Biochem. J. (1991) 275, 165–169 (Printed in Great Britain)

Proteins containing peptide sequences related to Lys-Phe-Glu-Arg-Gln are selectively depleted in liver and heart, but not skeletal muscle, of fasted rats

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In response to serum withdrawal, when overall rates of proteolysis increase in cultured fibroblasts, proteins containing peptide regions similar to Lys-Phe-Glu-Arg-Gln (KFERQ) are targeted to lysosomes for degradation, and the intracellular concentrations of these proteins decline [Chiang & Dice (1988) J. Biol. Chem. **263**, 6797–6805]. To test whether such proteins are also selectively depleted in mammalian tissues *in vivo*, we have used affinity-purified polyclonal antibodies to KFERQ to detect proteins containing such sequences in tissues of fed and fasted rats. Immunoreactive cytosolic proteins were partially depleted from liver and heart of fasted rats, but the time course differed for these two tissues. Immunoreactive proteins in liver were lost during days 2 and 3 of fasting, whereas such proteins in heart were depleted within day 1 of fasting. In the same fasted rats, levels of immunoreactive cytosolic proteins did not change in two skeletal muscles, the dark soleus and the pale extensor digitorum longus. Immunoreactive proteins in a myofibrillar fraction were also partially depleted in heart, but not in skeletal muscles, of fasted rats. The most likely explanation for these results is that the protein loss in different tissues upon fasting results from selective activation of different proteolytic pathways. The increased proteolysis in liver and heart of fasted animals includes activation of the KFERQ-selective lysosomal pathway, whereas increased proteolysis in skeletal muscle does not.

INTRODUCTION

In confluent cultures of primary human fibroblasts, degradation of certain proteins, but not others, is stimulated in response to serum withdrawal [1–3]. One such protein, pancreatic RNAase A, has been studied in detail by erythrocyte-mediated microinjection [4]. The degradative pathway is lysosomal [5,6], and residues 7–11 of RNAase A, KFERQ, are essential for entry into this pathway [3,7,8]. A cellular protein that is a member of the heat-shock 70 kDa protein family binds to the KFERQ sequence and promotes lysosomal degradation of RNAase A in two different cell-free systems [9].

Antibodies against KFERQ precipitate 25–30% of cytosolic proteins from fibroblasts, and only these immunoreactive proteins are degraded more rapidly in response to serum withdrawal [7]. The peptide regions recognized by the anti-KFERQ IgGs are not necessarily KFERQ, but are chemically related in ways that have been previously described in detail [3,8]. More than 80% of the immunoreactive proteins are lost within the first 24 h of serum deprivation [7].

This selective lysosomal pathway of protein degradation appeared to be operative in liver and kidney, but not in brain and testis, of fasted rats [7]. We presumed that these differences were related to the net protein loss by liver and kidney, but not brain or testis, during fasting. Skeletal muscle, which constitutes the major protein reserve in the body, also shows marked weight loss in response to nutritional deprivation, especially the pale muscles [10]. However, more recent evidence suggests that lysosomes play a minor role in the increased proteolysis and weight loss in skeletal muscle during fasting [11,12] and upon denervation [13]. Therefore it was decided to investigate the relative importance of the KFERQ-selective pathway of proteolysis in skeletal and cardiac muscles and in liver of rats in the fed state and at different times after nutritional deprivation. We have compared the levels of proteins containing KFERQ-like peptide regions initially and after fasting in liver, heart, soleus (a dark skeletal muscle) and extensor digitorum longus (EDL; a pale skeletal muscle).

MATERIALS AND METHODS

Animals

Male CD-strain rats weighing 60–70 g (Charles River Laboratories, Wilmington, MA, U.S.A.) were fed *ad libitum* with Purina lab chow for at least 3 days before experiments. Weights at death (means ± S.E.M.) were 84 ± 3.6 g ($n = 12$) for the fed animals and 53 ± 1.3 g ($n = 16$) 3 days after removal of food ($P < 0.001$).

