Insulin-like growth factor I stimulates degradation of an mRNA transcript encoding the 14 kDa ubiquitin-conjugating enzyme

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Upon fasting, the ubiquitin-dependent proteolytic system is activated in skeletal muscle in parallel with the increases in rates of proteolysis. Levels of mRNA encoding the 14 kDa ubiquitin-conjugating enzyme ($E2_{14k}$), which can catalyse the first irreversible reaction in this pathway, rise and fall in parallel with the rates of proteolysis [Wing and Banville (1994) *Am. J. Physiol.* **267**, E39–E48], indicating that the conjugation of ubiquitin to proteins is a regulated step. To characterize the mechanisms of this regulation, we have examined the effects of insulin, insulin-like growth factor I (IGF-I) and des(1–3) insulin-like growth factor I (DES-IGF-I), which does not bind IGF-binding proteins, on $E2_{14k}$ mRNA levels in L6 myotubes. Insulin suppressed levels of $E2_{14k}$ mRNA with an IC_{50} of 4×10^{-9} M, but had no effects on mRNAs encoding polyubiquitin and proteasome subunits C2 and C8, which, like $E2_{14k}$, also increase in skeletal muscle upon fasting. Reduction of $E2_{14k}$ mRNA levels was more sensitive to

IGF-I with an IC_{50} of approx. 5×10^{-10} M. During the incubation of these cells for 12 h there was significant secretion of IGF-I-binding proteins into the medium. DES-IGF-I, which has markedly reduced affinity for these binding proteins, was found to potently reduce $E2_{14k}$ mRNA levels with an IC_{50} of 3×10^{-11} M. DES-IGF-I did not alter rates of transcription of the $E2_{14k}$ gene, but enhanced the rate of degradation of the 1.2 kb mRNA transcript. The half-life of the 1.2 kb transcript was approximately one-third that of the 1.8 kb transcript and can explain the more marked regulation of this transcript observed previously. This indicates that the additional 3' non-coding sequence in the 1.8 kb transcript confers stability. These observations suggest that IGF-I is an important regulator of $E2_{14k}$ expression and demonstrate, for the first time, stimulation of degradation of a specific mRNA transcript by this hormone, while overall RNA accumulates.

INTRODUCTION

The degradation of muscle proteins to amino acids represents a critical first step in gluconeogenesis [1]. Like other processes in intermediary metabolism, muscle protein degradation is precisely regulated by hormones. For example, the anabolic hormone, insulin, suppresses muscle proteolysis [2,3], while catabolic hormones such as glucocorticoids [4] and tri-iodothyronine stimulate this process [5]. Although detailed studies of hormonal regulation of proteolysis have been carried out previously (reviewed in [6]), the biochemical pathway(s) by which muscle proteins are degraded and the exact steps in the proteolytic sequence regulated by hormones remain unknown.

Recent studies indicate that the ubiquitin-dependent proteolytic system is regulated in skeletal muscle during fasting and refeeding in parallel with the changes in rates of proteolysis, and therefore suggest that this system is involved in the muscle protein loss. In this degradative pathway, ubiquitin, an 8 kDa peptide, becomes covalently linked to proteins and targets them for recognition and degradation by a 1500 kDa protease, the 26 S proteasome [7]. Upon fasting, there is increased expression of ubiquitin [8], increased levels of ubiquitinated proteins [9], and increased levels of mRNAs encoding some proteasome subunits [8] in skeletal muscle, and these levels revert to normal on refeeding in parallel with the changes in the rates of proteolysis. Similar activation of the ubiquitin system has been observed in other conditions of muscle atrophy such as disuse atrophy [10], denervation [8,9], metabolic acidosis [11], cancer [12] and sepsis [13], further implicating the ubiquitin system in the degradation of muscle proteins.

Since multiple steps in the pathway appear to be regulated, the

rate-limiting step(s) remain to be defined. As metabolic pathways are often regulated at the first irreversible reaction [14], we have focused our attention on the regulation of conjugation of ubiquitin to proteins. In this process [15], ubiquitin is first activated by ubiquitin-activating enzyme (E1), which transfers the ubiquitin to one of a family of ubiquitin-conjugating enzymes (E2) (reviewed in [16]), which either ligates the ubiquitin directly to substrates or requires the presence of a third protein, ubiquitin protein ligase (E3). The first thermodynamically irreversible reaction is the conjugation of ubiquitin to the target protein catalysed by E2 with or without E3 [17]. *In vitro*, some E2s can conjugate ubiquitin directly to some substrates, but it appears that E3s are probably required for conjugation to most substrates *in vivo* [18]. Thus, we have begun to identify isoforms of these two enzymes that are expressed in skeletal muscle and to study their regulation.

