Journal of Cell Science 113, 2003-2010 (2000)
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JCS1231

Genetic defects in acetylcholine signalling promote protein degradation in muscle cells of *Caenorhabditis elegans*

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Accepted 24 March; published on WWW 10 May 2000

SUMMARY

A myosin-*lacZ* fusion, expressed in 103 muscle cells of *Caenorhabditis elegans*, reports on how proteolysis in muscle is controlled by neural and intramuscular signals. Upon acute starvation, the fusion protein is degraded in the posterior 63 cells of the body-wall muscle, but remains stable in 32 anterior body-wall muscles and 8 vulval muscle cells. This distinction correlates with differences in the innervation of these cells. Reporter protein in the head and vulval muscles becomes labile upon genetic 'denervation' in mutants that have blocks in pre-synaptic synthesis or release of acetylcholine (ACh) or post-synaptic reception at nicotinic ACh receptors (nAChR), whereas protein in all 103 muscles is stabilized by the nicotinic agonist levamisole in the absence of ACh production. Levamisole does not stabilize muscle protein in nAChR mutants that are behaviorally resistant to levamisole. Neural inputs thus exert negative control over the proteolytic process in muscle by stimulating muscle nicotinic ACh receptors.

Key words: Atrophy, Cholinergic, Denervation, Proteasome, Proteolysis, Ubiquitin

INTRODUCTION

The amount of any given protein in any cell or tissue is controlled by the balance between synthesis and proteolytic degradation. This balance is especially critical in muscle, which adapts markedly to physiological demand and is also a principal reservoir of metabolic resources, containing nearly 50% of total body protein in mammals (Rooyackers and Nair, 1997). Thus, the anabolic-catabolic balance must shift in favor of protein accumulation during growth, remodeling or adaptation to increased workload and, conversely, shifts locally or systemically in favor of protein catabolism upon fasting, when workload is reduced and in a variety of disease states. The loss of muscle protein may reach a pathological extreme (muscle atrophy) that compromises structural integrity and contractile function under conditions of malnutrition (Li and Goldberg, 1976), chronic disuse (Taillandier et al., 1996), denervation (Furuno et al., 1990), sepsis (Biolo et al., 1997; Cooney et al., 1997; Mitch, 1998), hormonal imbalance (Rooyackers and Nair, 1997) or cancer cachexia (Argiles and Lopez Soriano, 1996; Toomey et al., 1995). However, what appears to be 'pathological' protein breakdown from the perspective of muscle function may in some instances be 'adaptive' for the organism, by providing for metabolic resources derived from proteolysis to be redirected to repair, remodeling, wound healing, or strong immune response.

Although muscle cells contain a variety of lysosomal and cytosolic proteases, the ubiquitin-proteasome system (Baumeister et al., 1997; Coux et al., 1996; Hershko and Ciechanover, 1998) has been assigned a primary role in enhanced proteolysis (Attaix et al., 1998; Mitch and Goldberg, 1996; Solomon and Goldberg, 1996) from inhibitor-based studies (Hobler et al., 1998b; Taillandier et al., 1996; Tawa et al., 1997) of disuse, denervation or sepsis. Levels of polyubiquitin mRNA (Auclair et al., 1997; Garcia Martinez et al., 1995; Medina et al., 1991; Tiao et al., 1997), ubiquitinated protein (Medina et al., 1991), ubiquitin-conjugating enzymes (Hobler et al., 1999; Wing and Banville, 1994) and the activity of the proteasome itself (Attaix et al., 1997; Hobler et al., 1998a) have all been reported to increase in muscle under various conditions that promote abnormal proteolysis. However, we still know relatively little about the biochemical signals or the molecular mechanisms that control the rates of degradation of individual proteins or protein degradation in general in muscle cells. These signals and mechanisms are of interest because they may present potential targets for therapeutic intervention to slow or prevent muscle atrophy under pathological conditions.

We have been developing a model system to study these processes in muscle, using the soil nematode *Caenorhabditis elegans*. This organism has a relatively simple muscular system (Sulston and Horvitz, 1977), including 95 body-wall muscle cells that provide locomotion and 8 vulval muscle cells that control egg-laying. A great deal is known about the innervation of these muscles by a nervous system whose synaptic connectivities have been completely determined (White et al.,

1986). The body-wall muscle of nematodes is generally similar to the striated skeletal muscle of vertebrates, although there are a number of significant distinctions, most notably oblique rather than cross-striation (Rosenbluth, 1965), differences in the size and composition of thick and thin filaments (Epstein et al., 1974; Mackenzie and Epstein, 1980; Waterston et al., 1974), the fact that nematode muscle cells remain mononucleate rather than fusing to form syncytia (Sulston and Horvitz, 1977) and the extension of processes (muscle 'arms') to the motor neuron (White et al., 1986) rather than the receipt of highly branched axonal extensions from the neurons. Despite these differences, it is clear that the basic functional and molecular features of neuromuscular junctions in nematodes and mammals are generally similar. The wealth of mutants available in *C. elegans* and the essentially complete genomic DNA sequence (Consortium, 1998), present compelling advantages for the application of genetics to analyze the signals and signal transduction pathways that control muscle proteolysis.

To take advantage of this potential, we began (Zdinak et al., 1997) to study protein breakdown in muscle using a chimeric reporter protein encoded by a transgene, expressed specifically in muscle from a *C. elegans* muscle myosin heavy-chain (*unc-54* gene) promoter (Fire and Waterston, 1989; Okkema et al., 1993). The protein product fuses the N-terminal 298 amino acid fragment (only a fraction of the ATPase domain) of the myosin heavy-chain to β-galactosidase from *Escherichia coli*, a 146 kDa fusion polypeptide which forms active β-galactosidase tetramers that are not incorporated into myofibrils but remain soluble in muscle cytosol. Zdinak et al. (1997) showed that the 'reporter' was completely stable in well-fed animals, but that inactivation and degradation were triggered by starvation. Western blots indicated a general correspondence between inactivation and physical degradation of the reporter. This degradation must be catalyzed by pre-existing proteolytic enzyme(s), since the inactivation rate was the same when cycloheximide (CH) inhibited de novo protein synthesis. The same argument applies if something other than a protease (e.g. ubiquitin conjugation) is rate-limiting for inactivation of the 'reporter' protein. Ubiquitinated intermediates were detected in active β-galactosidase tetramers affinity-purified from starved animals (Zdinak et al., 1997), suggesting an early role for the ubiquitin-proteasome system. Furthermore, genetic and biochemical evidence indicated that lysosomal proteases were not an important factor in reporter breakdown (Zdinak et al., 1997).

In this paper we report that the starvation-induced degradation of the reporter protein does not occur uniformly in all muscle cells, but shows a striking cell-specific pattern of reporter stability and lability that corresponds to anatomical differences in the innervation of these muscle cells. Using both mutational blocks and chemical agonists, we show that degradation of the reporter protein is promoted by 'genetic denervation' that blocks pre-synaptic synthesis or release of acetylcholine (ACh) or its post-synaptic reception at nicotinic ACh receptors (nAChR) and, conversely, that stimulation of postsynaptic nAChR is sufficient to preserve protein in the denervated muscle of starved animals. These findings indicate that *C. elegans* can provide a useful model in which the power of genetics can be used to study the mechanisms by which denervation affects muscle protein metabolism.