Subcellular fractionation

After the rats were killed by cervical dislocation, the liver, heart and skeletal muscles were rapidly dissected and homogenized at 4 °C in a Dounce apparatus. Liver was homogenized in 20 mM-sodium phosphate-buffered saline, pH 7.4, containing 2 mM-EDTA and 1 mM-dithiothreitol. The homogenate was centrifuged at 10000 *g* for 10 min and then at 100000 *g* for 1 h. The final supernatant was considered the cytosolic fraction. Muscles were homogenized in 10 mM-Tris/maleate (pH 7.0)/100 mM-KCl/2 mM-$MgCl_2$/1 mM-EGTA/1 mM-dithiothreitol. The homogenate was centrifuged at 1500 *g* for 15 min. A myofibrillar fraction was prepared from the pellet by three washes with the muscle homogenization buffer containing 1% Triton X-100, followed by two washes with the same buffer without detergent [14]. The supernatant fraction from the initial 1500 *g* centrifugation was further centrifuged as described above for liver homogenates in order to obtain a cytosolic fraction.

Abbreviation used: EDL, extensor digitorum longus.

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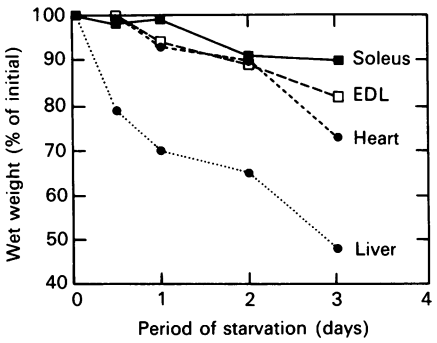

Fig. 1. Time course of loss of wet weight from rat tissues during fasting

See the legend to Table 1 and the Materials and methods section for experimental details. The data are from the first four fed and four fasted animals used for these studies.

Antibodies

Rabbit antibodies to KFERQ linked to BSA were prepared as described previously [7]. The IgGs were first isolated by affinity chromatography on a Protein A–Sepharose column, and specific IgGs were isolated by using an RNAase S-peptide–Sepharose column as described in [7].

Immunoblots

Protein concentrations of subcellular fractions were determined by the bicinchoninic acid method [15], and equal amounts of protein (100 μg) were applied to each lane of a slab gel. Proteins were separated by SDS/PAGE in 1.5 mm-thick 8%- or 10%-acrylamide slab gels as described in [16]. Duplicate gels were run, and one gel was stained with Amido Black. The protein was quantified by using a Bio-Rad 620 Video Densitometer in the transmission mode, and the total area under the absorbance scan was calculated by computer with Bio-Rad software. This procedure verified that equal amounts of protein were analysed, with the exception of a single lane in one experiment (see legend of Fig. 2).

Protein bands from the other gel were transferred to nitrocellulose paper in a Trans-Blot cell as suggested by the supplier (Bio-Rad), except that BSA (3%, w/v) or skim milk (5%, w/v) rather than gelatin was used in the blocking buffer. Anti-KFERQ IgGs were used at 5 μg/ml, and binding was detected by using pre-titred goat anti-rabbit IgGs coupled to horseradish peroxidase or alkaline phosphatase (Sigma) diluted 1:3000. Immunoreactivity was quantified with the densitometer operating in the reflectance mode. Only lanes within a single immunoblot were compared, so variability in efficiency of protein transfer or binding to nitrocellulose in different experiments cannot explain our results. Immunoblots of various amounts of cytosolic proteins (20, 50 and 100 μg) indicated that the assay was linear over this 5-fold range. Furthermore, no antibody binding was evident with preimmune IgGs or IgGs raised to an unrelated pentapeptide, RYLPT [7], under these conditions.