We previously reported the molecular cloning of a 14 kDa ubiquitin-conjugating enzyme ($E2_{14k}$) [19]. This E2 can support conjugation to a broad spectrum of endogenous proteins in a reticulocyte extract [18] and therefore probably plays an important role in the proteolytic functions of ubiquitin. Consistent with this, $E2_{14k}$ mRNA levels in skeletal muscle rise upon fasting and fall with refeeding in parallel with the changes in the rates of proteolysis [20]. $E2_{14k}$ protein levels appeared to be very low in muscle as they could not be detected immunochemically [20]. However, the rise and fall of $E2_{14k}$ mRNA levels did parallel similar changes in levels of ubiquitinated proteins [9], consistent with changes in conjugating activity occurring in these muscles. Furthermore, insulin, the key hormone regulating intermediary metabolism, can suppress $E2_{14k}$ mRNA levels in cultured muscle cells [20].

Abbreviations used: DES-IGF-I, des(1–3) insulin-like growth factor I; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin protein ligase; IGF-I, insulin-like growth factor I; polyUb, polyubiquitin.

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These observations pose several further questions regarding the regulation of ubiquitin-dependent proteolysis. First, since the expression of ubiquitin, E2_{14k} and several proteasome subunits are increased upon fasting [8], it remains unknown whether insulin regulates E2_{14k} expression specifically or modulates these other components co-ordinately. Secondly, because the levels of insulin observed to suppress E2_{14k} mRNA levels were high enough to produce binding to both insulin and insulin-like growth factor I (IGF-I) receptors, it is unclear which receptor is involved in mediating this effect. Since serum levels of both insulin and IGF-I decrease upon fasting [21], either hormone could mediate the increase in E2_{14k} mRNA levels in muscle observed upon fasting. Finally, as these studies did demonstrate a potent ability of IGF-I to suppress E2_{14k} mRNA levels, we have determined whether this occurs by inhibition of gene transcription and/or by stimulation of mRNA degradation.

EXPERIMENTAL

Cell culture

L6 myoblasts were maintained in culture in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum. To form myotubes, the myoblasts were grown to confluency in DMEM with 10% (v/v) horse serum. The medium was then changed to DMEM with 2% horse serum to induce fusion. On day 3 after the decrease in serum, more than 80% of the nuclei were in multinucleated cells. To test for the effects of hormones on E2_{14k} mRNA expression, the myotubes were rinsed twice with PBS and incubated for 12–16 h in DMEM without serum. The medium was then replaced and insulin (Lilly), recombinant human IGF-I or recombinant des(1–3) IGF-I (DES-IGF-I) (both from Gropep), or diluent was added as indicated in the Figures. To prevent non-specific binding of hormone to the plates, BSA (Sigma, RIA grade) was added to a final concentration of 1 mg/ml. Unless otherwise indicated, incubations with various hormones were for 12 h as previous studies demonstrated a maximal effect of insulin at this time [20]. Pilot studies using radioiodinated ligands indicated that IGF-I and DES-IGF-I were both stable in the medium during the incubation period. However, insulin was degraded in the medium with a half-life of approx. 11 h. Therefore, supplemental insulin (25% of the original amount) was added to the medium at 4 h intervals to maintain the concentration. Incubations were terminated by rinsing the cells with PBS followed by solubilization with guanidinium isothiocyanate as indicated below.

Quantification of mRNA levels

Levels of the various mRNA transcripts described in the results section were estimated by RNA blot hybridization (Northern analysis). Following treatment as indicated above, RNA was isolated by solubilizing the cells in guanidinium isothiocyanate followed by extraction with phenol/chloroform [22]. RNA blots were performed by electrophoresis of 10 µg of each RNA sample in 1% agarose containing formaldehyde followed by transfer on to nylon membranes and UV cross-linking of the RNA to the membrane. The membrane was hybridized at 65 °C with a ³²P-labelled cDNA of interest, washed at the same temperature, and then subjected to autoradiography. Quantification of the individual transcripts was by densitometric scanning of the autoradiographs (Gelscan, LKB) or by phosphorimager analysis (Fuji) of the blots. Smaller quantities of some of the samples were loaded on the same gel to verify that quantification of the bands was linear.