MATERIALS AND METHODS

Genetics

Strains were handled and maintained at 20°C (unless otherwise indicated) according to the methods of Brenner (1974). For most purposes, a transgene containing a fusion between the *C. elegans* major muscle myosin heavy-chain gene (*unc-54*) and the *E. coli lacZ* gene was obtained from strain PD55 (*tra-3*(e1107) IV; ccIs55 (*sup-7* (st5) *unc-54::lac Z*) V; Fire and Waterston, 1989; Okkema et al., 1993). The *sup-7* allele used as transformation marker is a temperature-sensitive trp-tRNA *amber* suppressor (Bolten et al., 1984). Where indicated, transgenes were obtained either from strain PD56 (*tra-3*(e1107) IV; ccIs56 (*sup-7*(st5) *unc-54::lacZ*)), which contains (at an unknown site of integration) the same fusion as PD55 but with the addition of a nuclear-localization sequence from SV40 virus (Fire and Waterston, 1989; Okkema et al., 1993); or from strain EH16 (lwIs16 (*act-4::lacZ*) X) which contains an X-linked small repeat of a fusion between the *C. elegans act-4* (cytoplasmic actin) gene and *E. coli lacZ* (Stone and Shaw, 1993).

The following mutant alleles were used: for LG I, *unc-13* (e51), *unc-29* (e1072), *unc-38* (e264); for LG II, *unc-4* (e120), *unc-52* (e669); for LG III, *unc-32* (e189), *unc-47* (e307), *unc-25* (e156); for LG IV, *cha-1* (p1182^{ts}), *him-8* (e1489), *unc-17* (e245), *unc-17* (e113), *dpy-13* (e184), *lev-1* (e211), *dpy-9* (e12), *unc-30* (e318), *unc-30* (e191); for LG V, *dpy-11* (e224), *unc-51* (e369), *unc-34* (e315), *unc-42* (e270); for LG X, *dpy-7* (e88), *unc-2* (e55).

The location of the *unc-54::lacZ* transgene ccIs55 on LG V was established by crossing males containing the transgene with hermaphrodites carrying visible markers on various single linkage groups. F_2 animals homozygous for the visible marker were stained for β-galactosidase activity (see below). F_2 individuals homozygous for markers on linkage groups I-IV and X showed β-galactosidase activity, but those homozygous for LG V markers showed much less than 1/4 $lacZ^+$ individuals. The transgene was then mapped by constructing a heterozygote of genotype *dpy-11 unc-42*/ccIs55; 7/7 Unc non-Dpy and 0/6 Dpy non-Unc progeny stained for β-galactosidase, thus placing ccIs55 on the left arm of LG V. Two-point crosses with *dpy-11* indicated a location approximately 14 centiMorgans from *dpy-11* on the left arm of LG V.

Strains homozygous for various mutant alleles and a transgene were constructed as follows: If an unlinked mutant allele could be readily scored by visual inspection, *him-8* males carrying the transgene were crossed with mutant hermaphrodites, and homozygous mutant F_2 stained for β-galactosidase activity; *lacZ*$^+$ animals were placed on individual plates and stained for β-galactosidase for several generations (subcloning if required) to ensure homozygosity for the *lacZ* transgene. Strains carrying linked visible markers on LG V were constructed similarly, except that large numbers of mutant F_2 animals sometimes had to be stained to detect rare *lacZ*$^+$ recombinants.

In some cases, mutant alleles of interest produced little or no visible phenotype. In one case (*cha-1* (p1182)) a visible phenotype could be scored after 1-2 hours. exposure to 25°C. Some non-visible markers were scored by resistance to a drug (e.g. *lev-1* confers levamisole resistance; Lewis et al., 1980a).

The resulting strains sometimes carry the *him-8* marker and thus segregate considerable numbers of male progeny. For strains carrying the ccIs55 transgene, we sometimes do not know if the parental *tra-3* marker has been carried into the new strains, since the pseudomale phenotype is suppressed by the *sup-7 amber* suppressor that remains closely linked to the *unc-54::lacZ* transgene. The presence of this mutant *sup-7* allele also causes many of these strains to grow significantly slower than their counterparts lacking the ccIs55 transgene.

Histochemical staining for β-galactosidase activity

X-Gal (5-Br-4-Cl-indolyl-β-D-galactopyranoside) staining after acetone fixation was performed by a modification (Zdinak et al., 1997)

of the protocol of Fire (1992). Slides were photographed on Kodak Ektachrome EH-160 transparency film under bright-field illumination with a red suppression filter. When it was necessary to recover viable progeny from the stained worms, egg-bearing hermaphrodites were individually picked into 0.6% sodium dodecyl sulfate (SDS), incubated for 5 minutes at room temperature and washed with BU buffer (70 mM potassium phosphate, 70 mM NaCl, pH 7), prior to staining with X-Gal as described (Zdinak et al., 1997). This procedure kills adult animals but leaves viable embryos inside contained eggs (Sebastiano et al., 1986). Stained individuals were individually picked to agar plates; progeny generally emerged within 1-2 days.

Other methods

Procedures for production of age-synchronous populations of early larvae and for acute starvation were as described (Zdinak et al., 1997). For wild-type animals, starvation was imposed in early adulthood, i.e. at or slightly before the appearance of fertilized eggs in the animals. Because a fixed time interval was allowed from synchronization to starvation, and growth rates vary slightly among mutant strains, starvation of various mutants was initiated at slightly different points of 'early adulthood'. In some later experiments we omitted the peptone from the starvation plates to prevent growth of contaminating bacteria. Starvation periods were 36 to 48 hours.

For many drug experiments, starvations were carried out in the wells of 24-well tissue culture dishes, either on 0.5 ml pads of NG agar (Brenner, 1974) or in BU buffer (0.25 ml/well) containing 200 μg/ml streptomycin. Drug concentrations were as follows: levamisole, 1 mM; aldicarb, 10 mM for wild-type and 40 mM for *cha-1* mutant animals; lactacystin, 1 μM; Z-Leu$_3$-CHO, 200 μM. When drugs were dissolved in DMSO, the final concentration of DMSO in the experiment was always 1% (v/v) or less, and controls with DMSO alone were included.

RESULTS AND DISCUSSION

Cell-specific patterns of proteolysis

When the wild-type strain PD55 is subjected to acute starvation, proteolysis of the previously stable reporter protein begins after a lag of about 8 hours and proceeds such that the apparent half-life of the protein is 17 hours (Zdinak et al., 1997). However, histochemical staining for β-galactosidase activity (Fig. 1) shows that inactivation is not uniform in all muscle cells. After 36-44 hours of starvation, activity is lost almost completely in the posterior 63 body-wall muscle cells, but retained in the 32 anterior-most head muscles and the 8 vulval muscle cells. The same cell-specific pattern was observed when cycloheximide was added at the onset of starvation at a concentration sufficient to completely inhibit protein synthesis, implying that the distinctions between cell groups are not the result of differential synthesis, but of differential protein stability. These cell groupings correspond to innervation differences: the posterior body-wall muscles are innervated only in the nerve cords, whereas the head muscles are also innervated in the nerve ring and the vulval muscles have distinct innervation (White et al., 1986). The correspondence suggests that the cell-specific differences in reporter stability are controlled by the nature and/or activity of neural inputs to specific muscle cells.

