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Degradation of transgene-coded and endogenous proteins in the muscles of *Caenorhabditis elegans*☆

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Abstract

To develop reporter systems to study the regulation of protein degradation in innervated muscle, we have used strains of the nematode *Caenorhabditis elegans* containing transgenes that fuse *lacZ* or green fluorescent protein (GFP) coding regions to muscle-specific promoter/enhancer regions, such that the fusion proteins are expressed exclusively in body-wall and vulval muscle cells. The starvation-induced degradation of the β-galactosidase reporter protein is quantitatively similar to that of two endogenous muscle proteins, arginine kinase and adenylate kinase. A soluble GFP in the muscle cytosol is degraded during starvation, but when GFP is fused to a full-length myosin heavy chain and incorporated into myofibrils, it is resistant to starvation-induced degradation. This suggests that under some conditions soluble muscle proteins may be extensively catabolized in preference to the proteins of the contractile fibers.

Keywords: Nematode; Proteolysis; Degradation; Starvation; Muscle; GFP; β-Galactosidase; Transgene

As a graduate student in the laboratory of I.C. Gunsalus, one of us (L.A.J) faced a recurring challenge in explaining to outsiders that we worked on the microbial catabolism of camphor. Reactions ranged from blank stares to a puzzled "Camphor? Why?" to a resigned shrug which usually signified "What can one do with such people?" Of course, Gunny's explanation was pellucid. He knew there was something important to be learned about oxygenases, and it was obvious (to him!) that such enzymes would be the fundamental instruments used by any microbe to subsist on such a greaseball molecule as camphor. Accordingly, enrichment cultures were done and with his chronic good judgment, Gunny chose to focus on the *Pseudomonas* that emerged [1], while more or less ignoring other less convenient isolates. The rest, as they say, is history.

Among the many kernels of scientific wisdom to be gleaned from Gunny's investigational "style" are these: Pick an important problem, make a shrewd choice of model system, and then exploit that system relentlessly.

Some years ago, our laboratory became interested in the regulation of protein catabolism in muscle. This is an essential problem both in normal adaptive physiology and in various pathologies. Muscle contains more than half the protein of an adult human, providing a valuable reserve that can be mobilized in nutritional need [2]. However, protein is costly to synthesize, and biochemical economy dictates that it be maintained only so long as it is used to provide contractile power. Muscle is consequently among the most plastic of tissues. Muscle proteins, including both the contractile apparatus (myofibrils) and the supporting enzymatic machinery that powers and regulates contraction, accumulate to meet demand (e.g., increased exercise), only to be readily recycled (catabolized) when that functional demand is diminished by disuse or impairment of neural inputs. In some cases the complex set of hormonal and other signals that control this balance [2] seems to go awry, to the point where extreme muscle wasting (cachexia) becomes the proximal cause of death in many cancer patients [3].

☆ Dedicated, with admiration and affection, to Prof. I.C. Gunsalus, a founding editor of this journal.

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We know surprisingly little about what extramuscular signals govern the rate of protein catabolism, or how these signals are transduced by muscle cells, or what might be the ultimate molecular effectors of such signal-transduction. To bring the power of genetics to bear on these questions, we chose as a model the nematode *Caenorhabditis elegans*, a small (~1.5 mm long) soil organism and the simplest animal that has both a well-characterized neuromuscular system [4] and exceptionally well-developed tools for both classical and molecular genetics [5]. Its cellular simplicity is a strong attraction; for example, only 95 body-wall muscle cells drive the worm's sinusoidal motility. At the cellular and molecular levels the neuromuscular system of *C. elegans* has powerful resemblances to those of higher animals [6].

But what proteins are we to follow in the muscles of such a tiny creature? To "report" on protein catabolism [7] we introduced the use of a transgene, a fusion between the promoter and enhancer regions of a nematode myosin heavy-chain gene (unc-54) and the lacZ gene of *Escherichia coli*. The fusion protein product was expressed from the integrated transgene only in 95 body-wall and 8 vulval muscle cells and formed active β-galactosidase tetramers, which did not associate with myofibrils, but remained soluble in the muscle cytosol. Because *C. elegans* has no endogenous β-galactosidase, it is easy to follow the fate of this fusion protein. The reporter was completely stable in well-fed wild-type animals [7], but was catabolized upon acute starvation [7] or upon diminution of neural signaling to nicotinic acetylcholine receptors [8] or upon mutational activation of the Ras-Raf-MEK-MAP kinase signaling cascade [9].