Determination of mRNA half-lives

Rates of degradation of specific mRNAs were determined by measuring their rates of disappearance in the presence of an inhibitor of RNA synthesis. After preincubation of L6 myotubes with or without hormone for 6 h, actinomycin D (1 µg/ml) was added to the medium and incubation continued for up to 8 h. Longer incubations were not possible due to loss of cells from prolonged deprivation of serum. Initial studies measuring the incorporation of [³H]uridine into RNA showed that RNA synthesis was inhibited by > 99% under these conditions (S. S. Wing and N. Bedard, unpublished work). At the time intervals indicated in Figure 6, cells were solubilized, and RNA was prepared. A constant fraction of the RNA recovered from the plates was subjected to Northern analysis, and mRNA transcripts quantified as indicated above.

Determination of rates of gene transcription

Rates of gene transcription were determined by measuring the incorporation of [³²P]UTP into nuclear RNA transcripts initiated by RNA polymerase. Nuclei were prepared from 15-cm-diam. plates of myotubes formed from fusion of approx. 10 × 10⁶ confluent myoblasts. Cells were detached from plates by incubation with 5 ml of 0.05% trypsin/0.02% EDTA in PBS. The detached cells were collected in 50 ml of PBS and pelleted by centrifugation at 500 g for 5 min at 4 °C. After washing twice with 25 ml of PBS, the cells were lysed by resuspension with a pipette in 1 ml of ice-cold 10 mM Tris, pH 7.4, containing 10 mM NaCl, 3 mM MgCl₂ and 0.5% Nonidet P-40. The cells were incubated on ice for 5 min with brief vortexing at low speed each minute. Nuclei were collected by centrifugation at 13000 g for 15 s at 4 °C and resuspended with a pipette in 150 µl of ice-cold 50 mM Tris, pH 7.4, containing 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol (DTT) and 40% (w/v) glycerol. The isolated nuclei were frozen in a solid CO₂/ethanol bath and kept in liquid nitrogen until used.

For transcriptional run-on assays, nuclei were rapidly thawed and collected by centrifugation at 10000 g for 15 s at 4 °C. Transcription was carried out by resuspending the pellet in 325 µl of transcription buffer {25 mM Hepes, pH 7.9, 0.1 M KCl, 10 mM magnesium acetate, 10 mM DTT, 4 mM creatine phosphate, 3.2 mM ATP, 1 mM CTP and GTP, 200 µCi/reaction [³²P]α-UTP (3000 Ci/mmol), 750 units/ml RNasin, 20 units/ml creatine phosphokinase and 5 mg/ml BSA} and then incubating for 30 min at 30 °C. The reaction was terminated by addition of 12.5 units of RNase-free DNase I (Promega) in 30 µl of digestion solution (0.1 M MgCl₂, 50 mM vanadyl ribonucleoside complex, 2 mg/ml tRNA). Following incubation for 30 min at 30 °C, proteins were digested by adding 150 µl of 5 × TES (1 × TES: 10 mM Tris, pH 7.5, 2.5 mM EDTA, 1% SDS) and 120 µg of proteinase K, and incubating for 30 min at 37 °C. The reaction mixture was extracted twice with phenol/chloroform/isoamyl alcohol. After extraction of the final aqueous phase with chloroform, the RNA in the aqueous phase was precipitated with 2 vol. of ethanol and left overnight at –80 °C. After centrifugation at 13000 g for 10 min, the pellet was dried and resuspended in 300 µl of redigestion buffer (20 mM Hepes, pH 7.9, 5 mM MgCl₂, 1 mM CaCl₂, 1 mM MnCl₂, 2 mM vanadyl ribonucleoside complex) containing 24 units of RNase-free DNase I. The reaction mixture was incubated for 1 h at 37 °C, mixed with 66 µl of 5 × TES and extracted with phenol/chloroform. The RNA in the aqueous phase was precipitated by making the aqueous phase 10% trichloroacetic acid and incubating on ice for 15 min. After centrifugation at 13000 g for 10 min, the pellet was washed twice with 5% trichloroacetic acid, once with 80% ethanol and

then dried. The pellet was redissolved in diethyl pyrocarbonate (DEPC)-treated water.

