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Review

Control of ubiquitination in skeletal muscle wasting

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Abstract

The ubiquitin proteasome system is now well recognized to play a role in mediating skeletal muscle protein wasting. Ubiquitin exerts its effects by covalent attachment to other proteins. Increased ubiquitination of muscle proteins has been observed in a number of conditions of atrophy suggesting that flux through the pathway may be regulated by controlling availability of ubiquitinated substrates for the proteasome. Therefore the enzymes that control ubiquitination of proteins likely play critical roles in regulating flux through the pathway, are sites of activation by catabolic stimuli and potentially good drug targets in the search for therapies for wasting disorders. In this article, the enzymes that can modulate ubiquitination are briefly reviewed and the current data regarding regulation of these enzymes in skeletal muscle are described. Physiological regulators of muscle size appear to modulate many of these enzymes and several of these regulators appear to do so via signaling pathways that involve Akt or NFκB. Further work needs to be done to identify all the enzymes that are involved in controlling ubiquitination in muscle, to characterize their regulation by non-transcriptional mechanisms also, and most importantly to identify their target substrates and to determine how these various pathways of ubiquitination work together to mediate the catabolic stimulus.

Keywords: Ubiquitin conjugating enzyme; Ubiquitin protein ligase; Deubiquitinating enzyme; Ubiquitin specific protease; Muscle proteolysis

Contents

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1. Introduction

The ubiquitin proteasome system was originally believed to be responsible primarily for degradation of short-lived and abnormal proteins. However, it is now clear that it also plays an important role in mediating the degradation of long-lived myofibrillar proteins in skeletal muscle. Although the system has been implicated in skeletal muscle wasting for over a decade, solid evidence to support this has only been obtained in the last few years. This article will focus on the current state of knowledge of the control of ubiquitination in skeletal muscle and highlight some of the major questions that remain unresolved. The regulation of the proteasome itself in atrophying skeletal muscle will not be discussed in this article. Readers are referred to other articles in this issue for a discussion of this area.

Ubiquitin is an abundant 8 kDa peptide found in all eukaryotic cells. To date, no function has been clearly ascribable to free ubiquitin. The functions of ubiquitin arise from its covalent attachment to other proteins (rev. in Glickman & Ciechanover, 2002). This attachment is in the form of an isopeptide bond between the carboxy group of the C-terminal glycine of ubiquitin and the ε-amino group found on the side chain of lysine residues of proteins. Occasionally the ubiquitination can occur on the α-amino group of the initial amino acid of the target protein (Ciechanover & Ben-Saadon, 2004). The protein may remain monoubiquitinated or additional ubiquitin moieties can be added to produce a chain of ubiquitin moieties in which each moiety is connected via an isopeptide bond between the C-terminal glycine of the distal ubiquitin and a lysine residue of the more proximal ubiquitin. Ubiquitin has seven lysine residues and a proteomic analysis in yeast indicates that all seven lysines can be used in ubiquitin–ubiquitin linkages, though to varying degrees (Peng et al., 2003). Use of different lysine residues results in different conformations of the chain and confers different functions. Chains of ubiquitin linked via lysine 48 target the substrate protein for degradation by the proteasome (Chau et al., 1989). Chains linked via lysine 63 appear not to confer degradation, but rather serve other signaling functions. For example, lysine 63 linked chains attached to signaling molecules downstream of cytokine receptors in the pathway to NFκB activation appear vital for positive signal transduction (Deng et al., 2000; Wertz et al., 2004) and also appear important in mediating DNA repair (Spence, Sadis, Haas, & Finley, 1995) and the stress response (Arnason & Ellison, 1994). The roles of ubiquitin–ubiquitin linkages via other lysine residues remain unknown.

Monoubiquitination also serves important functions. Recently, it has been shown to be an important positive signal for internalization and delivery of membrane proteins for degradation in the lysosome (rev. in Marmor & Yarden, 2004). The ubiquitin tag recruits proteins that are important for mediating trafficking to the multivesicular body which is an intermediate structure upstream of the lysosome. The interacting proteins contain one of several domains that bind ubiquitin (rev. in Di Fiore, Polo, & Hofmann, 2003). In addition histones have been known to be monoubiquitinated for many years (West & Bonner, 1980). This ubiquitination is regulated during the cell cycle. The function of this remains unclear, but is likely involved in modulating chromatin structure.

