription factor critical in muscle development and differentiation (Tintignac et al., 2004). Since muscle atrophy occurs in fully differentiated adult muscle, and animals lacking atrogin-1 develop normal muscle, the physiological role of this interaction is not clear.

Recent studies have elucidated one of the major signaling pathways responsible for activation of atrogin-1 during muscle atrophy (Lee et al., 2004; Sandri et al., 2004; Stitt et al., 2004). In conditions of muscle atrophy, signaling through the IGF-1/PI3K/Akt pathway is suppressed, which in turn maintains FoxO transcription factors in a dephosphorylated (active) state. FoxO factors are potent activators of atrogin-1 transcription, and bind directly to multiple sites within the atrogin-1 promoter (Sandri et al., 2004).

4. MuRF1

The genomic approach used by the workers at Regeneron also identified another E3, MuRF1, in their models of atrophying muscle (Bodine et al., 2001). Like atrogin-1, MuRF1 is also induced in atrophying muscles from fasted, diabetic, tumor-bearing and uremic animals as well as atrophying human muscles, suggesting that it plays a fundamental role in the development of muscle wasting (Jones et al., 2004; Lecker et al., 2004). Mice lacking MuRF1 are also protected from denervation-induced muscle atrophy, though to a somewhat lesser degree than mice lacking atrogin-1 (Bodine et al., 2001). MuRF1 is a 40kDa protein which contains a RING domain at its amino-terminal end, as well as two coiled-coil domains in its central region (Centner et al., 2001; Spencer et al., 2000). The RING domain of MuRF1 imparts ubiquitin conjugating activity, as was demonstrated in vitro by Bodine et al. (2001), though the targets of this activity are not yet known. Interestingly, MuRF1 and other members of the MuRF family have been found in association with...
the myofibrillar component, titin, at the M-line, raising the possibility that they might target components of the myofibrillar apparatus for degradation in atrophying muscle (Centner et al., 2001; McElhinny, Perry, Witt, Labeit, & Gregorio, 2004). On the other hand, there are also indications that MuRF1 can be found in the nucleus of muscle cells (McElhinny, Kakinuma, Sotimachi, Labeit, & Gregorio, 2002), though its location in atrophying muscle is not known. Recently, MuRF1 was also found to interact with glucocorticoid modulatory element binding protein-1 (GMEB-1), a nuclear protein that regulates transcription in response to glucocorticoids (McElhinny et al., 2002). It is intriguing that glucocorticoids are potent activators of muscle protein breakdown and that MuRF1 expression is increased in the presence of glucocorticoids (Bodine et al., 2001). Finally, MuRF1 has also been shown to degrade troponin I in cardiomyocytes (Kedar et al., 2004). The interaction between MuuRF1 and this myofibrillar protein in skeletal muscle and its potential role in the development of muscle atrophy is yet to be established.

5. Other muscle specific enzymes involved in ubiquitin conjugation

In addition to atrogin-1 and MuRF1, Kandarian and coworkers have evidence that mRNA for Nedd4, a HECT-domain E3 is increased in muscles atrophying from disuse (Jackman & Kandarian, 2004), though activation of this E3 has not been seen in other types of atrophying muscle. Pickart and coworkers have also identified a HECT-domain ubiquitin–protein ligase called KIAA10 that is highly expressed in skeletal muscle (You & Pickart, 2001). KIAA10 targets TIP120B, a muscle-specific TATA-box interacting protein, for ubiquitination and degradation by the 26S proteasome (You, Wang, Aoki, Tamura, & Pickart, 2003). The role of KIAA10 in normal or atrophying muscle is, however not yet known. Additionally, Oxx, a muscle specific RING finger ubiquitin ligase (Nastasi et al., 2004), has recently been shown to ubiquitinate membrane-bound beta-catenin and may play an important role in sarcomere organization and possibly dystrophic states (Wu, Liu, & He, 2004). U box-containing E3s, such as UFD2 and CHN-1, the C. elegans orthologue of CHIP, form an E3/E4 complex that enhances the ubiquitination of UNC-45, a myosin chaperone that is involved in myosin assembly (Hoppe et al., 2004). These ubiquitination enzymes could also potentially play roles in muscle protein breakdown in atrophying muscle.

Two other Ub-carrier proteins (E2s) also demonstrate muscle specific patterns of expression. Chrysis and Underwood showed that transcripts of E2G, first identified by Watanabe et al. (1996), and a UBC4/UBC5 isoform are increased in muscles of rats treated with glucocorticoids (Chrysis & Underwood, 1999). Li et al. (2003) have recently demonstrated that UbH2 is also predominantly expressed in muscle, and that its levels rise in response to TNFs, another mediator of muscle atrophy. Whether these E2s act in combination with atrogin-1 or MuRF1 or, alternatively, with some as yet undefined additional E3 is not known.

6. Conclusions

One concept that seems to be emerging is that multiple distinct ubiquitin conjugation pathways, and possibly other proteolytic systems, are activated in muscle as it atrophies. This coordinate regulation of proteolytic enzymes suggests that in atrophy each functions to remove distinct cellular components. Such parallel pathways acting on different muscle proteins might explain why the gene knockouts of E3s/UBR1 and E2_14k in mice are still able to undergo atrophy, and why atrogin-1/MAFbx and MuRF1 knockouts are only partially effective at preventing the development of atrophy. Mat ing these animals to test if the effects of deleting individual enzymes are additive in diminishing atrophy should help to clarify this important issue. It is also possible that the function of one or more proteolytic systems are linked. For instance, a caspase or a member of the calpain family could be the elusive protease able to generate proteins with the novel N-terminal amino groups recognized by E3s/UBR1/UBR2. In such a model, atrophy of muscle would be a multi-step proteolytic process. Conclusive evidence for this model will require careful biochemical studies and identification of the critical proteins degraded by these enzymes. Until the activities and cellular substrates of these proteases and enzymes of the ubiquitin conjugating system are more fully characterized, it is difficult to predict if these various proteolytic systems work in series or in parallel. Although much work lies ahead, the major...
players in this excessive proteolysis now appear to be coming into focus.

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