To identify the transcripts of interest, the RNA was hybridized with nitrocellulose membranes, on to which had been dot-blotted linearized plasmids (3 μ g) containing the genes of interest. After prehybridization at 42 °C in 50 % formamide/0.5 M NaCl/50 mM sodium phosphate (pH 6.5)/2 mM EDTA/0.2 % SDS/1 $\times$ Denhardt's solution/100 μ g/ml each of *Escherichia coli* tRNA and polyA, the RNA (approx. 10⁶ c.p.m.) was added and hybridization continued for 48 h. The membranes were washed with 2 $\times$ SSC (SSC: 150 mM NaCl, 15 mM sodium citrate), 0.1 % SDS for 15 min at room temperature, then for 30 min at 60 °C, treated with RNase A (20 μ g/ml) in 2 $\times$ SSC for 1 h at room temperature, then washed with 2 $\times$ SSC/0.1 % SDS for 15 min at 60 °C. The filters were subjected to autoradiography and also to quantitative analysis with a phosphorimager.

Detection of IGF-I-binding proteins in media

Following incubation with cells, samples (45 μ l) of the medium were removed and subjected to SDS/PAGE under non-reducing conditions on 12.5 % gels. Following transfer to nitrocellulose, the membranes were hybridized as described previously with IGF-I labelled with Na¹²⁵I [23]. After washing, the membranes were subjected to autoradiography and quantitative densitometry.

Statistical analyses

The means of two samples were compared by Student's *t*-test. Means of multiple samples were compared by one-way analysis of variance. Degradation rates of mRNA transcripts were determined by linear regression analysis.

Figure 1 Effects of insulin on mRNA levels of E2_{14k}, polyUb, proteasome subunits C2 and C8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

L6 myotubes were prepared as described in the Experimental section and incubated in the presence or absence of insulin (1.7×10^{-7} M). RNA was isolated from the cells and 10 μ g samples were subjected to electrophoresis on an agarose gel and transferred to a nylon membrane. Following hybridization with ³²P-labelled cDNA probes encoding the indicated genes, the membranes were washed and exposed to film. Representative samples are shown. Similar results were seen in the three other replicate samples from the same experiment. Shown to the right are estimates of the sizes of the transcripts in kilobases.

RESULTS

Effects of insulin on levels of mRNA encoding E2_{14k}, polyubiquitin (polyUb), and proteasome subunits C2 and C8

Since hormones can regulate multiple steps in a metabolic pathway, we determined whether insulin might regulate expression of other components of the ubiquitin system, particularly the genes encoding polyUb and the C2 and C8 subunits of the proteasome, which were previously shown to be activated also upon fasting [8]. L6 myotubes were exposed to insulin and the RNA analysed by RNA blotting with cDNA probes encoding either E2_{14k}, polyUb, or proteasome subunits C2 and C8 (Figure 1). As demonstrated previously, insulin suppresses E2_{14k} mRNA levels. However, no changes were seen in levels of mRNAs encoding ubiquitin, and the proteasome subunits C2 and C8.

Sensitivity of suppression of E2_{14k} mRNA levels to different concentrations of insulin and IGF-I

To determine whether the insulin-mediated suppression of E2_{14k} mRNA levels was likely to occur through binding to the insulin

Figure 2 Effects of various doses of (A) insulin or (B) IGF-I on levels of E2_{14k} mRNA

L6 myotubes were prepared as described in the Experimental section and exposed to the indicated concentrations of the hormones. RNA was then isolated and analysed by Northern blotting with a cDNA probe encoding E2_{14k} as indicated in Figure 1. The two transcripts were quantified by densitometry, summed, and plotted above as percentages of the value obtained in the absence of treatment with hormone. Each point represents the mean $\pm$ S.E.M. of 3–4 determinations.