Worms which had been starved for 40-48 hours and then re-fed for 24 hours showed a complete regain of β-galactosidase activity; regain was prevented by cycloheximide. This confirms the inference (Zdinak et al., 1997) that adult animals retain the ability to carry out de novo expression of the *lacZ* fusion construct from the *unc-54* promoter.

When a nuclear-localization sequence from virus SV40 was added to the reporter construct (Fig. 2), the fully-fed animals showed β-galactosidase activity in muscle nuclei and cytoplasm. After starvation, the cytoplasmic activity was gone, but nuclear activity remained. It is evident that fusion protein is protected from proteolysis by the nuclear membrane, which remains intact in these non-cycling cells.

Although it is clear that *lacZ* fusion protein is 'reporting' on the activation of a muscle proteolytic system that presumably has other endogenous protein substrates, a matter of continuing concern is the extent to which the inactivation and degradation of a myosin-*lacZ* fusion protein are representative of the analogous processes for normal muscle proteins. We have found (L. P. Benner and L. A. Jacobson, unpublished) that two native muscle enzymes, arginine kinase and adenylate kinase, are inactivated upon starvation to approximately the same extent as the *unc-54::lacZ* reporter, and that their inactivation is also catalyzed by pre-existing proteolytic systems. We have also carried out limited studies with an *act-4::lacZ* fusion that contains only 16 N-terminal amino acid residues derived from the *act-4* (cytoplasmic actin) gene (Stone and Shaw, 1993), and found that the cell-specific pattern of β-galactosidase stability and instability in the muscles of starved worms is generally similar to that of the myosin-*lacZ* fusion. This implies that reporter inactivation by proteolysis is not strongly determined by the 298 N-terminal residues of myosin sequence in the *unc-54::lacZ* fusion. Studies with reporter proteins physically incorporated into myofibrils are now in progress.

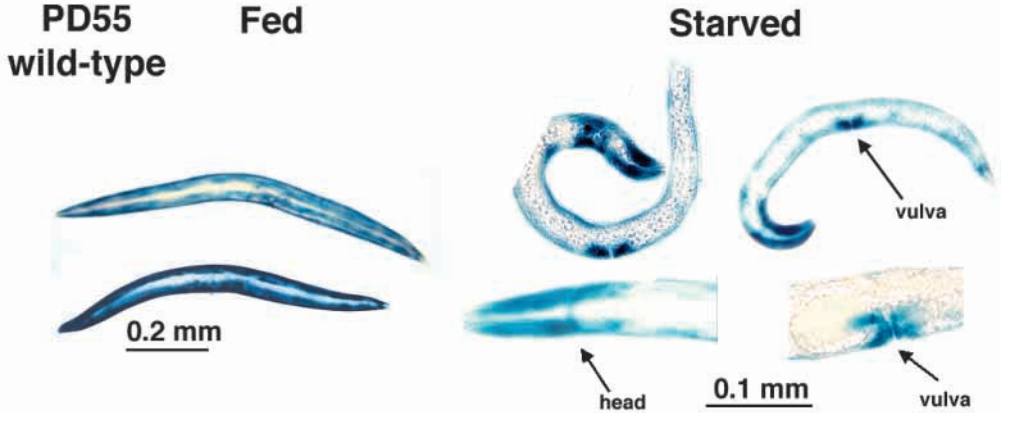

Fig. 1. Stability of cytosolic *unc-54::lacZ* fusion proteins in wild-type *C. elegans*. Strain PD55 contains a transgene consisting of an in-frame fusion of *lacZ* to 5′-portions and 3′-portions of the *unc-54* gene (See Materials and Methods). Where indicated, worms were starved from early adulthood for 36-44 hours at 20°C. Worms in all figures are shown with anterior at left and ventral side down.

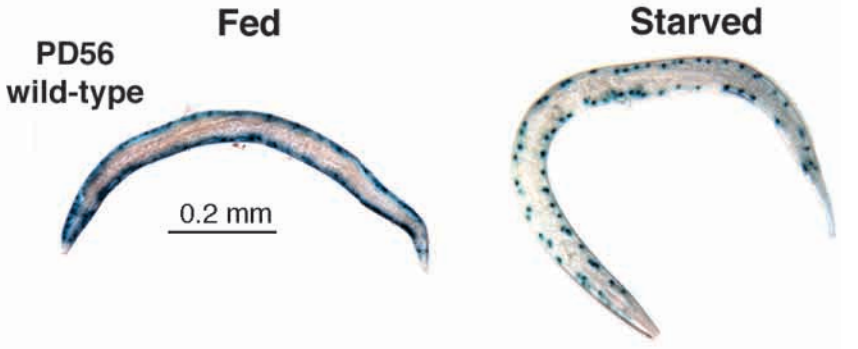

Fig. 2. Stability of nuclear-localized *unc-54::lacZ* fusion proteins in wild-type *C. elegans*. Strain PD56 contains the same reporter construct as PD55, except that a nuclear localization sequence from SV40 is interposed immediately upstream of *lacZ*. Where indicated, worms were starved from early adulthood for 36-44 hours at 20°C.

Acetylcholine input is required for muscle protein stability

To test the hypothesis that neural inputs control proteolysis in muscle, we used a variety of mutants deficient in proteins involved in signal generation in nerve or signal reception in muscle (Table 1). Unless otherwise noted, all mutants when fully fed showed *lacZ* expression indistinguishable from that of fed wild-type animals. It should not be assumed that any mutation completely eliminates the function of the gene product unless explicitly stated.

The body-wall muscles of *C. elegans* receive excitatory signals from cholinergic motor neurons. To test if cholinergic stimulation is responsible for controlling protein stability in muscle, we constructed a strain carrying the myosin-*lacZ* reporter and a temperature-sensitive mutation in the *cha-1* gene, which encodes choline acetyltransferase (Alfonso et al., 1994; Table 1). When the *cha-1*^{ts} mutants are shifted to nonpermissive temperature (25°C), choline acetyltransferase is (reversibly) inactivated (Rand, 1989) within less than an hour, extremely little ACh is produced (see below) and the animals become paralyzed as a result of 'genetic denervation'. When a *cha-1*^{ts} mutant is starved at permissive temperature (20°C), the distinction between the head and posterior cells with respect to protein stability is identical to that in wild-type, but starvation at 25°C leads to protein instability in all muscles (Fig. 3). This shows that ACh input is necessary for protein stability in the head muscles. Note that in slightly older animals starved at 25°C, stained embryos are often observed, thus providing an internal positive control for the X-Gal staining reaction. These embryos may retain β-galactosidase activity because they are nourished by nutrients in the egg and thus not starved, or the relevant proteolytic systems may not yet exist in embryos.