2. Increased ubiquitination in atrophying skeletal muscle

One of the early findings implicating a role for the ubiquitin system in atrophying skeletal muscle was the presence of increased levels of ubiquitinated proteins in muscles in the catabolic state. These were originally described in muscles atrophying in response to denervation or starvation (Wing, Haas, & Goldberg, 1995) and subsequently has been reported to occur in other conditions such as sepsis (Tiao et al., 1996) and

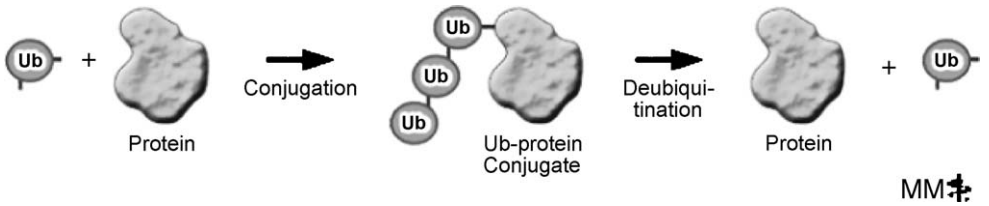

Fig. 1. Steady state levels of ubiquitination are increased in muscle atrophying in response to many catabolic stimuli. This increased ubiquitination can be mediated by regulation of enzymes involved in conjugation or deubiquitination.

cancer (Baracos, DeVivo, Hoyle, & Goldberg, 1995; Llovera, Garcia-Martinez, Agell, Lopez-Soriano, & Argiles, 1995; Lorite, Thompson, Drake, Carling, & Tisdale, 1998). In addition, studies have demonstrated increased rates of ubiquitination in extracts prepared from atrophying muscles (Combaret et al., 2004; Kee et al., 2003; Solomon, Baracos, Sarraf, & Goldberg, 1998a). This accumulation of ubiquitinated proteins suggests a simple model in which flux through the pathway is regulated by the availability of ubiquitinated substrates for the proteasome. In this scenario, the enzymes which can control the degree of ubiquitination of proteins become potential important sites of activation by stimuli of atrophy. They also become potential targets for modulation by drugs in the quest for therapies to prevent wasting in the many clinical disorders in which it is seen. The enzymes that control ubiquitination can be divided into two main categories—enzymes involved in conjugation and those that are involved in deubiquitination (Fig. 1).

3. Regulation of enzymes involved in conjugation (Table 1)

The conjugation of ubiquitin to proteins is mediated by a series of sequential reactions (rev. in Pickart, 2001; Weissman, 2001). Ubiquitin is first activated by ubiquitin activating enzyme (E1). Ubiquitin activation is a two-step process. In the first step, ATP is hydrolyzed and the energy is coupled to adenylation of ubiquitin on its C-terminal glycine. In the second step, the ubiquitin moiety is transferred to the cysteine residue of the active site of E1 to form a ubiquitin–thiol ester linkage. Subsequently, E1 transfers the activated ubiquitin onto the active site cysteine of a ubiquitin conjugating enzyme (E2). Substrates are recognized by ubiquitin protein ligases (E3s) which also bind E2s and thereby permit transfer of ubiquitin onto proteins. In some situations, a fourth enzyme (E4, also known as U box type E3), is involved in elongating short ubiquitin chains already attached to the substrate (Koegl et al., 1999).

There is only one ubiquitin activating enzyme in the eukaryotic cell. Another mammalian gene with sequence similarity to E1, UBE1L, has now been shown to be the activating enzyme for the ubiquitin like protein ISG15 (Yuan & Krug, 2001). The single ubiquitin activating enzyme supplies activated ubiquitin to a large family of ubiquitin conjugating enzymes. There are 11 ubiquitin conjugating enzymes in yeast. Analysis of sequence databases indicates that each of these yeast E2s has at least one and often multiple orthologues in mammalian genomes. In addition, there are ubiquitin conjugating enzymes that appear unique to higher eukaryotes. These are generally large enzymes and appear to combine both the E2 and E3 functions in one protein

Table 1
List of genes involved in ubiquitination currently known to be regulated in skeletal muscle wasting (see text for citations)

Ubiquitin genes	Ubiquitin conjugating enzymes	Ubiquitin–protein ligases	Deubiquitinating enzymes
UbB	$E2_{14k}$/HR6B/UBC2	E3α/UBR1	USP19
UbC	$E2_{20K}$	E3α-II/UBR2	USP14
UBA52	UBC4/UBC5 isoforms	UBR3	
Ub protein S27A		Atrogin-1/MAFbx, MURF-1, E4	