Figure 3 IGF-I stimulates rapid secretion of binding proteins

L6 myotubes were exposed to the indicated concentrations of IGF-I for 12 h. Aliquots (45 μ l) of the medium were subjected to electrophoresis on a 12.5% non-reducing SDS/polyacrylamide gel and the resolved proteins were transferred to a nitrocellulose membrane. A partially purified fraction [22], containing binding proteins from 230 μ l of serum, was treated as described in [22] and electrophoresed on the same gel as a positive control. The membrane was then hybridized with 125 I-IGF-I, washed and subjected to autoradiography. (A) A representative autoradiogram. (B) Quantification by densitometry. Shown are the means $\pm$ S.E.M. of 3–4 determinations.

receptor, the ability of various concentrations of insulin to mediate this effect was determined (Figure 2A). The suppression by insulin was maximal at concentrations beyond 2×10^{-7} M with a half-maximal effect at approx. 4×10^{-9} M.

Previous studies in L6 myotubes have demonstrated the presence of an insulin receptor with a K_D for insulin of 1.8×10^{-9} M and the ability of insulin to stimulate glucose uptake with a half-maximal rate at a concentration of approximately 10^{-9} M [24]. Since the K_D for receptor binding and the IC_{50} for suppression of $E2_{14k}$ mRNA levels are of the same order of magnitude, insulin could be acting by binding to its receptor. However, the maximal effect would require complete occupancy of the receptors by the hormone. Since many biological effects, including glucose uptake in L6 myotubes, are maximal at fractional occupancy of the receptor, it raised the possibility that the ligand could be acting through the IGF-I receptor. To evaluate this possibility, the suppression of $E2_{14k}$ mRNA levels by various concentrations of IGF-I was determined (Figure 2B). Suppression was indeed achieved at lower concentrations with maximal suppression achieved at $> 10^{-8}$ M and a half-maximal effect at approx. 5×10^{-10} M.

Previous reports have indicated that muscle cells secrete IGF-

Figure 4 Effects of various doses of DES-IGF-I on levels of $E2_{14k}$ mRNA

L6 myotubes were prepared as described in the Experimental section and exposed to the indicated concentrations of DES-IGF-I. RNA was then isolated and analysed by Northern blotting with a cDNA probe encoding $E2_{14k}$ as indicated in Figure 1. The two transcripts were quantified by densitometry, summed, and plotted above as percentages of the value obtained in the absence of treatment with DES-IGF-I. Each point represents the mean $\pm$ S.E.M. of 3–4 determinations.

binding proteins [25]. Since these proteins have high affinity for IGF-I, they could interfere with such dose-response analysis. Indeed, much greater variability was seen in the results with IGF-I than in our previous studies with insulin and could be explained by variability introduced by the secretion of binding proteins into the medium. Since these previous reports demonstrated production of binding proteins after at least 24 h of incubation with the cells, it remained unknown whether the relatively short incubations (12 h) used in these studies were long enough to permit secretion of significant amounts of these proteins. Therefore, samples of the medium were subjected to analysis by ligand blotting with 125 I-IGF-I (Figure 3A). The medium did indeed contain binding proteins the production of which increased with increasing concentrations of IGF-I (Figure 3B). The major protein produced was approx. 24 kDa in size. When compared with the corresponding band in a sample containing the equivalent of 230 μ l of rat serum applied to the same gel, it appeared that exposure of the cells to 1.3×10^{-7} M (1 μ g/ml) IGF-I for 12 h resulted in secretion of this binding protein in the medium to a concentration that was approx. 4-fold greater than that in rat serum (Figure 3A). Small amounts of approx. 30 kDa and 46 kDa bands could also be seen, particularly when the blot was overexposed. These data indicate that IGF-I-binding proteins are secreted into the medium at high concentrations within 12 h and therefore could bind significant amounts of the added hormone.

Thus, to evaluate more precisely the ability of IGF-I to suppress $E2_{14k}$ mRNA levels, the effect of DES-IGF-I, an analogue of IGF-I which only binds to IGF-binding proteins with 100-fold less affinity [26], was studied (Figure 4). Indeed, the dose-response curve was markedly shifted towards lower concentrations, indicating that native IGF-I was being sequestered by secreted binding proteins relatively early during the incubation.