Since we shifted the mutants to nonpermissive temperature only at the initiation of starvation, this experiment also shows that ACh acts in the short term to inhibit muscle proteolysis, rather than by exerting a long-term 'developmental' effect on the target muscle. Furthermore, the same observations were made when *cha-1*^{ts} mutants were grown at 20°C, then shifted to 25°C and starved in the presence of cycloheximide to prevent de novo protein synthesis, implying that ACh deficiency causes reporter instability in the head muscles without the requirement for any newly synthesized protein. That is, the state or level of acetylcholine inputs must control the state of one or more molecular 'switches' inside muscle; the 'switch state' in turn controls whether or not muscle protein is rapidly degraded.

Note that it is impossible to have 'fed' *cha-1*^{ts} mutants at 25°C, since they become paralyzed at the non-permissive temperature, there is no visible pumping of the pharynx and they cannot eat. Such animals lose reporter activity in all cells and at about the same rate as those starved at 25°C. This appears to eliminate the formal possibility that the triggering of muscle

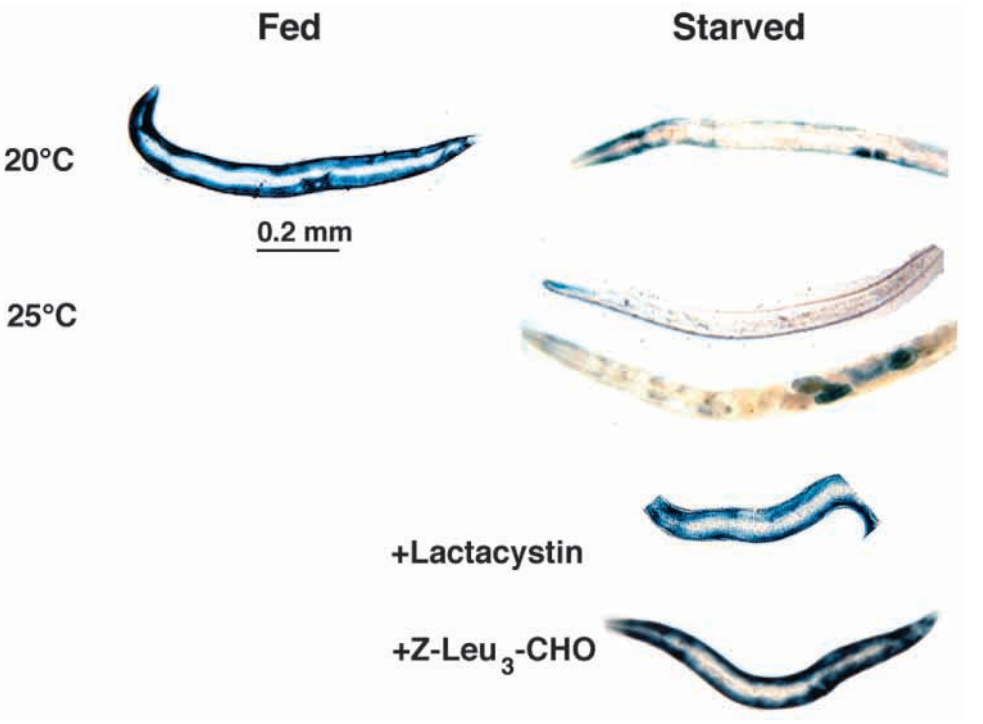

Fig. 3. Stability of reporter protein in mutants deficient in ACh synthesis. Mutants (*cha-1* (p1182^{ts})) containing the *unc-54::lacZ* transgene derived from strain PD55 were roughly age-synchronized and grown to early adulthood at 20°C, then starved for 40-44 hours at 20°C or 36-40 hours at 25°C, as indicated. Proteasome inhibitors as indicated (1 μM lactacystin or 200 μM Z-Leu_3-CHO) were added at the start of starvation at 25°C.

Table 1. Mutant alleles and their products

Gene (allele)	Gene product
cha-1 (p1182^{ts})	Choline acetyltransferase
unc-13 (e51)	Presynaptic phorbol-ester binding protein
unc-17 (e245)	Vesicular ACh transporter
unc-25 (e156)	Glutamic acid decarboxylase
unc-47 (e307)	Vesicular GABA transporter
lev-1 (e211)	nAChR non-alpha subunit
unc-29 (e1072^{am})	nAChR non-alpha subunit
unc-38 (e264)	nAChR alpha subunit

proteolysis by starvation is a chemosensory response to the absence of external food rather than a metabolic response.

When the *cha-1*^{ts} mutants were starved at non-permissive temperature in the presence of the acetylcholinesterase inhibitor aldicarb (Opperman and Chang, 1991), protein stability was restored in the head but not posterior body muscles (Fig. 4). This indicates that the *cha-1*^{ts} mutants still produce some very low level of acetylcholine (ACh) even at nonpermissive temperature, such that a more normal level of ACh can be restored when acetylcholinesterase is inhibited, and this normal level of ACh is sufficient to maintain normal protein stability. We also observed (Fig. 4) that aldicarb did not promote protein stability (although it did induce hypercontraction) in the posterior body-wall muscles of wild-type animals, implying that the anterior-posterior distinctions may reflect differences in the intrinsic levels of ACh signaling by the neurons that innervate these two groups of cells.

Two observations show that ACh must be released at neuromuscular junctions to promote muscle protein stability. First, a mutation in the *unc-17* gene (which encodes the ACh vesicular transporter for presynaptic release (Alfonso et al., 1993; Table 1) evokes more rapid inactivation of the reporter protein in starved animals (Fig. 5), although stability in the head is still greater than in the rear body. Second, we studied a mutation in the *unc-13* gene, which encodes a phorbol ester binding protein (Maruyama and Brenner, 1991; Table 1) that regulates syntaxin (Sassa et al., 1999); mutants accumulate excess ACh presynaptically (Hosono et al., 1989), indicating a presynaptic defect in ACh release. An *unc-13* mutation destabilizes lacZ reporter throughout the starved animal (Fig. 5). Thus ACh accumulation, even to a level higher than that in wild-type, does not promote muscle protein stability if the ACh is not released to the neuromuscular junction.

We can rule out the alternative possibility that the posterior body-wall muscle cells are receiving higher levels of inhibitory γ-aminobutyric acid (GABA) inputs (McIntire et al., 1993) on the basis of studies with mutants (Table 1) that have altered GABA synthesis or

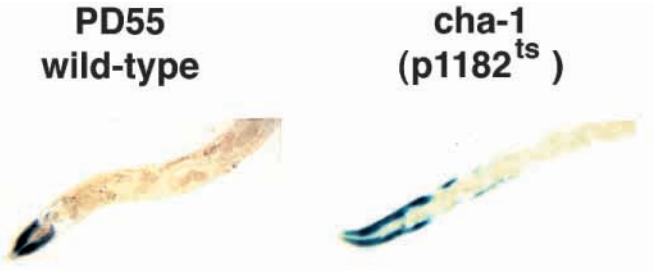

Fig. 4. Effect of an acetylcholinesterase inhibitor on reporter protein stability in starved animals. Animals were roughly age-synchronized and grown to early adulthood at 20°C, then starved for 36-40 hours at 25°C in the presence of 10 mM aldicarb (wild-type) or 40 mM aldicarb (*cha-1*^{ts} mutants). Compare the untreated wild-type (Fig. 1) and untreated *cha-1*^{ts} mutants (Fig. 3). Muscles in the head of the wild-type animal are more strongly hypercontracted than those in the *cha-1* mutant.