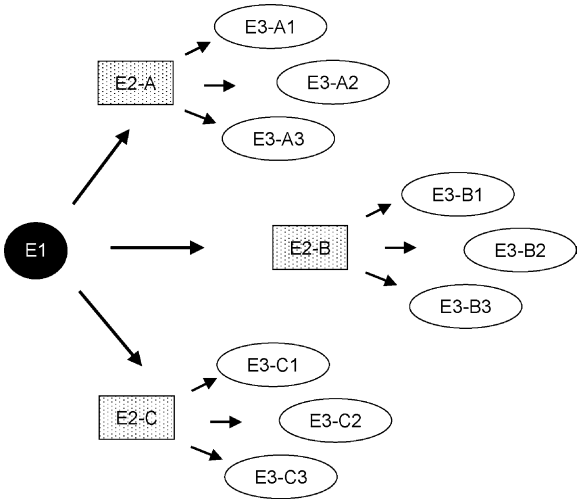

Fig. 2. Multiple pathways of ubiquitin conjugation permit fine regulation of ubiquitination to specific protein targets. Ubiquitin activation enzyme (E1) supplies activated ubiquitin to a family of ubiquitin conjugating enzymes (E2s). Each ubiquitin conjugating enzyme interacts with a specific subset of ubiquitin protein ligases (E3s) which in turn bind specific protein substrates.

(Bartke, Pohl, Pyrowolakis, & Jentsch, 2004; Berleth & Pickart, 1996). Each conjugating enzyme appears to interact with specific subsets of E3s which in turn recognize specific substrates. E3 enzymes known to date are characterized by the presence of one of two major sequence motifs. One group contains a ∼350 residue HECT domain at the C-terminus and these enzymes are generally monomeric. The other contains a RING finger domain or variants of it such as a PH domain or U box. Screening the human genome database suggests that there are ∼90 HECT domain E3s and ∼800 RING finger E3s. Many of these RING E3s are multisubunit complexes where substrate binding and E2 binding can occur on distinct subunits. Thus, the major enzymes involved in ubiquitination are organized in a hierarchical fashion and result in multiple pathways of ubiquitin conjugation and the potential for very fine control of ubiquitination, particularly through regulation at the level of E3s (Fig. 2).

3.1. Ubiquitin activating enzyme

Intuitively, the presence of a unique ubiquitin activating enzyme renders it unlikely to be a major site of control of ubiquitination as its regulation would influence all pathways of conjugation. Indeed, to date, regulation of E1 in atrophying mammalian skeletal muscle has not been reported. In vitro studies are also consistent with E1 not being an important locus of regulation. Its intrinsic activity appears to be high, being capable of charging an excess of ubiquitin conjugating enzymes (Haas, Warms, Hershko, & Rose, 1982). In addition, in many tissues, including muscle, supplementation of extracts with additional E1 does not increase the in vitro rate of ubiquitin conjugation (Rajapurohitam, Bedard, & Wing, 2002). However, E1 can be upregulated in conditions of markedly elevated proteolysis. In the programmed cell death of abdominal muscles of the insect *Manduca sexta*, the marked upregulation of ubiquitin conjugation is associated with an increased level of ubiquitin activating enzyme (Haas, Baboshina, Williams, & Schwartz, 1995).

3.2. Ubiquitin conjugating enzymes

In yeast, inactivation of many ubiquitin conjugating enzymes results in specific phenotypes indicating that they play a role in determining the specificity of function of ubiquitination. Therefore, they could be an important locus of regulation. In vitro studies also support a potential role for E2s in the control of ubiquitination. Supplementation of muscle extracts with additional UBC2 or UBC4/UBC5, two major subclasses of ubiquitin conjugating enzymes, can indeed increase the rate of ubiquitination of overall proteins (Rajapurohitam et al., 2002). Therefore, we have explored whether isoforms of these two ubiquitin conjugating enzymes are induced in atrophying skeletal muscle. UBC4/UBC5 is a particularly attractive candidate since in yeast these two nearly identical genes are responsible for the bulk of steady state ubiquitination (Seufert & Jentsch, 1990). In muscle atrophy, a broad spectrum of proteins is also likely being degraded. We previously cloned three mammalian orthologues of yeast UBC4/UBC5 (Wing & Jain, 1995). Although our work suggested that none of these isoforms appear to be upregulated in skeletal muscle atrophying in response to fasting or denervation (Wing, unpublished data), the UBC4-1 isoform (human UBCH5B) is reported to be induced at the mRNA level in muscles from glucocorticoid treated rats (Chrysis & Underwood, 1999). In addition, a recent gene array analysis has revealed that another isoform, UBC4-2,

is induced in muscle from fasted, diabetic, uremic and tumor bearing rats (Lecker et al., 2004).

