MAFbx/Atrogin-1 Controls the Activity of the Initiation Factor eIF3-f in Skeletal Muscle Atrophy by Targeting Multiple C-terminal Lysines*§

Received for publication, October 2, 2008, and in revised form, December 9, 2008. Published, JBC Papers in Press, December 10, 2008, DOI 10.1074/jbc.M807641200

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We recently presented evidence that the subunit eIF3-f of the eukaryotic initiation translation factor eIF3 that interacts with the E3-ligase Atrogin-1/muscle atrophy F-box (MAFbx) for polyubiquitination and proteasome-mediated degradation is a key target that accounts for MAFbx function during muscle atrophy. To understand this process, deletion analysis was used to identify the region of eIF3-f that is required for its proteolysis. Here, we report that the highly conserved C-terminal domain of eIF3-f is implicated for MAFbx-directed polyubiquitination and proteasomal degradation. Site-directed mutagenesis of eIF3-f revealed that the six lysine residues within this domain are required for full polyubiquitination and degradation by the proteasome. In addition, mutation of these six lysines (mutant K5–10R) displayed hypertrophic activity in cellulo and in vivo and was able to protect against starvation-induced muscle atrophy. Taken together, our data demonstrate that the C-terminal modifications, believed to be critical for proper eIF3-f regulation, are essential and contribute to a fine-tuning mechanism that plays an important role for eIF3-f function in skeletal muscle.

Skeletal muscle is a dynamic tissue that has the capacity to continuously regulate its size in response to a variety of external cues including mechanical load, neural activity, hormones/growth factors, stress, and nutritional status. In addition, skeletal muscle serves as the most significant repository for protein in the body, a source that is tapped to provide a pool of amino acids for tissue repair and gluconeogenesis under conditions of starvation and other stresses. The maintenance of muscle mass is controlled by a fine balance between catabolic and anabolic processes, which determine the level of muscle proteins and the diameter of muscle fibers. Muscle loss occurs as the result of a number of disparate conditions including cancer, diabetes, AIDS, sepsis, renal failure, aging, cachexia, and other systemic diseases (1). These diverse conditions result in reduced protein synthesis and increased protein breakdown. The process of atrophy is characterized by the activation of the ATP-dependent ubiquitin-proteasome proteolysis pathway (2). Proteins destined for degradation by the ubiquitin-proteasome pathway are marked by covalent linkage with a chain of ubiquitin molecules (Ub) on lysine residue(s) for further degradation into short peptides by the 26 S proteasome. This process requires an Ub-activating enzyme (E1), an Ub-conjugating enzyme (E2), and an Ub ligase (E3) that acts as the last step of the cascade (3). E3 proteins regulate the timing and the substrate specificity in protein degradation. In multiple models of skeletal muscle atrophy, the muscle-specific F-box protein MAFbx/Atrogin-1 (MAFbx) is up-regulated and appears to be essential for accelerated muscle protein loss (4–6). MAFbx mRNA increases 8–40-fold in all types of atrophy studied, and this increase precedes the onset of muscle weight loss. Moreover, knock-out animals lacking MAFbx show a reduced rate of muscle atrophy after denervation (5). These observations suggest a major role for MAFbx in regulating muscle size via its participation in a muscle-specific ubiquitin ligase complex. Recently, we demonstrated that MAFbx interacts physically with the f-subunit of the eukaryotic initiation factor eIF3 (eIF3-f) for polyubiquitination and proteasome-mediated degradation during skeletal muscle atrophy (7). eIF3 is a multisubunit complex with an apparent molecular mass of ~650 kDa. It plays an important role in translation by binding directly to the 40 S ribosomal subunit and promoting the formation of the 43 S preinitiation complex, consisting of the Met-tRNA-eIF2-GTP ternary complex, eIF1, eIF1A, and the 40 S ribosomal subunit (8, 9). Furthermore, eIF3 interacts with the eIF4F-mRNA complex, thus

* This work was supported by grants from the “Association Française contre les Myopathies” (AFM), the department PHASE from INRA, and INSERM.

1 The recipient of graduate AFM and INRA fellowships.

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3 The abbreviations used are: Ub, ubiquitin; MAFbx, muscle atrophy F-box; eIF3, eukaryotic initiation factor 3; eIF3-f, eukaryotic initiation factor 3 subunit f; eIF1, eukaryotic initiation factor 1; eIF4F, eukaryotic initiation factor 4F; eIF4E, eukaryotic initiation factor 4E; MyHC, myosin heavy chain; MCK, muscle creatine kinase; PBS, phosphate buffered saline; TA, tibialis anterior; PI3K, phosphatidylinositol-3-phosphate kinase; mTOR, mammalian target of rapamycin; 56K1, ribosomal 56 S kinase 1; rp56, ribosomal protein S6; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; MEK, MAPK/ERK kinase; 4E-BP1, eIF4E-binding protein 1; CHX, cycloheximide; E1, Ub-activating enzyme; E2, Ub-conjugating enzyme; E3, Ub ligase; GFP, green fluorescent protein; HA, hemagglutinin; WT, wild type.