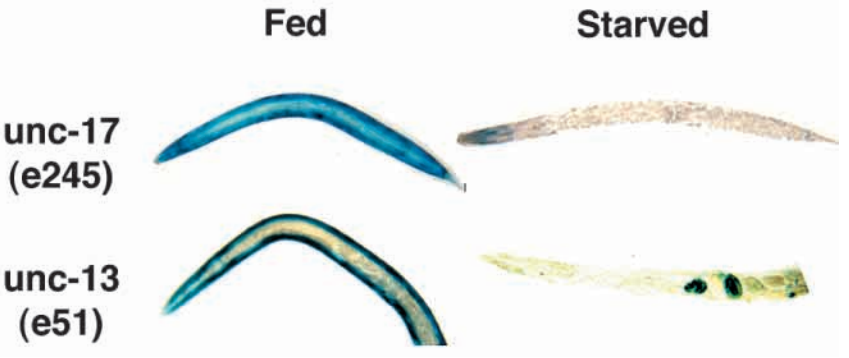

Fig. 5. Stability of reporter protein is altered by mutations affecting presynaptic ACh release. Animals shown contained the *unc-54::lacZ* transgene derived from strain PD55, and were homozygous for recessive mutations as indicated. Starvation times were as in Fig. 1. Compare the wild-type controls in Fig. 1.

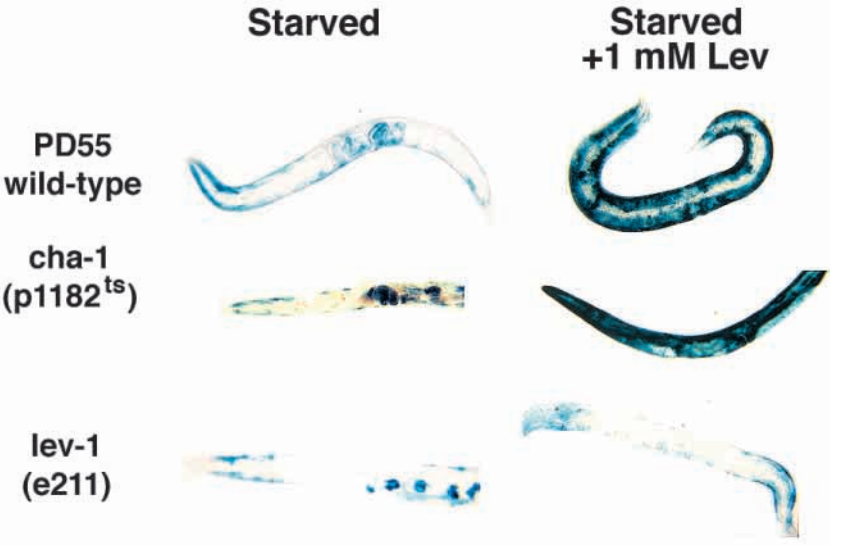

Fig. 6. Reporter protein stability is increased by stimulation of muscle nicotinic ACh receptors. Animals were grown as in Fig. 4, then starved at 25°C in the presence or absence of 1 mM levamisole.

release. The *unc-25* gene encodes glutamic acid decarboxylase (Jin et al., 1999); mutant homozygotes do not make GABA. The *unc-47* gene encodes the vesicular GABA transporter (McIntire et al., 1997); mutant homozygotes accumulate GABA presynaptically, but do not release it. We examined both *unc-25* and *unc-47* mutant homozygotes and found (data not shown) that muscle-specific expression of the lacZ reporter in fed animals was similar to that in fed wild-type animals. Furthermore, both mutants were the same as the wild-type in the starvation-induced pattern of anterior-posterior differences in protein stability (data not shown).

Control of proteolysis is mediated by nicotinic ACh receptors

Motor function in the body wall muscles of *C. elegans* is primarily mediated by nicotinic-type ACh receptors (nAChR; Ballivet et al., 1996; Fleming et al., 1993; Squire et al., 1995). To determine if stimulation of these receptors is sufficient to promote protein stability in muscle, we treated either wild-type animals or *cha-1*^{ts} mutants with the nicotine analogue levamisole (Lewis et al., 1980b) which stimulates muscle to hypercontract. As shown in Fig. 6, levamisole treatment of starved wild-type animals promotes nearly complete stability of reporter protein throughout the animals, including all posterior body-wall muscles. A similar observation was made in *cha-1*^{ts} mutants which were starved and levamisole-treated at the non-permissive temperature (Fig. 6). Since the *cha-1*^{ts} mutation should largely (but not completely) abolish nerve-to-muscle signaling by ACh, this observation suggests that levamisole promotes protein stability by stimulating muscle rather than neuronal nAChR. Similar effects of levamisole were also observed in *unc-17* and *unc-13* mutants (data not shown), consistent with a post-synaptic site of action of levamisole.

The *lev-1* gene encodes a subunit of the nAChR (Fleming et al., 1997). Animals homozygous for mutations in *lev-1* are resistant to the behavioral effects of levamisole (Lewis et al., 1980a), and in these mutants treatment with levamisole did not stabilize the reporter protein (Fig. 6). This shows that levamisole acts to stabilize reporter protein in muscle solely by virtue of its action on nAChR, rather than some other pharmacological effect (e.g. inhibition of a phosphatase; Farley et al., 1982).

To further confirm the role of nAChR, we studied mutations in two additional genes (*unc-29*, and *unc-38*) that encode other subunits of the nAChR (Fleming et al., 1997; Table 1). These mutations also confer resistance to the behavioral effects (muscle hypercontraction) of levamisole (Lewis et al., 1980a) and are themselves associated with recessive 'uncoordinated' phenotypes, presumably because the mutant nAChRs have subnormal function. The mutation we studied in *unc-38* is unconditional; it causes the reporter protein in starved animals to be less stable than in wild-type such that there is no significant retention in either head or vulval muscles (Fig. 7). The *unc-29* allele we examined is an amber mutation, so the *sup-7* amber suppressor incorporated as a transformation marker with the *unc-54::lacZ* transgene (see Materials and Methods) affects UNC-29 expression. When the mutants were grown at 20°C, *sup-7* suppression was effective, the uncoordinated phenotype of the *unc-29*^{am} mutant was suppressed, and reporter stability during starvation was very similar to that in wild-type animals (Fig. 7). When the same mutants were grown at 25°C, *sup-7* did not suppress the uncoordinated phenotype and the reporter was significantly more unstable in the head and vulval muscles than is the case in wild-type animals (Fig. 7).