UBC2 has two orthologues in mammalian cells, known as HR6A and HR6B (Koken et al., 1991). The HR6A isoform does not appear to be regulated in skeletal muscle atrophying in response to fasting or denervation (Adegoke, Bedard, Roest, & Wing, 2002; and unpublished data). The HR6B isoform was identified earlier as a major ubiquitin conjugating enzyme in rabbit reticulocytes and named $E2_{14k}$ (Wing, Dumas, & Banville, 1992). In contrast to HR6A, the HR6B/$E2_{14k}$ isoform is induced at the mRNA level in many forms of muscle wasting (Chrysis & Underwood, 1999; Fang et al., 2000; Hobler et al., 1999; Lecker et al., 1999; Lorite et al., 2001; Temparis et al., 1994; Wing & Banville, 1994). Furthermore, expression of this enzyme is suppressed by the major anabolic factor IGF-1 both in muscle cells (Wing & Banville, 1994) and in vivo (Chrysis & Underwood, 1999). In many situations, $E2_{14k}$ mRNA expression correlates very well with rates of muscle proteolysis (Kee et al., 2003; Taillandier, Aurousseau, Combaret, Guezennec, & Attaix, 2003; Wing & Banville, 1994). Specific measurements of HR6B/$E2_{14k}$ protein have been difficult as it is 96% identical to the HR6A isoform. However, use of an antibody specific to the C-terminal end of HR6B/$E2_{14k}$ (Koken et al., 1996) on muscles from fed and fasted animals, indicated that HR6B/$E2_{14k}$ protein levels are unchanged (Adegoke et al., 2002). This suggested that the induction at the mRNA level serves to maintain protein levels constant under conditions when many other muscle proteins are being degraded. However, we have recently characterized skeletal muscle protein degradation in mice lacking the HR6B/$E2_{14k}$ gene (Adegoke et al., 2002). These mice appear to have muscle size, protein content and rates of proteolysis similar to that of wild type mice. Furthermore, the loss of muscle protein upon fasting (Adegoke et al., 2002) and upon glucocorticoid treatment (Adegoke & Wing, unpublished data) also occurs in the absence of this gene. This is not due to compensatory induction of the HR6A isoform whose expression remains stable. The lack of effect can be explained though by the fact that the remaining HR6A protein levels were still sufficiently greater than apparent Km to support conjugation by major E3s in skeletal muscle. The possible complementary role of the HR6A isoform is difficult to test critically as HR6A/HR6B double mutants are not viable (Roest et al., 2004).

Recently, another E2, $E2_{20K}$ has been shown to be induced in skeletal muscle atrophying in response to TNF administration (Li et al., 2003b). TNF also stimulates expression of $E2_{20K}$ in cultured myotubes (Li et al., 2003b), consistent with a direct effect of the cytokine on skeletal muscle. Whether this E2 is upregulated in other forms of muscle wasting remains unknown. These findings together do demonstrate that regulation of ubiquitination can occur at the level of ubiquitin conjugating enzymes. However, to date, the most dramatic regulation of expression appears to occur at the level of ubiquitin protein ligases.

3.3. Ubiquitin protein ligases

Since HR6B/$E2_{14k}$ appears induced in many forms of muscle wasting, various groups have explored the roles of E3s that interact with this E2. The best characterized such E3 is E3α/UBR1. E3α/UBR1 is the ligase that recognizes substrates for the N-end rule pathway (Bartel, Wunning, & Varshavsky, 1990; Reiss, Kaim, & Hershko, 1988). In this pathway, the E3 recognizes substrates with specific N-termini. E3α/UBR1 has been reported to be upregulated in muscles atrophying as a result of sepsis (Fischer, Sun, Gang, Pritts, & Hasselgren, 2000) or insulin deficiency of diabetes (Lecker et al., 1999) or fasting (Kwon, Xia, Davydov, Lecker, & Varshavsky, 2001). Studies of a fraction of soluble muscle extracts indicated that most of the ubiquitination in this fraction was dependent on the N-end rule pathway (Solomon, Lecker, & Goldberg, 1998b). Furthermore, this N-end rule dependent conjugation was activated in fractions prepared from muscle extracts from septic or tumor bearing animals (Solomon et al., 1998a). However, as was the case for HR6B/$E2_{14k}$, E3α/UBR1 protein levels were unchanged in the insulin deficient states (Kwon et al., 2001; Lecker et al., 1999). Mice lacking E3α/UBR1 have been created and do not appear to have a defect in muscle catabolism (Kwon et al., 2001). Again, the situation is complicated by the presence of multiple genes encoding UBR1 like proteins in mammals. In fact, mRNA levels of two other isoforms UBR2/E3α-II and UBR3 are also induced in muscle upon fasting (Kwon et al., 2001) and in the case of UBR2/E3α-II in tumor bearing animals (Kwak et al., 2004). UBR2 knockout mice have also been described (Kwon et al., 2003), but the phenotypes observed are female lethality and male infertility. The muscle wasting

response of either UBR1 or UBR2 knockout mice to various catabolic stimuli have not been reported. Given the presence of regulation of multiple UBR isoforms, analyses of additional mouse mutants and perhaps mice with multiple isoforms inactivated may be required to critically evaluate the role of the N-end rule pathway in muscle wasting. Interestingly, although upregulation has been seen in many conditions by different investigators for $E2_{14k}$ and in some for UBR1/UBR2, none of these changes in these N-end rule pathway genes were detected in a microarray analysis of RNA from several catabolic conditions (Lecker et al., 2004).