RESULTS

After food deprivation the weight of the liver decreased in the rats more rapidly than did weights of heart or skeletal muscles (Fig. 1). Loss of tissue protein closely paralleled the loss of wet weight for these four tissues throughout the fasting period

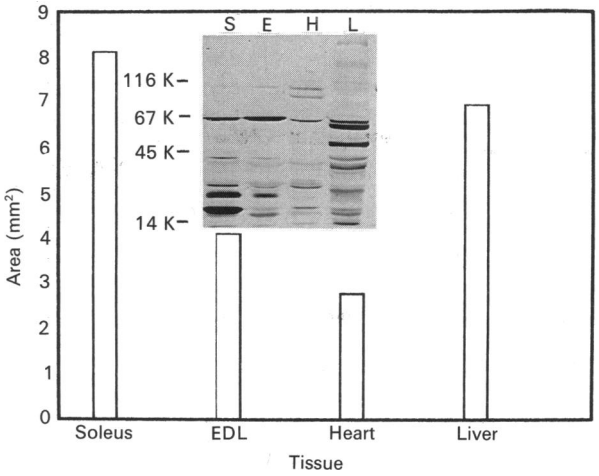

Fig. 2. Reactivity of cytosolic proteins from different rat tissues with anti-KFERQ IgGs

All tissues were from fed animals. The inset shows immunoblots of cytosolic proteins from soleus (S), EDL (E), heart (H) and liver (L) after separation on a 1.5 mm-thick 8%-polyacrylamide gel. Quantification of the protein applied to each lane from a duplicate gel stained with Amido Black indicated approx. 100 μg of protein for soleus, EDL and liver, but only 60 μg for heart. We corrected for this under-representation of heart protein in quantifying the immunoreactive proteins. The positions of molecular-mass markers (K = kDa) are shown on the left: 116 K, *Escherichia coli* β-galactosidase; 67 K, BSA; 45 K, ovalbumin; 14 K, lysozyme. Quantification of the immunoblots after correction for the lesser amount of protein applied for heart gave the values shown in the bar graph. Similar results were obtained in two additional experiments using tissues from other rats.

Table 1. Tissue wet weights and protein content before and after fasting for 3 days

Tissues were quickly dissected, weighed, and placed in phosphate-buffered saline at 0 °C. After subcellular fractionation as described in the Materials and methods section, protein concentrations were determined by the bicinchoninic acid method [15]. Data are means ± S.E.M. from 3 separate experiments using a total of 12 fed and 16 fasted animals. All tissues had statistically significant losses of wet weight after fasting ($P < 0.001$). Losses in protein content were not statistically significant (presumably owing to the small sample size), except for liver ($P < 0.025$).

	Wet weight (mg)			Cytosolic protein content (mg/tissue)		
Tissue	Fed	Fasted	(% loss)	Fed	Fasted	(% loss)
Soleus	36 ± 2	28 ± 1	22	0.98 ± 0.21	0.74 ± 0.13	24
EDL	41 ± 2	28 ± 1	32	1.27 ± 0.21	0.96 ± 0.13	24
Heart	403 ± 19	266 ± 7	34	10.5 ± 1.6	7.6 ± 1.2	28
Liver	4627 ± 200	2063 ± 106	55	270 ± 25	131 ± 10	51