Taken together, these observations show that stimulation of muscle nAChR is both necessary and sufficient to promote muscle protein stability during starvation. Although our data do not establish definitively that the cell-specific distinctions in muscle protein stability result directly from corresponding differences in the endogenous level of ACh signaling to these cells, that is the simplest explanation consistent with the data.

The ubiquitin-proteasome system is necessary for reporter inactivation

Under starvation conditions the reporter protein is degraded with the accumulation of various degradation intermediates (Zdinak et al., 1997), some of which are conjugated to ubiquitin (Ub). These Ub-conjugated intermediates were found in affinity-purified, enzymatically active β-galactosidase tetramers, implying that Ub conjugation may be an early step in reporter protein degradation.

Ub conjugation is usually associated with proteolysis by the proteasome (Hasselgren and Fischer, 1997; Hershko and Ciechanover, 1998; Varshavsky, 1997), although a possible

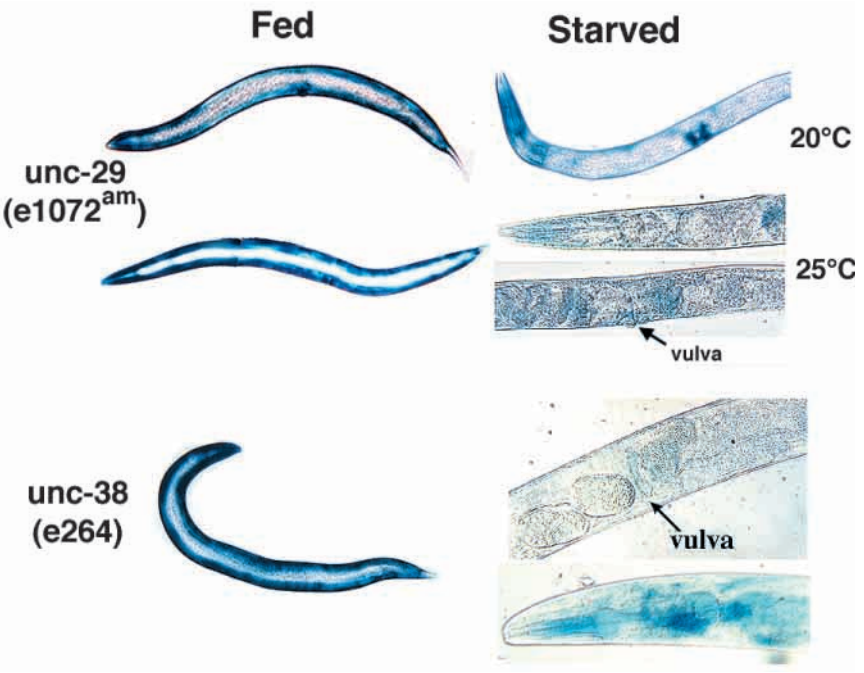

Fig. 7. Stability of reporter protein is decreased by mutations affecting nicotinic ACh receptor. Animals shown contained the *unc-54::lacZ* transgene derived from strain PD55, and were homozygous for recessive mutations as indicated. Growth and starvation conditions were as in Fig. 3. Compare the wild-type controls in Fig. 1.

association with lysosomal proteolysis has also been suggested (Gropper et al., 1991). We have found that lactacystin and Z-(leu)$_3$-CHO, which are relatively specific inhibitors of proteasome activity (Craiu et al., 1997; Lee and Goldberg, 1996), markedly inhibit reporter degradation in vivo in starved, genetically denervated *cha-1^{ts}* mutants (Fig. 3). Proteasome inhibitors also protect protein in denervated mammalian muscle (Tawa et al., 1997). Formally, such inhibitor-based observations show that proteasome activity is necessary for reporter inactivation in nematode muscle and for protein degradation in mammalian muscle, but do not prove that the proteasome acts directly on the target protein in either case.

Acetylcholine signaling negatively regulates muscle proteolysis

The objectives of this investigation were to determine if nerve-muscle signaling affects protein stability in nematode muscle as it is known to do in mammalian muscle, and if mutations affecting known gene products could be used to illuminate the mechanism by which neurons influence muscle protein stability. Our data show that excitatory ACh signals from neurons negatively regulate the inactivation of a reporter protein in muscle. The consistent effects of mutations that affect known pre-synaptic or post-synaptic products allow us to exclude a variety of alternative hypotheses for the signaling mechanism. When we disrupted nerve-muscle signaling acutely with a temperature-sensitive mutation in the *cha-1* gene, synaptic connections had already been formed under permissive conditions and were sufficiently normal to support normal locomotion, feeding and egg-laying. We can thus infer that intact neuromuscular junctions per se are not sufficient to promote protein stability in muscle. Similarly, our experiments with GABA-deficient mutants demonstrate that inhibitory GABA signaling is not required to promote protein degradation in muscle.

By examining enzymatic activity of the reporter protein, we are almost certainly detecting those proteolytic events that lead to inactivation, rather than any steps that merely serve to physically degrade inactive protein. These (presumed early) inactivating events require proteasome activity and are known to involve ubiquitination of the reporter protein (Zdinak et al., 1997). Note, however, that some early proteolytic cleavages do not necessarily inactivate the β-galactosidase tetramers (Zdinak et al., 1997).

We showed previously (Zdinak et al., 1997) that the reporter protein is completely stable in fully-fed animals and that its degradation is triggered by acute starvation in a process that requires only the activation of pre-existing but formerly inactive proteolytic systems. In the present work, we have used this starvation-induced proteolysis to expose the influence of innervation on proteolytic processes in muscle, and have shown that in starved animals the default state of muscle proteolysis is 'on', unless it is turned 'off' by excitatory signals from motor neurons, or by a substitute for such signal, an externally supplied nicotinic agonist. That is, rapid protein degradation in muscle requires both starvation and the absence of ACh signal from innervation.

In the muscle cells of *C. elegans*, denervation thus has the consequence of promoting protein degradation, as it also does in mammalian muscle. The simplicity of the nematode and the availability of mutants has enabled us to show that denervation releases a pre-existing proteolytic system to act upon protein in muscle. Although the influence of innervation on muscle protein degradation has evidently been conserved from *C. elegans* to mammals, it remains to be seen if neural signals are mediated by the same intramuscular signal-transduction pathways in nematode and mammalian muscle. The use of the reporter-protein system in a genetically tractable organism allows us to bring to bear the power of genetic analysis to identify the intramuscular signal transduction mechanisms by which neural activity negatively regulates proteolysis.

This work was supported by grants IBN-9218839 and MCB-9630841 from the National Science Foundation. We are grateful to A. Fire, H. R. Horvitz, J. Rand and J. Shaw for the gift of mutant strains. Many strains were obtained from the *Caenorhabditis* Genetics Center, which is supported by the NIH National Center for Research Resources. We thank K. Poydence and C. Ravin for technical assistance and C. J. Walsh for the use of his microscope.

REFERENCES

Alfonso, A., Grundahl, K., Duerr, J. S., Han, H. P. and Rand, J. B. (1993). The Caenorhabditis elegans unc-17 gene: a putative vesicular acetylcholine transporter. *Science* **261**, 617-619.