Recently, two other ubiquitin protein ligases have been identified and shown to be markedly induced in skeletal muscles atrophying in response to a wide array of catabolic conditions. One is a RING finger containing protein MURF-1 (Bodine et al., 2001). Interestingly, this protein was previously identified in a yeast two hybrid screen as an interacting protein of titin (Centner et al., 2001). Titin is a large ∼3500 kDa protein that spans half of the sarcomere and so raises the interesting possibility that ubiquitination and degradation of titin might alter sarcomeric structure to release myofibrillar proteins for degradation. The other is MAFbx/atrogin-1 (Bodine et al., 2001; Gomes, Lecker, Jagoe, Navon, & Goldberg, 2001). This protein contains an F-box motif which is found in the substrate recognition subunit of the SCF family of multisubunit complex type E3s. Levels of protein for these E3s in muscle wasting have not been reported to date. However, importantly, mice lacking either gene show a blunted response to muscle wasting in response to denervation (Bodine et al., 2001). This key finding represents the first solid evidence for a critical role of the ubiquitin pathway in mediating the atrophic process.

E4 is an enzyme which can elongate short ubiquitin chains (Koegl et al., 1999). This enzyme may play an important role in promoting degradation of substrates as the proteasome appears to only efficiently recognize chains of at least four ubiquitins (Thrower, Hoffman, Rechsteiner, & Pickart, 2000). Since there are many deubiquitinating enzymes in the cell, longer chains increase the probability of maintaining adequate chain length for recognition by the proteasome. Interestingly, microarray analysis has identified E4 as being upregulated in muscle from diabetic, fasted, uremic and tumor bearing animals (Lecker et al., 2004).

4. Regulation of deubiquitinating enzymes

A large number of deubiquitinating enzymes is now recognized to exist and is growing as more sequence motifs that define this activity become known. There are presently three major classes of deubiquitinating enzymes identified (rev. in Wing, 2003). Although all are cysteine proteases, the particular conserved sequences allow them to be segregated into either the large UBP/USP family or the smaller UCH and otubain domain containing families. In addition there is within the regulatory subunit of the proteasome, a metalloproteinase that possesses an essential deubiquitinating activity (Verma et al., 2002; Yao & Cohen, 2002). Since these enzymes remove ubiquitin from proteins, their downregulation could be a mechanism by which ubiquitination of proteins is enhanced and proteolysis is activated. Such a possibility is supported by in vitro observations that ubiquitin aldehyde, an inhibitor of many deubiquitinating enzymes, can increase the level of ubiquitination of proteins in various tissue extracts including skeletal muscle (Rajapurohitam et al., 2002). This regulation has been demonstrated to apply to specific proteins. HAUSP is a deubiquitinating enzyme that can remove ubiquitin from p53 (Li et al., 2002). Overexpression of HAUSP indeed does result in stabilization of p53 while depletion of HAUSP by siRNA can lower levels of p53.

For this reason, we began to identify deubiquitinating enzymes that are expressed in skeletal muscle. Using a degenerate oligonucleotide encoding the conserved CYS box in the USP/UBP family of deubiquitinating enzymes in a modified 3′RACE reaction, we were able to identify four different deubiquitinating enzymes in skeletal muscle (Combaret et al., in press). None of them were found to be down regulated in various conditions of muscle wasting. Interestingly though, one of them, USP19, was found to be upregulated at the mRNA level in muscles wasting in response to fasting, diabetes, cancer and glucocorticoid therapy (Combaret et al., in press). However, levels of expression of the protein appear to be very low and not detectable by western blotting and so we have not been able to confirm whether induction occurs at the protein level.