We now pose two crucial questions. First, how well does the LacZ reporter reflect the fate of soluble proteins that are normally produced in muscle? Second, do soluble cytosolic proteins and insoluble proteins in myofibrils have similar susceptibilities to proteolysis, or do their different physical states govern their fates? We report here that during acute starvation, the rate of degradation of the LacZ reporter protein is similar to the rates for two muscle marker enzymes, adenylate kinase and arginine kinase (the nematode analogue of vertebrate creatine kinase). We also use transgenes expressing green fluorescent protein (GFP) as either a soluble cytosolic form or fused upstream of a full-length myosin heavy-chain (myo-3 product) so as to be incorporated into myofibrils. This comparison shows that during starvation the soluble GFP is degraded, but the myofibrillar GFP remains stable. Our findings indicate that under some conditions soluble proteins in muscle are extensively catabolized in preference to the proteins in the contractile fibers.

Materials and methods

Nematodes. C. elegans strains were maintained and grown at 20 °C and roughly age-synchronized [7]. In our experiments, $t = 0$ is designated in early adulthood 40–44 h after age synchrony. The strain PD55 (tra-3(e1107) IV; ccIs55 (unc-54::lacZ, sup-7) V) was used as the "wild-type" strain for enzyme assays. An extrachromosomal array (stEx27, gift of P. Hoppe and R. Waterston) consisting of the GFP gene from *Aequorea victoria* [10] inserted in-frame between the promoter and coding region of the myo-3 (minor myosin heavy-chain) gene and a dominant rol-6(su1006) marker was integrated by irradiation (4300 Rad from a ^{137}Cs source) followed by clonal screening for progeny that no longer produced any nonrollers. The resulting strain carrying the integrated transgene jIs01 (myo-3::GFP, rol-6) was outcrossed three times to wild-type and placed in a genetic background containing ccIs55 to give strain PJ727. Strain PJ1145 expressing soluble GFP specifically in body-wall and vulval muscle was constructed by crossing the extrachromosomal array njEx38 (PG_o::GFP, rol-6, Punc-54::daf-2^+), a gift from C. Wolkow and G. Ruvkun, into a genetic background containing ccIs55.

Enzyme assays. β-Galactosidase was assayed fluorimetrically in 10-worm lysates [7]. For arginine kinase (APK) and adenylate kinase (AK) assays, 20-worm samples were picked into 27 μL of buffer (0.25 M glycylglycine, 0.1% bovine serum albumin, 0.1% (v/v) Triton X-100, pH 7.4), lysed by six freeze–thaw cycles, and made 10 mM in *N*-acetylcysteine. Spectrophotometric assays of AK and APK were based upon the system of [11], in which ATP production is coupled to the formation of NADPH via hexokinase and glucose-6-phosphate dehydrogenase:

$$\text{adenylate kinase}: \quad 2\text{ADP} \rightarrow \text{ATP} + \text{AMP}$$

$$\text{arginine kinase}: \quad \text{Arg} \sim \text{P} + \text{ADP} \rightarrow \text{Arg} + \text{ATP}$$

$$\text{hexokinase}: \quad \text{ATP} + \text{glucose} \rightarrow \text{glucose 6-phosphate} + \text{ADP}$$

$$\text{G6PDH}: \quad \text{glucose 6-phosphate} + \text{NADP}^+ \rightarrow 6-\text{phosphogluconate} + \text{NADPH} + \text{H}^+$$

The reaction mixture consisted of 50 mM glycylglycine, pH 7.4; 34 mM D-glucose; 4 mM Mg acetate; 0.4 mM NADP; 10 units hexokinase (Sigma); 5 units glucose-6-phosphate dehydrogenase (Sigma); 0.02% bovine serum albumin and worm lysate as indicated. Adenylate kinase activity was measured from the rate of change of absorbance (340 nm) after the addition of 1.4 mM ADP. Arginine phosphate was then added to a final concentration of 13 mM and the incremental rate of change in absorbance measured. Arginine kinase activity was calculated as the rate (+Arg~P + ADP) minus the rate (+ADP). There was no observable rate in reactions with Arg~P but without ADP. Assays were linear with time and with amount of crude worm extract added (Fig. 1).