Alfonso, A., Grundahl, K., McManus, J. R. and Rand, J. B. (1994). Cloning and characterization of the choline acetyltransferase structural gene (cha-1) from *C. elegans*. *J. Neurosci.* **14**, 2290-2300.

Argiles, J. M. and Lopez Soriano, F. J. (1996). The ubiquitin-dependent proteolytic pathway in skeletal muscle: its role in pathological states. *Trends Pharmacol. Sci.* **17**, 223-226.

Attaix, D., Taillandier, D., Combaret, L., Ralliere, C., Larbaud, D., Aurousseau, E. and Tanaka, K. (1997). Expression of subunits of the 19S complex and of the PA28 activator in rat skeletal muscle. *Mol. Biol. Rep.* **24**, 95-98.

Attaix, D., Aurousseau, E., Combaret, L., Kee, A., Larbaud, D., Ralliere, C., Souweine, B., Taillandier, D. and Tilignac, T. (1998). Ubiquitin-proteasome-dependent proteolysis in skeletal muscle. *Reprod. Nutr. Dev.* **38**, 153-165.

Auclair, D., Garrel, D. R., Chaouki Zerouala, A. and Ferland, L. H. (1997). Activation of the ubiquitin pathway in rat skeletal muscle by catabolic doses of glucocorticoids. *Am. J. Physiol.* **272**, C1007-1016.

Ballivet, M., Alliod, C., Bertrand, S. and Bertrand, D. (1996). Nicotinic acetylcholine receptors in the nematode Caenorhabditis elegans. *J. Mol. Biol.* **258**, 261-269.

Baumeister, W., Cejka, Z., Kania, M. and Seemueller, E. (1997). The proteasome: A macromolecular assembly designed to confine proteolysis to a nanocompartment. *Biol. Chem.* **378**, 121-130.

Biolo, G., Toigo, G., Ciocchi, B., Situlin, R., Iscra, F., Gullo, A. and Guarnieri, G. (1997). Metabolic response to injury and sepsis: changes in protein metabolism. *Nutrition* **13**, 52s-57s.

Bolten, S. L., Powell Abel, P., Fischhoff, D. A. and Waterston, R. H. (1984). The sup-7(st5) X gene of Caenorhabditis elegans encodes a $tRNA^{Trp}_{UAG}$ amber suppressor. *Proc. Nat. Acad. Sci. USA* **81**, 6784-6788.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics* **77**, 71-94.

Consortium, The C. elegans Sequencing (1998). Genome sequence of the nematode C. elegans: a platform for investigating biology. *Science* **282**, 2012-2018.

Cooney, R. N., Kimball, S. R. and Vary, T. C. (1997). Regulation of skeletal muscle protein turnover during sepsis: mechanisms and mediators. *Shock* **7**, 1-16.

Coux, O., Tanaka, K. and Goldberg, A. (1996). Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.* **65**, 801-847.

Craiu, A., Gaczynska, M., Akopian, T., Gramm, C. F., Fenteany, G., Goldberg, A. L. and Rock, K. L. (1997). Lactacystin and clasto-lactacystin beta-lactone modify multiple proteasome beta-subunits and inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation. *J. Biol. Chem.* **272**, 13437-13445.

Epstein, H. F., Waterston, R. H. and Brenner, S. (1974). A mutant affecting the heavy chain of myosin in Caenorhabditis elegans. *J. Mol. Biol.* **90**, 291-300.

Farley, J. R., Puzas, J. E. and Baylink, D. J. (1982). Effect of skeletal alkaline phosphatase inhibitors on bone cell proliferation in vitro. *Miner. Electrolyte Metab.* **7**, 316-323.

Fire, A. and Waterston, R. H. (1989). Proper expression of myosin genes in transgenic nematodes. *EMBO J.* **8**, 3419-3428.

Fire, A. (1992). Histochemical technqiues for locating *Escherichia col*i β-galactosidase activity in transgenic organisms. *Gene Anal. Tech. Appl.* **9**, 151-158.

Fleming, J. T., Tornoe, C., Riina, H. A., Coadwell, J., Lewis, J. A. and Sattelle, D. B. (1993). Acetylcholine receptor molecules of the nematode Caenorhabditis elegans. In *Comparative Molecular Neurobiology* (ed. Y. Pichon), pp. 65-80. Basel: Birkhauser Verlag.

Fleming, J. T., Squire, M. D., Barnes, T. M., Tornoe, C., Matsuda, K., Ahnn, J., Fire, A., Sulston, J. E., Barnard, E. A., Sattelle, D. B. et al. (1997). Caenorhabditis elegans levamisole resistance genes lev-1, unc-29, and unc-38 encode functional nicotinic acetylcholine receptor subunits. *J. Neurosci.* **17**, 5843-5857.

Furuno, K., Goodman, M. N. and Goldberg, A. L. (1990). Role of different proteolytic systems in the degradation of muscle proteins during denervation atrophy. *J. Biol. Chem.* **265**, 8550-8557.

Garcia Martinez, C., Llovera, M., Agell, N., Lopez Soriano, F. J. and Argiles, J. M. (1995). Ubiquitin gene expression in skeletal muscle is increased during sepsis: Involvement of TNF-alpha but not IL-1. *Biochem. Biophys. Res. Commun.* **217**, 839-844.

Gropper, R., Brandt, R. A., Elias, S., Bearer, C. F., Mayer, A., Schwartz, A. L. and Ciechanover, A. (1991). The ubiquitin-activating enzyme, E1, is required for stress-induced lysosomal degradation of cellular proteins. *J. Biol. Chem.* **266**, 3602-3610.

Hasselgren, P. O. and Fischer, J. E. (1997). The ubiquitin-proteasome pathway: review of a novel intracellular mechanism of muscle protein breakdown during sepsis and other catabolic conditions. *Ann. Surg.* **225**, 307-316.

Hershko, A. and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425-479.

Hobler, S., Tiao, G., Fischer, J. E. and Hasselgren, P. O. (1998a). The sepsis-induced increase in muscle proteolysis is associated with increased 20S proteasome activity and is blocked by 20S proteasome inhibitors. *Surgical Forum* **47**, 14-17.

Hobler, S. C., Tiao, G., Fischer, J. E., Monaco, J. and Hasselgren, P. O. (1998b). Sepsis-induced increase in muscle proteolysis is blocked by specific proteasome inhibitors. *Am. J. Physiol.* **274**, R30-37.

Hobler, S. C., Wang, J. J., Williams, A. B., Melandri, F., Sun, X., Fischer, J. E. and Hasselgren, P. O. (1999). Sepsis is associated with increased ubiquitin conjugating enzyme E214k mRNA in skeletal muscle. *Am. J. Physiol.* **276**, R468-473.

Hosono, R., Sassa, T. and Kuno, S. (1989). Spontaneous mutations of trichlorfon resistance in the nematode, Caenorhabditis elegans. *Zool. Sci.* **6**, 697-708.