The increased rather than decreased expression suggests that the deubiquitinating enzyme is functioning in a manner other than in negative regulation of substrate degradation. Deubiquitinating enzymes have in-

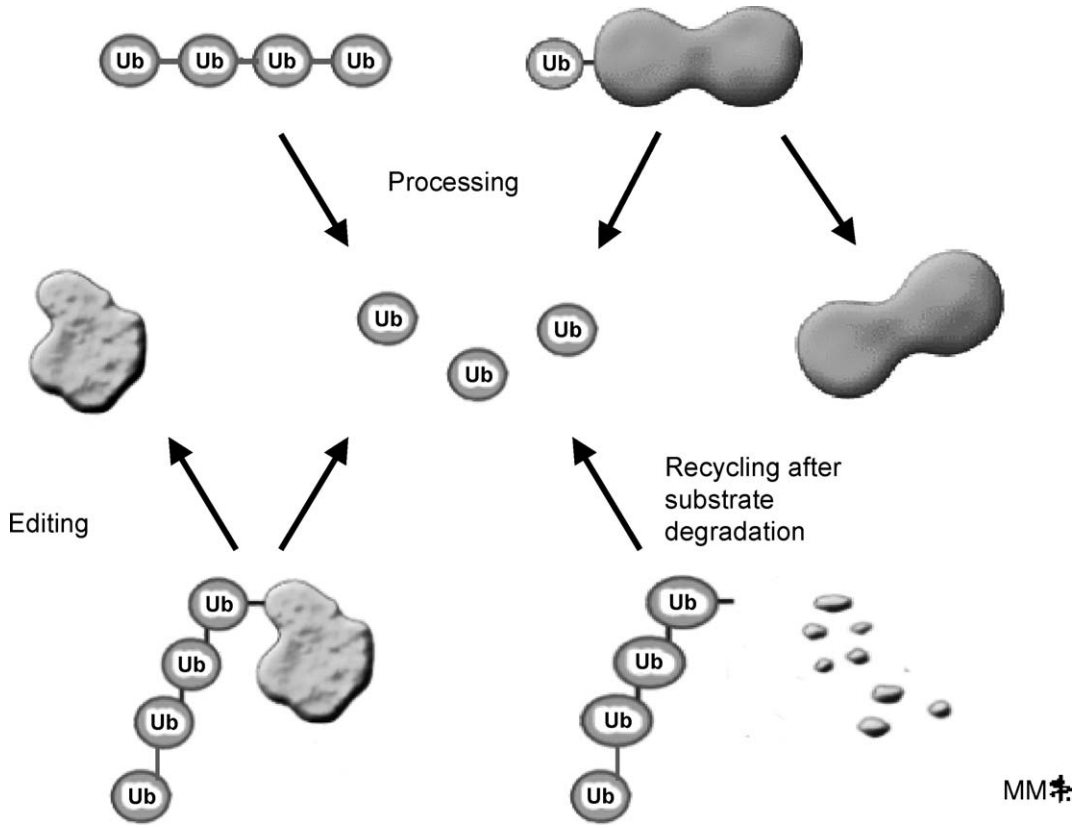

Fig. 3. Multiple functions of deubiquitinating enzymes. Deubiquitinating enzymes can *process* the translational products of the polyubiquitin genes and ubiquitin–fusion protein genes to their mature products. The enzymes can also *edit* ubiquitination and thereby rescue inappropriately ubiquitinated proteins from degradation. They also play important roles in *recycling* ubiquitin from the polyubiquitin chain after the target protein has been committed to degradation by the proteasome. They may also regenerate ubiquitin from adducts formed by reaction of intracellular nucleophiles (thiols, amines) with ubiquitin linked via reactive thiolester bonds in enzymes involved in conjugation.

deed other roles (rev. in Wing, 2003) (Fig. 3). These enzymes can act simultaneously or subsequently to the proteasome to recycle ubiquitin from substrates that have been committed to degradation by the proteasome. Accumulation of chains can inhibit proteasome function (Amerik, Swaminathan, Krantz, Wilkinson, & Hochstrasser, 1997; Piotrowski et al., 1997) and so increased expression of the deubiquitinating enzyme may accelerate proteolysis by clearing these chains. In addition, deubiquitinating enzymes may function as processing proteases to produce monomeric ubiquitin from the linear polyubiquitin products of the polyubiquitin and ubiquitin–fusion protein genes. They may also be involved in regenerating ubiquitin from putative non-productive adducts with abundant small molecule nucleophiles in the cell such as glutathione. Thus, USP19 may function to produce free ubiquitin and in this way increased expression may support ubiquitination and degradation. Of course, one cannot rule out the possibility that the increased USP19 expression serves to stabilize a specific regulatory protein which functions to activate muscle proteolysis.

Using microarray analysis, another deubiquitinating enzyme, USP14, has also been identified as being upregulated at the mRNA level in various conditions of muscle wasting (Lecker et al., 2004). To date, there are no reports of USP14 protein levels in these conditions. However, this enzyme has been characterized. It is associated with the proteasome (Borodovsky et al., 2001) and mutational studies of the yeast homologue UBP6 indicate that it functions to regenerate free ubiq-

uitin from substrates targeted for degradation (Leggett et al., 2002). Thus its increased expression may serve to increase efficiency of the proteasome.