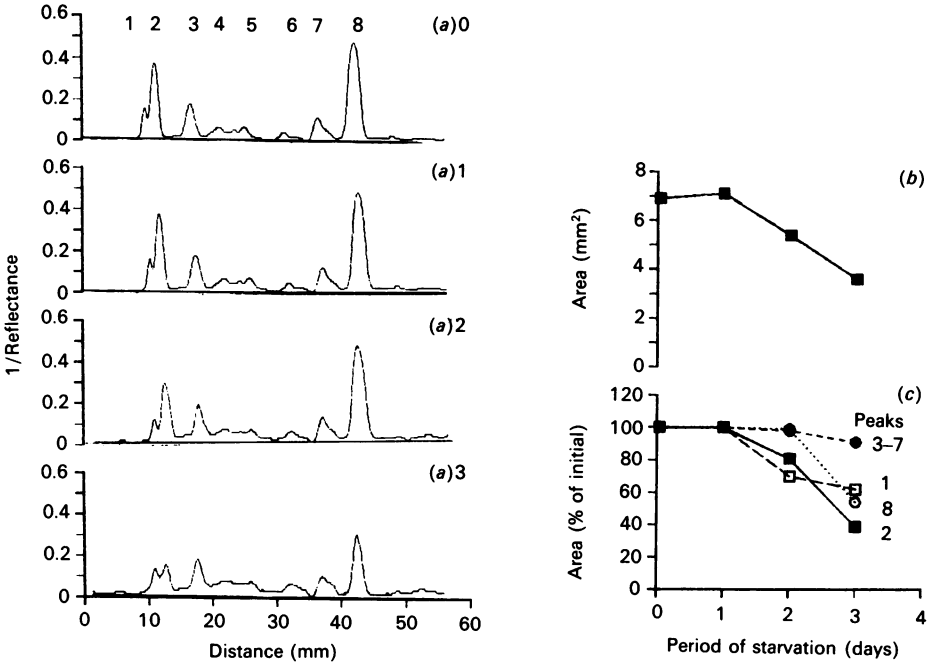

Fig. 3. Effect of fasting on immunoreactive cytosolic proteins from liver

Proteins were separated on 10%-polyacrylamide gels and transferred to nitrocellulose filters. Panels (*a*)0–(*a*)3 show the reflectance scans of liver proteins from rats fasted for 0, 1, 2 or 3 days respectively. Identifiable peaks of 1/reflectance are numbered in (*a*)0. Panel (*b*) shows the quantification of immunoreactive proteins with increasing times of fasting, and panel (*c*) shows quantitative results for individual peaks. Similar results were obtained in four additional experiments.

(results not shown). By 3 days after food deprivation, soleus, EDL, heart and liver decreased significantly in wet weight (Table 1). During this time, the liver lost approx. 50% and heart, soleus and EDL lost 24–28%, of their cytosolic protein content.

Immunoblots of the tissue proteins that react with anti-KFERQ IgGs are shown in Fig. 2. The patterns of the immunoreactive proteins are distinctive for the soleus, EDL, heart and liver, although certain proteins may be present in all four tissues. The total amount of immunoreactive proteins was 2–3-fold higher in liver and soleus than in heart and EDL (Fig. 2).

Fig. 3 shows the loss of proteins that react with anti-KFERQ IgGs from rat liver cytosol upon fasting. The migration positions of immunoreactive bands do not exactly correspond to those shown in Fig. 2, owing to differences in polyacrylamide-gel concentrations (8% for Fig. 2 and 10% for the other Figures). Fig. 3(*a*) compares the reflectance scans of liver immunoreactive proteins for fed rats and at different times after food deprivation (panels 0–3). Quantification of these scans indicates no loss of immunoreactive protein during day 1 of fasting, 26% loss by day 2, and 53% loss by day 3 (Fig. 3*b*). Four additional experiments confirmed that immunoreactive proteins were lost primarily on days 2 and 3 after food deprivation, and showed a range of 40–55% loss of immunoreactive proteins at 3 days of fasting. Fig. 3(*c*) demonstrates that different bands in the immunoblot are depleted to different extents. Bands 1, 2 and 8 decreased by 40–60%, whereas bands 3–7 decreased by only 10%. The significance of their heterogeneous kinetics is not known.

Immunoreactive proteins from heart cytosol are also decreased with fasting (Fig. 4*a*). In contrast, no loss of immunoreactive proteins was evident for proteins from EDL (Fig. 4*b*) or soleus (Fig. 4*c*). The time course of changes in immunoreactive proteins is quantified in Fig. 5. Most of the immunoreactive proteins from heart were lost between 12 and 24 h of fasting.