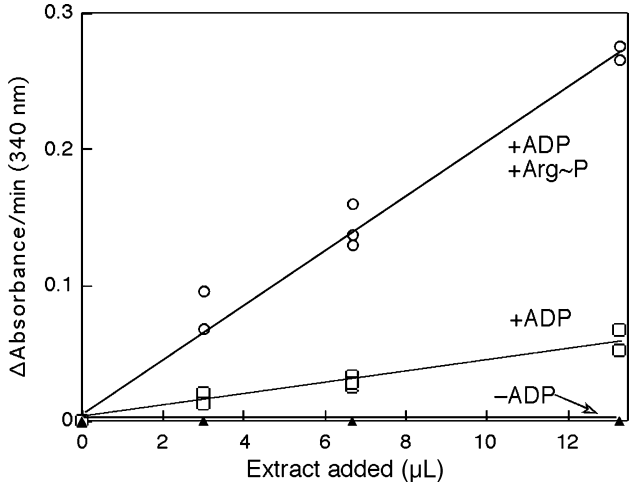

Fig. 1. Spectrophotometric assay of adenylate kinase (AK) and arginine kinase (APK). AK activity is calculated from the rate (+ADP). APK activity is calculated from the rate (+Arg~P + ADP) minus the AK activity.

Immunoblotting. Quantitative measurements of the UNC-54::LacZ fusion protein were carried out as described [7] by electrophoresis on 7.5% polyacrylamide gels followed by immunoblotting with monoclonal anti-β-galactosidase antibody (Promega Z-378A). GFP was measured similarly, except that a rabbit polyclonal anti-GFP antibody (Abcam Ltd. ab290) was used after electrophoresis on 15% gels (soluble GFP) or 4–12% gradient gels (MYO-3::GFP).

Photomicrography. Fixed animals stained for β-galactosidase activity with X-gal [7] were photographed with brightfield illumination. Live animals containing GFP were photographed using epifluorescence illumination with a Chroma 41001 filter set. Images were recorded with a Leica DFC-300F digital camera and post-processed using Adobe Photoshop software.

Results and discussion

Starvation-induced degradation of endogenous muscle proteins

Reporter proteins are convenient for following proteolysis in specific cells, but there are potential pitfalls. Attack by proteases is sensitive to the conformational properties of the target proteins [12,13] and proteins expressed from fusion transgenes may have unusual conformations, particularly at the points of fusion. For example, the unc-54::lacZ transgene begins with the first four exons of the *C. elegans* unc-54 (myosin heavy-chain) gene [14], followed by a short "stuffer" fragment, followed by the entire *E. coli* lacZ coding region. The UNC-54 fragment is certainly large enough to form a folded structure, but as it constitutes only part of the myosin ATPase domain, the protein does not associate with myofibrils. The LacZ region must fold correctly, since the fusion protein assembles into tetramers with β-galactosidase activity [7]. The intervening region is likely too small to form an independently folded domain and may be disordered. In consequence, the 149-kDa fusion protein can undergo limited proteolytic processing to remove the N-terminal myosin and stuffer regions, leaving an active 116-kDa product the size of *E. coli* β-galactosidase [7]. The fusion protein is stable in well-fed wild-type animals, but following acute starvation or genetic denervation it is ubiquitinylated and degraded by the proteasome [7,8].

It is therefore necessary to ask whether the in vivo degradation of such a reporter protein "represents" the fates of other proteins in the same cell. *C. elegans* is too small to permit dissection of muscle tissue, so instead we compared to two endogenous muscle enzymes. Adenylate kinase (AK, also known as myokinase for its role in muscle) provides for "scavenger" ATP synthesis from ADP. Arginine kinase (APK) catalyzes phosphorylation of ADP by the phosphagen arginine phosphate; thus, Arg∼P and APK buffer the ATP concentration in invertebrate muscle, much as their homologues creatine phosphate and creatine kinase do in vertebrate muscle.