5. Substrate availability

To this point of our analysis of the control of ubiquitination, I have focused the discussion on regulation of the enzymes involved. However, flux through many metabolic pathways is also regulated by substrate availability. This also occurs in the ubiquitin pathway where post-translational modification can render the protein 'available' for recognition by ubiquitin protein ligases (rev. in Pickart, 2004). The most common modification that can lead to ubiquitination is phosphorylation and is the mechanism by which key regulatory proteins such as IκBα, p27, and some membrane receptors are targeted. Other post-translational modifications that can act similarly are hydroxylation, glycosylation, deacetylation and protein binding or unbinding. Less frequent, but interesting are examples in which proteolytic cleavage renders the substrate available for ubiquitination. The clearest example of this is the SCC1 subunit of cohesion in *S. cerevisiae* which, upon cleavage by separin, exposes a destabilizing amino acid at the N-terminus and allows it to be recognized by the N-end rule pathway E3 UBR1 (Rao, Uhlmann, Nasmyth, & Varshavsky, 2001).

It has long been speculated that myofibrillar proteins must be released from their highly organized structure in the sarcomere in order to be available for degradation. Soluble, but not myofibrillar bound actin and myosin can be degraded by the ubiquitin proteasome pathway in vitro (Solomon & Goldberg, 1996). Indeed, a pool of easily releasable myofilaments can be identified in skeletal muscle (Etlinger, Zak, Fischman, & Rabinowitz, 1975) and has been observed to be increased in skeletal muscle from fasted or glucocorticoid treated rats (Dahlmann, Rutschmann, & Reinauer, 1986). Calpains have been implicated in the release of myofilaments from myofibrils (Belcastro, Gilchrist, Scrubb, & Arthur, 1994). Furthermore sepsis increases expression of several calpains as well as releasable myofilaments in skeletal muscle (Williams et al., 1999). In addition overexpression of the calpain inhibitor calpastatin in skeletal muscles results in blunted atrophy in response to muscle disuse (Tidball & Spencer, 2002). Intriguingly, it has been recently reported that caspase 3 can release a fragment of actin from sarcomeric proteins and this fragment appears to accumulate in some conditions of muscle wasting. Furthermore, caspase 3 activity is increased in muscles from diabetic rats and a caspase inhibitor can decrease the rate of proteolysis in these isolated muscles (Du et al., 2004). Thus, elements of the apoptotic pathway may be involved in providing substrates to the ubiquitin proteasome system.

Ubiquitin can be a viewed as a substrate in the conjugation process. However, its expression level in cells (Haas & Bright, 1987) studied to date as well as in skeletal muscle (Haas & Riley, 1988; Wing et al., 1995) suggest that its levels are high and saturating for ubiquitin activating enzyme in the basal state (Haas & Rose, 1982). In most catabolic conditions, induction of polyubiquitin genes is observed. A recent study also indicates that ubiquitin–fusion protein genes are also induced (Lecker et al., 2004). These inductions probably serve to ensure that free ubiquitin levels do not become limiting for conjugation.

6. Mechanisms of physiological regulation

A variety of physiological factors can activate muscle protein degradation. Interestingly, many of these regulators can modulate the expression of several of the enzymes controlling ubiquitination. Hormones such as insulin, IGF-1, glucocorticoids and thyroid hormone are well known to influence protein degradation. Insulin deficiency as produced by fasting or by streptozotocin in rodents results in increased expression of ubiquitin (Price et al., 1996; Wing & Goldberg, 1993), $E2_{14k}$ (Mitch et al., 1999; Wing & Banville, 1994), atrogin-1/MAFbx E3 (Lecker et al., 2004), UBR1, UBR2, UBR3 (Kwon et al., 2001; Lecker et al., 1999) and increased expression of USP19 (Combaret et al., in press) and USP14 (Lecker et al., 2004) deubiquitinating enzymes. Glucocorticoids, which promote catabolism, can increase expression of many of these components of the ubiquitin pathway (Chrysis & Underwood, 1999; Combaret et al., in press; Dehoux et al., 2004; Wing & Goldberg, 1993). The glucocorticoid stimulated expression can be blunted by administration of IGF-1, the major post-natal growth factor (Chrysis & Underwood, 1999; Dehoux et al., 2004; Wing & Bedard, 1996).

Cytokines play important roles in mediating the catabolic response to sepsis and in cancer cachexia. TNF-α can stimulate production of ubiquitin, ubiquitinated proteins (Garcia-Martinez, Agell, Llovera, Lopez-Soriano, & Argiles, 1993; Garcia-Martinez, Llovera, Agell, Lopez-Soriano, & Argiles, 1994) and $E2_{20K}$ (Li et al., 2003a). PIF, the proteolysis inducing factor identified in serum and urine of the MAC16 colon carcinoma bearing mice and in patients suffering from pancreatic cancer stimulates expression of ubiquitin and $E2_{14k}$ (Lorite et al., 2001).