Because myofibrillar proteins comprise most of the protein in cardiac and skeletal muscle, we also analysed this fraction from heart, EDL and soleus in fed and fasted (3 days) animals. The amount of immunoreactive protein in these fractions was less than in the cytosolic fractions (cf. Figs. 4 and 6). Also, the major actin and myosin bands were not recognized by the antibody. The proteins were modestly decreased in heart, but were unchanged in soleus and EDL from the fasted animals. Because of the low level of immunoreactive proteins in myofibrils, we cannot rule out the possibility that the immunoreactive proteins are actually contaminating cytosolic proteins.

DISCUSSION

We have observed that proteins containing KFERQ-like peptide regions exist in all tissues studied to date. However, only in certain tissues, such as liver and heart, are these proteins preferentially depleted upon nutritional deprivation. The depletion of these proteins most likely reflects an increased activity of the KFERQ-selective lysosomal pathway of proteolysis. This has been well documented in cultured fibroblasts by quantitative immunoprecipitation of a small amount of protein labelled to high specific radioactivity with [^{3}H]leucine [7]. We monitored loss of radioactivity from immunoreactive and non-immunoreactive cellular proteins after incubating cells in unlabelled medium containing excess leucine to prevent re-utilization of the radioisotope [7]. The observed enhancement in degradation of immunoreactive proteins in response to serum withdrawal predicted a 77% loss of such proteins in 24 h if there was no change in the synthesis rate of such proteins. The depletion measured by immunoblots was 80% [7].

Similar experiments are not possible *in vivo*, owing to the much lower specific radioactivity of protein that is attainable and the subsequent requirement for prohibitively large amounts of antibody for the quantitative immunoprecipitation. For example, our calculations show that each rat would have to be injected

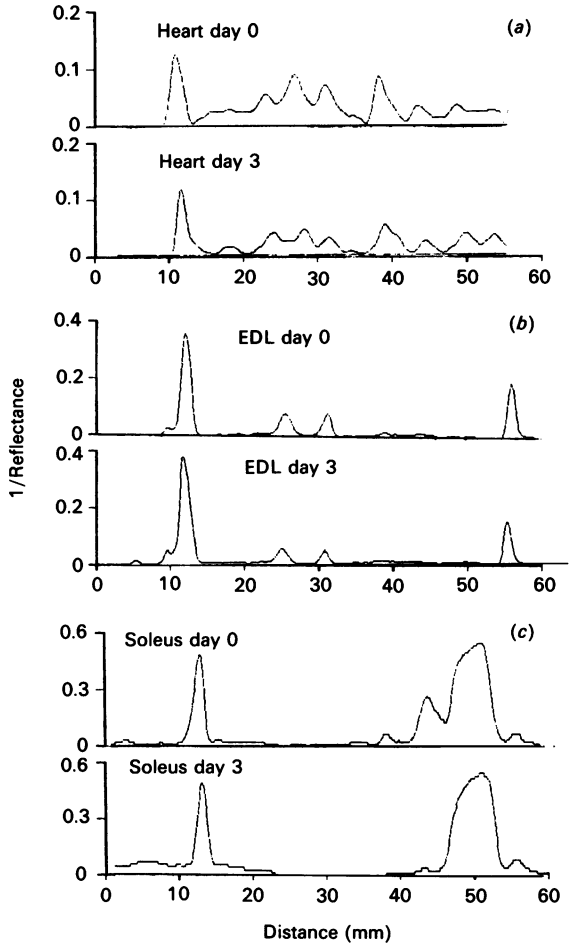

Fig. 4. Effect of fasting on immunoreactive cytosolic proteins from heart (*a*), EDL (*b*) and soleus (*c*)

Proteins from rats fasted for 0 and 3 days were separated as described for liver in the legend of Fig. 3.