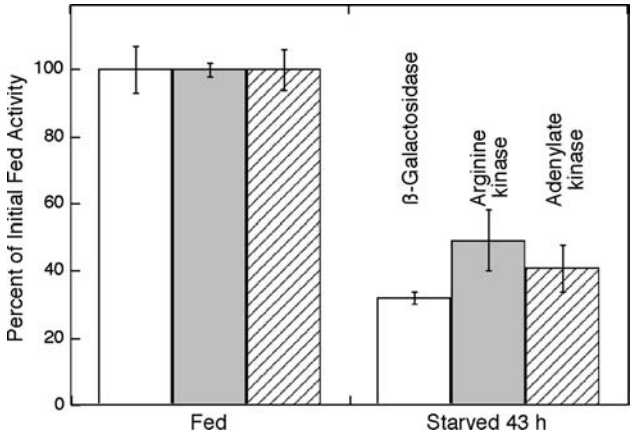

Fig. 2. Decline in activities of LacZ reporter, arginine kinase (APK), and adenylate kinase (APK) during 43-h starvation of wild-type animals. Data shown are means ± SD of quadruplicate assays of 10-worm lysates (β-galactosidase) or 20-worm lysates (AK and APK).

Activity measurements (Fig. 2) after 43 h of acute starvation indicate that AK and APK are degraded to about the same extent as the LacZ fusion protein. The observed declines in activities we take to represent degradation of preexisting protein molecules, as has been directly verified for the LacZ reporter [7] (Fig. 4, below). Minor differences in extents of degradation (Fig. 2) are reasonable, because each soluble, globular protein will have its own characteristic susceptibility to proteolysis, deriving from its amino acid sequence and folded conformation. These data therefore confirm that the LacZ reporter is as likely as any protein to provide "representative" information about the degradation of soluble muscle proteins.

Differential degradation of soluble and myofibrillar proteins

From in vitro experiments [15] it was proposed that proteins in myofibrils are less susceptible to proteolytic degradation than are soluble proteins of the muscle cytosol. To explore this further, we have used two different forms of GFP expressed specifically in muscle. One is GFP alone, soluble in the muscle cytosol. The second form is a fusion of GFP to a complete myosin heavy-chain (myo-3 product). This fusion protein is incorporated into myofibrils, so that fluorescence is visible only in the myofibrils (Fig. 3B) and not elsewhere in muscle cells.

Upon acute starvation, the soluble GFP appears by microscopy to be degraded much as is the soluble LacZ reporter protein in the same animals (Fig. 3A). In both cases, the remaining protein after 48 h of starvation is located exclusively in the contained embryos, which are not effectively starved while nourished by the yolk protein in the eggs. By contrast, the myofibrillar GFP appears to be undiminished even after 72 h of starvation

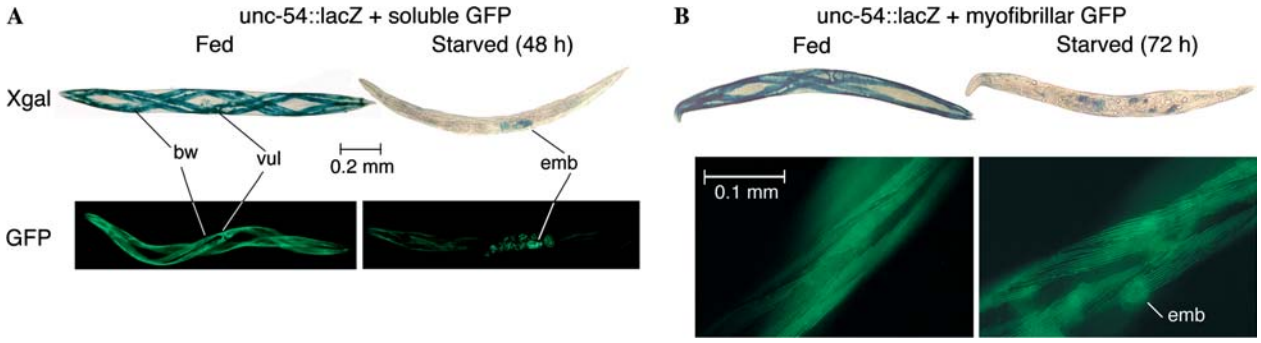

Fig. 3. Reporter protein distribution in fed and starved worms. Upper row shows X-gal staining (blue) of β-galactosidase activity in 95 body-wall (bw) and 8 vulval (vul) muscle cells. Lower row shows GFP fluorescence. Each pair of animals in a fed-starved comparison was photographed at the same exposure. The blue objects in the X-gal stained starved animals are embryos (emb) within the adult animals, also visible in the starved GFP photo at lower right. The helical twisting of the body-wall muscle bands is a result of the "roller" phenotype associated with the rol-6 transformation marker. (A) Strain PJ1145, containing soluble GFP in muscles; (B) strain PJ727, containing myofibrillar GFP.