Table 1 Effects of insulin and DES-IGF-I on levels of E2_{14k} mRNA

L6 myotubes were incubated in the absence of any hormone (control) or in the presence of insulin (3.5×10^{-7} M), DES-IGF-I (6.8×10^{-9} M), or both for 12 h. RNA was then isolated and E2_{14k} mRNA levels quantified by Northern blotting and densitometry as indicated in Figure 2. Shown are means $\pm$ S.E.M. for four samples. Means of each hormone treatment were significantly different from controls by analysis of variance ($P < 0.025$). Means of each hormone treatment were not significantly different from each other.

	E2 _{14k} mRNA (units/ μ g of RNA)
Control (no hormone)	2.27 ± 0.32
Insulin	1.15 ± 0.21
DES-IGF-I	1.17 ± 0.24
Insulin + DES-IGF-I	1.08 ± 0.17

Figure 5 Effect of DES-IGF-I on transcription of the E2_{14k} gene

L6 myotubes were incubated in the absence or presence of DES-IGF-I (6.8×10^{-9} M) for 6 h. Nuclei were isolated and associated RNA transcripts extended *in vitro* in the presence of [³²P]UTP as described in the Experimental section. The RNA was then purified and incubated with nitrocellulose membranes on to which had been applied plasmid DNA containing the 3.6 kb *Eco*RI genomic fragment of the E2_{14k} gene, a cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or no insert. After washing, the membranes were subjected to autoradiography and to quantitative analysis using a phosphorimager. Upper panel: representative autoradiogram. Lower panel: quantification (means $\pm$ S.E.M.) of samples from four separate preparations of nuclei from cells exposed to each condition. Differences between means ($\pm$ IGF-I) are not significant.

Maximal suppression was achieved at concentrations greater than 7×10^{-10} M with a half-maximal effect at 3×10^{-11} M.

To determine whether IGF-I and insulin could have additive effects, the myotubes were exposed to concentrations of each ligand capable of producing maximal effects (Table 1). The maximal effects of each individual hormone were similar and the combination of the two did not produce any further suppression.

Figure 6 Effects of DES-IGF-I on rates of degradation of E2_{14k} mRNA

L6 myotubes were incubated in the presence (open symbols) or absence (filled symbols) of DES-IGF-I for 6 h. Actinomycin D was then added to a final concentration of 1 μ g/ml. At the indicated times, cells were lysed. The RNA was isolated and levels of E2_{14k} mRNA measured as indicated in Figures 2 and 4 except that a phosphorimager was used for quantification. Each point represents the mean $\pm$ S.E.M. of 5–7 determinations. The half-life ($t_{1/2}$) was determined as $0.693/k$, where k is the slope of the lines obtained by plotting $\ln$ (mRNA level) versus time. Upper panel: rates of decay of the 1.2 kb transcript. Lower panel: rates of decay of the 1.8 kb transcript. Values of k obtained from samples treated with or without DES-IGF-I are significantly different at $P < 0.001$ for the 1.2 kb transcript; $P < 0.1$ for the 1.8 kb transcript.

Effects of IGF-I on E2_{14k} mRNA synthesis and degradation

Studies were carried out to determine whether the suppression of E2_{14k} mRNA was due to diminished rates of gene transcription and/or increased rates of mRNA degradation. Cells were pre-treated with or without DES-IGF-I for 6 h prior to analysis because our previous studies indicated that the maximal rate of decline of the mRNA occurred at this time [20]. To determine whether the suppression of E2_{14k} mRNA levels was due to suppression of gene transcription, rates of incorporation of nucleotides into nascent E2_{14k} mRNA transcripts were deter-

mined in myotube nuclei from cells incubated with or without DES-IGF-I (Figure 5). There were no apparent changes in the rates of transcription in the hormone-treated cells. Rates of transcription of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, were also similar in the two conditions, confirming that similar amounts of RNA were used in the hybridizations. No hybridization was detectable to the plasmid vector alone, indicating that the radioactive binding observed was specific.