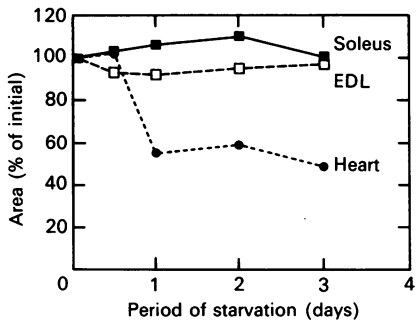

Fig. 5. Quantification and time course of loss of immunoreactive cytosolic proteins from heart, soleus and EDL

Quantification of scans shown in Fig. 4 and additional scans of immunoreactive proteins at 0.5, 1 and 2 days of fasting are shown. Similar results were obtained in two additional experiments.

with 500 mCi of [^{3}H]leucine to obtain liver proteins radiolabelled to the same extent as the fibroblast proteins used for the quantitative immunoprecipitation [7]. Alternatively, rats could be injected with only 1 mCi of [^{3}H]leucine, but then 40 mg of IgGs would be required for each quantitative immunoprecipitation.

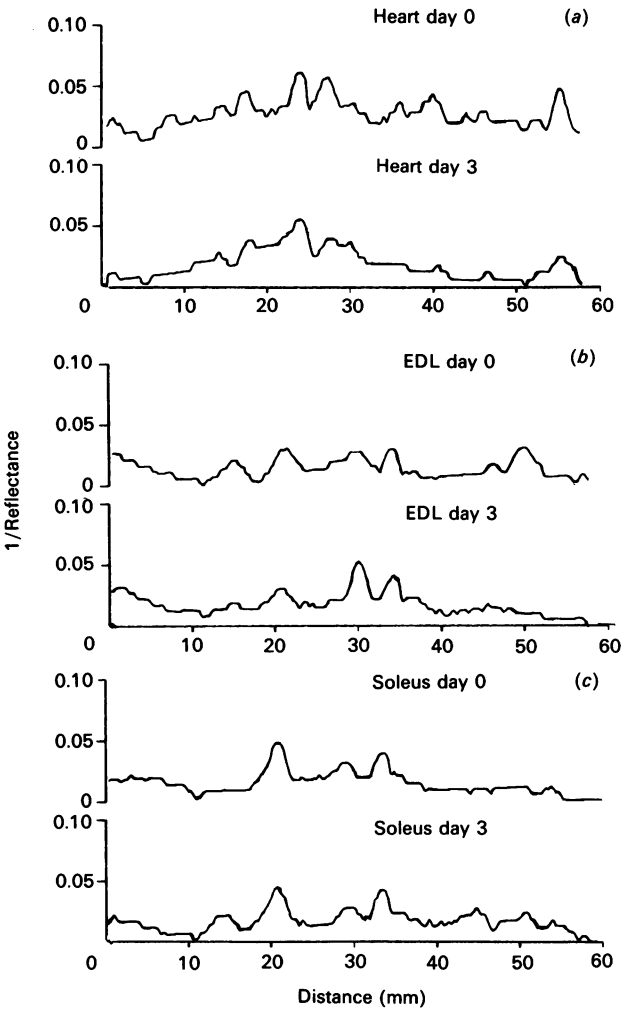

Fig. 6. Effect of fasting on immunoreactive myofibrillar proteins from heart (*a*), EDL (*b*) and soleus (*c*)

Myofibrillar proteins were isolated from fed and fasted (3 days) rats and were separated and analysed as described in the legend of Fig. 3.

Because of these experimental limitations, we cannot completely rule out the possibility that depletion of immunoreactive proteins from tissues of fasted animals results from a preferential decline in rates of synthesis of such proteins. In this case, the maintenance of immunoreactive proteins in skeletal muscle of fasted animals might be due to tissue-specific maintenance of synthesis rates of these proteins during fasting. We think it is more likely that loss of proteins containing KFERQ-like peptide regions reflects activation of the selective lysosomal proteolytic pathway, because there is growing evidence that different degradative pathways are activated in liver compared with skeletal muscle in response to fasting (see below).