(Fig. 3B). To quantitate these observations, we measured the amounts of LacZ, soluble GFP and myofibrillar GFP (in appropriate strains) by immunoblotting (Fig. 4). All three proteins are stable in fed animals, whereas LacZ and the soluble GFP are degraded during starvation. Although GFP forms an exceptionally compact monomer [16] that contains few accessible surface loops and presents a seemingly less inviting proteolytic target than the much larger β-galactosidase tetramer [17], the two proteins in soluble form are degraded at similar rates. By contrast, the GFP-myosin fusion protein in myofibrils remains almost entirely stable up to 72 h of starvation (Fig. 4).

The simplest interpretation is that myofibrillar proteins are largely resistant to proteolytic attack by virtue of their semi-insoluble state in the supramolecular complexes of the myofibrils. It seems highly unlikely that the GFP fused to the N-terminus of a myosin heavy-chain would be protected by any "specific" interactions with other proteins of the myofibrils. This simple interpretation is consistent with the proposal [15] that myofibrillar proteins are at risk of degradation only when they dissociate from the myofibrils. However, there are other more complex interpretations. The proteolytic susceptibility of any protein may depend upon the primary sequence and/or conformational context

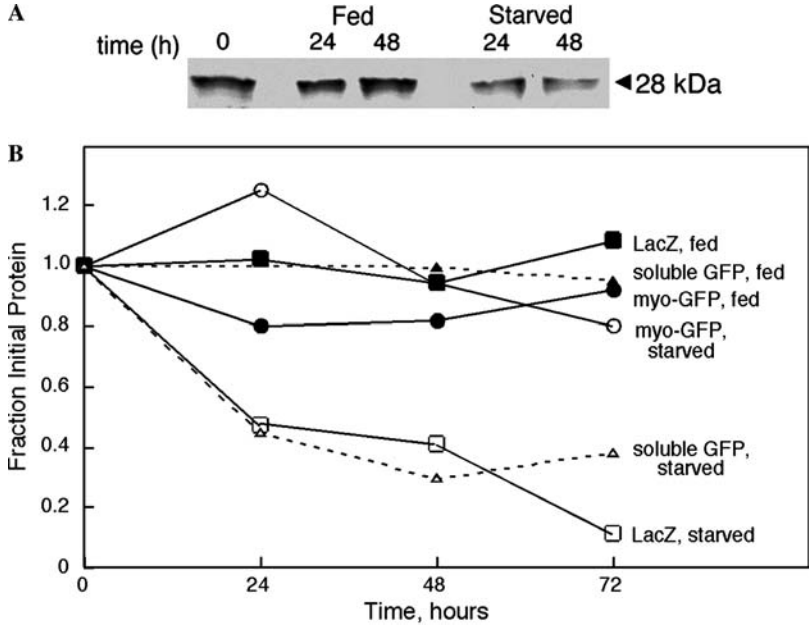

Fig. 4. Protein degradation in fed and starved worms assayed by immunoblotting. Worms at $t = 0$ had been grown to early adulthood (40 h at 20 °C) from age-synchronized populations. (A) Example immunoblot of soluble GFP, using 30 worms per lane, detection with polyclonal anti-GFP antibody. (B) Animals were fed or starved for the indicated times and immunoblots made as described in Materials and methods. Band intensities were integrated with NIH Image software.

[12,13,18,19]. The N-terminus of GFP is unmodified in this fusion, but proteolytic susceptibility might be affected by the presence of the large myosin moiety at the C-terminus rather than by the assembly of the fusion protein into myofibrils.

Reporter proteins as tools for genetics

One of our motives in developing these reporter systems is their potential use in identifying mutations that affect the regulation and/or execution of intramuscular proteolysis. For example, the LacZ reporter permitted rapid screening of a variety of existing single and double mutants with altered Ras-MAP kinase signaling [9], eventuating in the finding that activation of this pathway promotes protein degradation. The GFP-based reporter systems are even more convenient in this regard, since they allow the state of muscle protein degradation to be evaluated in live animals viewed under a dissecting microscope with epifluorescence illumination. We are currently using GFP reporter strains to isolate new mutations that affect muscle proteolysis.

Acknowledgments

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