Since rates of gene transcription did not appear to be altered, rates of mRNA degradation were measured to ascertain whether this might explain the lowering of E2_{14k} mRNA levels in DES-IGF-I-treated cells (Figure 6). After DES-IGF-I treatment for 6 h, but before addition of actinomycin D, the levels of the two transcripts were significantly lower than those from untreated cells, confirming efficacy of the hormone in the experiment. After addition of actinomycin D, the levels of all transcripts declined. In untreated cells, the half-lives of the two E2_{14k} mRNA transcripts differed (Figure 6, upper and lower panels). The 1.8 kb transcript turned over slowly with a $t_{1/2}$ of approx. 71 h, while the smaller 1.2 kb transcript turned over with a $t_{1/2}$ of 28 h. These different decay rates confirmed that the loss of mRNA was not due to non-specific degradation of total RNA. After exposure to DES-IGF-I, the $t_{1/2}$ of the 1.2 kb transcript decreased to 12 h. The $t_{1/2}$ of the 1.8 kb transcript appeared to decrease to 24 h, but the difference was only statistically significant at $P < 0.1$. In contrast, total RNA disappeared with a half-life of approx. 38 h in the absence of DES-IGF-I, but remained constant in the presence of the hormone (results not shown). Thus, this acceleration of degradation of both transcripts was not due to a non-specific enhancement of degradation of total RNA.

DISCUSSION

These results provide several new insights into the regulation of ubiquitin-dependent proteolysis. First, we have shown that IGF-I is an important regulator of E2_{14k} expression. DES-IGF-I potently suppressed levels of E2_{14k} mRNA in L6 myotubes (Figure 4) at low concentrations (IC_{50} : 3×10^{-11} M), consistent with action through a specific high-affinity IGF-I receptor. IGF-I receptors are abundant in L6 myotubes and the IC_{50} is much lower than the K_D for the receptor (5×10^{-8} M [27]), indicating that occupancy of a small fraction of the receptors is sufficient for maximal effect. Interestingly, insulin was relatively ineffective (Figure 2), requiring 100-fold greater, supraphysiological, concentrations to exert a similar suppressive effect. Thus, in these cells, IGF-I appears to be the physiologically relevant regulator of E2_{14k} expression. Since insulin at high concentrations can bind to the IGF-I receptor in L6 myotubes [27], our results are consistent with insulin exerting its effects through cross-binding to the IGF-I receptor. Furthermore, exposing cells to a combination of maximally effective concentrations of insulin and IGF-I did not generate a larger response than with the individual hormones (Table 1), thereby also suggesting that the two ligands act through a common pathway, beginning at the receptor or more distally.

Interestingly, the IC_{50} s of insulin, IGF-I and DES-IGF-I in suppressing E2_{14k} mRNA levels were very similar to those previously seen for suppression of protein degradation in confluent L6 myoblasts [28]. Although these cells are slightly less differentiated than those used in our studies, this correlation is striking and is similar to what we have previously demonstrated *in vivo* during fasting and refeeding where E2_{14k} mRNA levels are regulated in parallel with rates of proteolysis and levels of ubiquitinated proteins in skeletal muscle [20]. These results

provide further evidence of a tight linkage between activation of the ubiquitin system and activation of muscle proteolysis.

Secondly, the regulatory effect of IGF-I appears to be specific for E2_{14k} (Figure 1). Previous studies *in vivo* have indicated that, upon fasting, there are increases not only in E2_{14k} mRNA levels, but also in polyUb gene transcripts and in mRNA transcripts for some of the proteasome subunits [8]. However, none of these other genes appeared to be regulated by IGF-I. Because of the inability to perform prolonged incubations of L6 myotubes in the absence of serum, we cannot exclude the possibility of regulation of these other components of the pathway at later time points. Nevertheless, our data indicating that the conjugating enzyme is the first component in the ubiquitin system to be regulated, as well as the tight correlation between E2_{14k} expression and rates of proteolysis, would support our hypothesis that E2s catalyse an important site of regulation and possibly the rate-limiting step in the pathway. Further evaluation of this hypothesis will require determination of the effects of altering E2_{14k} levels on rates of proteolysis, by adding or removing E2_{14k} in muscle extracts and by using genetic approaches to manipulate E2_{14k} levels in muscle cells. Parallel changes in E2_{14k} expression and proteolysis have also been seen in muscles from dexamethasone-treated [29] and tumour-bearing rats [12]. However, rat muscles atrophying in response to acidosis [30] demonstrated increases in polyUb and proteasome subunits C2 and C9 mRNAs, but not in E2_{14k}-encoding transcripts. Since changes in E2_{14k} mRNA levels are generally smaller in magnitude than those of polyUb and proteasome subunits, the inability to demonstrate regulation of E2_{14k} in response to acidosis may simply reflect the small changes (<100%) in polyUb/proteasome transcripts seen in this study. Of course, acidosis may activate proteolysis through a slightly different mechanism.