Most of the lysosomal degradation of liver proteins under acute food deprivation conditions is due to macroautophagy [17], a process that appears to be rather non-selective [18,19]. Activation of the selective lysosomal pathway of degradation appears to be distinct from macroautophagy, on the basis of the selective pathway being activated later in fasting. In addition, macroautophagy results in preferential degradation of smooth endoplasmic reticulum [17,20], whereas the selective pathway results in preferential degradation of cytosolic proteins [7,21]. Perhaps macroautophagy accounts for the early loss of protein

from liver, whereas the pathway that is selective for the proteins with KFERQ-like sequences is responsible for much of the protein loss during days 2 and 3 of fasting (Fig. 1).

Approx. 15% of liver weight and protein are lost between days 2 and 3 of fasting (Fig. 1), and this loss represents 50% of the immunoreactive protein (Fig. 3). Therefore the proportion of liver cytosolic proteins that are immunoreactive may be approx. 30%, a value similar to that experimentally measured for cytosolic proteins from fibroblasts [7]. Heart contains 2–3 times less immunoreactive proteins than liver (Fig. 2), and half of these are lost between 12 and 24 h of fasting (Fig. 5). Therefore, the 7% loss of weight in the heart after a 24 h fast (Fig. 1) may be entirely due to the loss of proteins containing KFERQ-like peptide regions.

There are several possible explanations for the incomplete loss of immunoreactive proteins from liver after 3 days of fasting (Fig. 3). For example, the remaining immunoreactive proteins may be depleted only at later stages of fasting. It is also possible that some KFERQ-like sequences are exposed after protein denaturation in the SDS gels, but are inaccessible in proteins found *in vivo*.

The loss of immunoreactive proteins from liver (Fig. 3) and heart (Figs. 4–6), but not skeletal muscle (Figs. 4–6), of fasted animals suggests that different tissues *in vivo* enhance protein breakdown in distinct fashions. The selective uptake and degradation by lysosomes of proteins containing KFERQ-like sequences is the predominant increased proteolytic pathway in fibroblasts deprived of serum [7], and appears to be a component of the enhanced degradation in liver and heart of fasted animals. In contrast, loss of skeletal-muscle protein during fasting does not involve activation of this proteolytic pathway, even though skeletal muscles contain lysosomes [22] and proteins with KFERQ-like sequences (Fig. 2).

Whether or not lysosomes are responsible for the enhanced protein degradation in skeletal muscle during fasting or denervation atrophy has been a controversial issue. Lysosomes have been implicated in some studies [11,23], but not others [11–13]. More recent studies ([11]; H. Q. Han, K. Furuno & A. L. Goldberg, unpublished work; S. S. Wing & A. L. Goldberg, unpublished work) indicate that most of the increase in protein breakdown in skeletal muscle in fasting and denervation atrophy is due to activation of a non-lysosomal ATP-dependent proteolytic process. Furthermore, levels of ubiquitin–protein conjugates (S. S. Wing, A. L. Haas & A. L. Goldberg, unpublished work) and of polyubiquitin mRNAs (R. Medina, S. S. Wing & A. L. Goldberg, unpublished work) increase in skeletal muscle in fasting and fall on re-feeding. These observations suggest that the ATP–ubiquitin-dependent proteolytic system is responsible for most of the protein breakdown in the atrophying muscles. In contrast, polyubiquitin-gene expression does not change in liver, and increased only slightly in heart from the same fasted animals (R. Medina, S. S. Wing & A. L. Goldberg, unpublished work). These results support the idea that distinct proteolytic systems are activated in different tissues of fasted animals.

We thank Jerry Faust for many helpful suggestions throughout these experiments. This work was supported by grants from the National Institute on Aging to J. F. D., and from the National Institutes of Neurological, Communicative Diseases and Stroke and the Muscular Dystrophy Association of America to A. L. G. During the course of this work S. S. W. held postdoctoral fellowships from the Medical Research Council of Canada and the Fonds de la Recherche en Sante du Quebec, Canada.

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Received 6 September 1990/30 November 1990; accepted 10 December 1990