Jin, Y., Jorgensen, E., Hartwieg, E. and Horvitz, H. R. (1999). The Caenorhabditis elegans gene unc-25 encodes glutamic acid decarboxylase and is required for synaptic transmission but not synaptic development. *J. Neurosci.* **19**, 539-548.

Lee, D. H. and Goldberg, A. L. (1996). Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in Saccharomyces cerevisiae. *J. Biol. Chem.* **271**, 27280-27284.

Lewis, J. A., Wu, C.-H., Berg, H. and Levine, J. H. (1980a). The genetics of levamisole resistance in the nematode *Caenorhabditis elegans*. *Genetics* **95**, 905-928.

Lewis, J. A., Wu, C.-H., Levine, J. H. and Berg, H. (1980b). Levamisole-resistant mutants of the nematode *Caenorhabditis elegans* appear to lack pharmacological acetylcholine receptors. *Neuroscience* **5**, 967-989.

Li, J. B. and Goldberg, A. L. (1976). Effects of food deprivation on protein synthesis and degradation in rat skeletal muscles. *Am. J. Physiol.* **231**, 441-448.

Mackenzie, J. M. Jr and Epstein, H. F. (1980). Paramyosin is neccessary for determination of nematode thick filament in vivo. *Cell* **22**, 747-755.

Maruyama, I. N. and Brenner, S. (1991). A phorbol ester/diacylglycerol-binding protein encoded by the unc-13 gene of Caenorhabditis elegans. *Proc. Nat. Acad. Sci. USA* **88**, 5729-5733.

McIntire, S. L., Jorgensen, E., Kaplan, J. and Horvitz, H. R. (1993). The GABAergic nervous system of Caenorhabditis elegans. *Nature* **364**, 337-341.

McIntire, S. L., Reimer, R. J., Schuske, K., Edwards, R. H. and Jorgensen, E. M. (1997). Identification and characterization of the vesicular GABA transporter. *Nature* **389**, 870-876.

Medina, R., Wing, S. W., Haas, A. and Goldberg, A. L. (1991). Activation of the ubiquitin-ATP-dependent proteolytic system in skeletal muscle during fasting and denervation atrophy. *Biomed. Biochim. Acta* **50**, 347-356.

Mitch, W. E. and Goldberg, A. L. (1996). Mechanisms of muscle wasting: The role of the ubiquitin-proteasome pathway. *New Engl. J. Med.* **335**, 1897-1905.

Mitch, W. E. (1998). Robert H Herman Memorial Award in Clinical Nutrition Lecture, 1997. Mechanisms causing loss of lean body mass in kidney disease. *Am. J. Clin. Nutr.* **67**, 359-366.

Okkema, P. G., Harrison, S. W., Plunger, V., Aryana, A. and Fire, A. (1993). Sequence requirements for myosin gene expression and regulation in Caenorhabditis elegans. *Genetics* **135**, 385-404.

Opperman, C. H. and Chang, S. (1991). Effects of aldicarb and fenamiphos on acetylcholinesterase and motility of Caenorhabditis elegans. *J. Nematol.* **23**, 20-27.

Rand, J. B. (1989). Genetic analysis of the cha-1 – unc-17 gene complex in *Caenorhabditis*. *Genetics* **122**, 73-80.

Rooyackers, O. E. and Nair, K. S. (1997). Hormonal regulation of human muscle protein metabolism. *Annu. Rev. Nutr.* **17**, 457-485.

Rosenbluth, J. (1965). Structural organization of obliquely striated muscle fibers in *Ascaris lumbricoides*. *J. Cell Biol.* **25**, 495-515.

Sassa, T., Harada, S., Ogawa, H., Rand, J. B., Maruyama, I. N. and Hosono, R. (1999). Regulation of the UNC-18-Caenorhabditis elegans syntaxin complex by UNC-13. *J. Neurosci.* **19**, 4772-4777.

Sebastiano, M., D'Alessio, M. and Bazzicalupo, P. (1986). Beta-glucuronidase mutants of the nematode Caenorhabditis elegans. *Genetics* **112**, 459-468.

Solomon, V. and Goldberg, A. L. (1996). Importance of the ATP-ubiquitin-proteasome pathway in the degradation of soluble and myofibrillar proteins in rabbit muscle extracts. *J. Biol. Chem.* **271**, 26690-26697.

Squire, M. D., Tornoe, C., Baylis, H. A., Fleming, J. T., Barnard, E. A. and Sattelle, D. B. (1995). Molecular cloning and functional co-expression of a Caenorhabditis elegans nicotinic acetylcholine receptor subunit (acr-2). *Receptors Channels* **3**, 107-115.

Stone, S. and Shaw, J. E. (1993). A Caenorhabditis elegans act-4::lacZ fusion: use as a transformation marker and analysis of tissue-specific expression. *Gene* **131**, 167-173.

Sulston, J. E. and Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. *Dev. Biol.* **56**, 110-156.

Taillandier, D., Aurousseau, E., Meynial Denis, D., Bechet, D., Ferrara, M., Cottin, P., Ducastaing, A., Bigard, X., Guezennec, C. Y., Schmid, H. P. et al. (1996). Coordinate activation of lysosomal, Ca^{2+}-activated and ATP-ubiquitin-dependent proteinases in the unweighted rat soleus muscle. *Biochem. J.* **316**, 65-72.

Tawa, N. E. Jr, Odessey, R. and Goldberg, A. L. (1997). Inhibitors of the proteasome reduce the accelerated proteolysis in atrophying rat skeletal muscles. *J. Clin. Invest.* **100**, 197-203.

Tiao, G., Hobler, S., Wang, J. J., Meyer, T. A., Luchette, F. A., Fisher, J. E. and Hasselgren, P. O. (1997). Sepsis is associated with increased mRNAs of the ubiquitin-proteasome proteolytic pathway in human skeletal muscle. *J. Clin. Invest.* **99**, 163-168.

Toomey, D., Redmond, H. P. and Bouchier Hayes, D. (1995). Mechanisms mediating cancer cachexia. *Cancer* **76**, 2418-2426.

Varshavsky, A. (1997). The ubiquitin system. *Trends Biochem. Sci.* **22**, 383-387.

Waterston, R. H., Epstein, H. F. and Brenner, S. (1974). Paramyosin of Caenorhabditis elegans. *J. Mol. Biol.* **90**, 285-290.

White, J. G., Southgate, E., Thomson, J. N. and Brenner, S. (1986). The structure of the nervous system of *Caenorhabditis elegans*. *Phil. Trans. Roy. Soc. Ser. B* **314**, 1-340.

Wing, S. S. and Banville, D. (1994). 14-kDa ubiquitin-conjugating enzyme: structure of the rat gene and regulation upon fasting and by insulin. *Am. J. Physiol.* **267**, E39-E48.

Zdinak, L. A., Greenberg, I. B., Szewczyk, N. J., Barmada, S. J., Cardamone Rayner, M., Hartman, J. J. and Jacobson, L. A. (1997). Transgene-coded chimeric proteins as reporters of intracellular proteolysis: starvation-induced catabolism of a lacZ fusion protein in muscle cells of Caenorhabditis elegans. *J. Cell Biochem.* **67**, 143-153.