These factors can be regulated together in various pathological conditions to stimulate wasting. Indeed, activation of ubiquitination process has been observed in humans suffering from head trauma (Mansoor et al., 1996), sepsis (Tiao et al., 1997), and immobilization (Jones et al., 2004).

Progress has recently been made in identifying the signaling mechanisms involved in mediating a number of the effects described above. The PI3-kinase and Akt molecules play critical roles in mediating the ability of IGF-1 to both stimulate protein synthesis and inhibit ubiquitin dependent protein degradation (Sandri et al., 2004; Stitt et al., 2004). Signaling downstream from Akt ultimately suppresses the activity of FOXO transcription factors which are critical in activating the expression of both atrogin-1/MAFbx and MURF-1 E3s. Glucocorticoids stimulate polyubiquitin gene transcription, but via a novel MEK1, Sp1 dependent mechanism (Marinovic, Zheng, Mitch, & Price, 2002). Cytokines activate the NFκB transcription factor by stimulating the phosphorylation of the IκBα inhibitor of NFκB. This phosphorylation leads to ubiquitination and degradation of the inhibitor and release of NFκB. The central role of NFκB in mediating the muscle wasting process has recently been shown by the observation of severe wasting and increased expression of MURF-1 in mice overexpressing activated IκB kinase beta in skeletal muscle (Cai et al., 2004). Furthermore, overexpressing a non-activatable form of IκBα prevents the wasting in these mice as well as in a mouse model of denervation atrophy. PIF appears to activate NFκB, but this effect appears mediated by an eicosanoid, 15-hydroxyeicosatetraenoic acid (HETE) (Whitehouse, Khal, & Tisdale, 2003). Interestingly, inhibition of HETE formation by administration of eicosapentaenoic acid not only inhibits PIF stimulated proteolysis, but also that due to fasting (Whitehouse & Tisdale, 2001). These studies together suggest critical roles for Akt and NFκB as intermediate molecules in mediating the effects of many physiological regulators of ubiquitination.

7. Outstanding questions

Much progress has been made in this area recently. However, many questions remain outstanding of which I have selected a few to discuss.

Are there still other genes controlling ubiquitination that are involved in mediating muscle wasting? Over the past few years, the identification of sequence motifs specific for the various enzymes involved in modulating ubiquitination and the comparison of such motifs to genome sequences have together revealed the large number of enzymes involved. The use of general, but incomplete microarrays, has already permitted the identification of a number of genes that are commonly regulated in many forms of muscle atrophy (Lecker et al., 2004). Repeating such analyses with arrays providing more complete coverage of the genome will quite likely identify additional involved enzymes. Although such analyses have been used to describe genes regulated in multiple forms of atrophy, it will also be of interest to identify ubiquitin system genes regulated in specific forms of atrophy and that may play roles in signaling from those specific stimuli of atrophy.

What are the non-transcriptional mechanisms of regulation of these enzymes controlling ubiquitination? Although the transcriptional regulation of these components is presently determinable, other forms of enzyme regulation such as post-translational modifications, rates of degradation, compartmentalization or allosteric regulation remain poorly explored, but are likely to be also important.

What are the substrates of these enzymes controlling ubiquitination? Although the myofibrillar proteins are the main proteins degraded in muscle and can be degraded in vitro in a proteasome dependent manner, it remains to be firmly established that they are ubiquitinated and degraded in a proteasome dependent manner in vivo. It remains conceivable that the ubiquitin system is activated to degrade specific regulatory proteins or to modulate their activity and that such degradation or modulation activates another proteolytic system to

directly degrade these myofibrillar proteins. Although many protein interaction assays are now available to potentially identify substrates, it remains quite challenging to convincingly demonstrate that an identified interacting protein is a target of ubiquitination or deubiquitination.

What are the roles of each of these pathways of ubiquitination/deubiquitination in mediating the response to the catabolic stimulus? To date, a number of ligases have been identified to be induced in muscle wasting. Are some of these pathways redundant by ubiquitinating the same protein(s)? It would seem more likely that each of these pathways acts on distinct proteins which raises the question as to the temporal order in which these ubiquitination reactions occur in order to mediate the response to the catabolic stimulus. These responses may include not only protein degradation, but also other homeostatic responses such as changes in non-proteolysis related metabolism or changes related to alterations in cell size or shape.

The answers to these questions will not only produce a more complete picture of the intracellular molecular processes that underly muscle wasting, but will be important in determining what are the best targets for developing drugs in the search for therapies of wasting disorders.

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