Thirdly, we demonstrated that this regulation by IGF-I occurs not, as most commonly occurs, by altering gene transcription, but by stimulating mRNA degradation (Figure 6). This was clearly demonstrated for the 1.2 kb transcript. Although the IGF-I-stimulated change in the half-life of the 1.8 kb transcript was only of borderline significance, it probably does respond in a similar fashion since there were no changes in the rate of gene transcription (Figure 5). The levels of the 1.8 kb transcript were much lower than the 1.2 kb transcript (Figure 1) and decayed with a low half-life even when stimulated (24 h), characteristics which would render demonstration of statistically significant changes difficult. Since the hormones lowered E2_{14k} mRNA levels by 50–60% within 12 h (Figures 2 and 4), and most of the mRNA is 1.2 kb in length, the changes in half-life induced by IGF-I could account for the changes in steady-state levels. Thus, although IGF-I, like insulin [31], enhances overall RNA stability, specific mRNAs can be targeted for degradation. This is the first example that we are aware of whereby IGF-I has been demonstrated to stimulate such degradation. Our previous studies demonstrated that the suppression of E2_{14k} mRNA occurs over a period of 12 h. Recently, more rapid destabilization (15 min) of a specific mRNA by insulin has been observed [32]. The longer time prior to onset of the decline in E2_{14k} mRNA suggests that new protein synthesis may be required for this destabilizing effect of IGF-I.

We demonstrated that, under both basal or IGF-I-stimulated conditions, the half-life of the 1.8 kb transcript is approx. 2.5- to 3-fold longer than that of the 1.2 kb transcript (Figure 6). This therefore provides a mechanistic basis for previous observations that the smaller transcript demonstrates more marked changes in response to various stimuli including fasting, insulin/IGF-I treatment [20], dexamethasone-treated rats [29], and cancer-induced catabolism [12]. Since the larger transcript arises solely

from use of a more distal polyadenylation signal in exon 6 of the gene [20], this indicates that the extra 3' non-coding region in this transcript conveys structural elements which enhance stability of the mRNA. The exact nature of these structural elements remains unclear. Analysis of the sequence does not predict any secondary structure for this 3' non-coding region.

The absence of regulation at the level of gene transcription (Figure 5) is consistent with current knowledge of the structure of the E2_{14k} gene [20]. The 5' region of this gene has no TATA box and is relatively GC-rich, features consistent with this gene being constitutively transcribed. Furthermore, within the 5' upstream region, we did not observe consensus sequences for insulin or serum response elements.

These studies in myotubes indicate that IGF-I and not insulin is responsible for regulation of E2_{14k} mRNA expression. Whether this is true *in vivo* remains to be determined. The primacy of IGF-I in myotubes could be due to the relatively low number of insulin receptors compared with IGF-I receptors as discussed above. Nevertheless, these findings are intriguing in that available evidence does support an important role for IGF-I in the *in vivo* regulation of muscle protein turnover. Upon fasting, serum levels of both insulin and IGF-I decline [21] and return to normal with refeeding. Levels of muscle IGF-I mRNA also fall upon fasting [33], indicating that local production of this growth factor is also similarly regulated. Interestingly, refeeding fasted humans with an isocaloric, but protein-deficient diet which stimulates insulin release, but inhibits IGF-I production, prevents the normal improvement in nitrogen balance seen during refeeding with a protein-replete diet [34,35]. Studies involving pharmacological use of growth hormone and IGF-I also support the importance of IGF-I as a stimulator of muscle growth. For example, IGF-I can restore growth to diabetic animals in the absence of insulin therapy [36]. Administration of growth hormone to humans in catabolic states results in improved nitrogen balance concomitant with increased serum IGF-I levels [37,38]. Administration of IGF-I to humans [39], or of analogues of IGF-I [40] which do not bind to binding proteins to rats, also improves nitrogen balance. IGF-I, both circulating and locally produced, is therefore very likely to be an important regulator of muscle protein balance and our data indicate that its regulation of E2_{14k} may be an important mechanism for mediating these effects on protein balance.

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