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facilitating the binding of mRNA to the 43 S ribosomal complex, thereby forming the 48 S complex. Mammalian eIF3 is composed of at least 13 non-identical subunits, among which is the regulatory subunit eIF3-f. eIF3-f is a member of the Mov34 family, containing an Mpr1/Pad N-terminal (MPN) motif, which is found in certain subunits in two macromolecular complexes, homologous to eIF3, the COP9 signalosome, and the lid complex remains to be defined. However, eIF3-f is essential for the 19 S proteasome (10). eIF3-f function within the eIF3 complex remains to be defined. However, eIF3-f is essential for Schizosaccharomyces pombe viability, and its depletion markedly decreases the global protein synthesis in fission yeast (11). The same effect on protein translation was recently described in cells infected with the coronavirus SARS coV and coronavirus infectious bronchitis virus (12). In humans, down-regulation of eIF3-f is associated with several tumors (13, 14), and when overexpressed, eIF3-f negatively regulates cell growth by affecting translation efficiency and activation of apoptosis (15). In skeletal muscle, overexpression of eIF3-f induces a marked hypertrophy associated with an increase of sarcomeric proteins such as myosin heavy chain, troponin T, and desmin (7). Thus, the specific targeting of eIF3-f by the SCFMAFbx may account for the decreased protein synthesis observed in multiple types of skeletal muscle atrophy by specific translation inhibition of key muscle proteins expression (reviewed in Ref. 16). In the present work, we have mapped the regions of eIF3-f responsible for its MAFbx-mediated ubiquitination. We show that multiple lysine residues located in the C-terminal domain of eIF3-f are required for its polyubiquitination. Preventing polyubiquitination of eIF3-f by point mutations (K5–10R) leads to stabilization of the protein. (K5–10R indicates point mutations in which lysines are sequentially replaced by arginine (Lys → Arg) starting by lysine 218 (K5) to lysine 357 (K10).) We also demonstrate that the eIF3-f mutant K5–10R induces a strong hypertrophic phenotype in cellulo and in vivo. Finally, overexpression of eIF3-f K5–10R shows an enhanced ability in avoiding skeletal muscle atrophy. Taken together, these data suggest that the ubiquitination of eIF3-f on C-terminal lysine residues may serve two functions: in addressing protein degradation specifically by the SCFMAFbx during skeletal muscle atrophy and in regulating the translation of specific pool of mRNA of key sarcomeric proteins.

EXPERIMENTAL PROCEDURES

Reagents—MG132 (N-carbobenzyloxyl-Leu-Leu-leucinal) and cycloheximide (CHX) were purchased from Sigma, and 35S translabel was obtained from Valeant Pharmaceuticals.

Plasmid Constructs and Site-specific Mutagenesis of eIF3-f—eIF3-f constructs have been described previously (7). eIF3-f mutants were obtained by oligonucleotide-directed mutagenesis using a QuikChange multi site-directed PCR-based mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The mutations were confirmed by sequencing.

Cell Cultures—The mouse skeletal muscle cell line C2C12 was grown in Dulbecco’s modified Eagle’s medium supplemented with 20% of fetal calf serum and antibiotics. Myoblast fusion and differentiation was induced in subconfluent cells by replacing the medium with Dulbecco’s modified Eagle’s medium 2% fetal calf serum. Primary cultures were prepared from male mice from our own breeding stocks. All animals were treated in accordance with institutional and national guidelines. Briefly, mice satellite cells were isolated from the whole muscles of the paw. Cells were plated at a density of 2 × 10^4 cell/cm^2 on Matrigel-coated Petri dishes (BD Biosciences) in 80% Ham’s-F10 medium containing glucose, penicillin, and amphotericin B (Invitrogen), supplemented with 20% horse serum. After 2 days, cells were washed with Ham’s-F10 and placed in complete medium supplemented with 5 ng/ml basic fibroblast growth factor. Atrophy was induced in cultured myotubes by switching the medium to PBS (100 mM NaCl, 5 mM KCl, 1.5 mM MgSO4, 50 mM NaHCO3, 1 mM NaH2PO4, 2 mM CaCl2) for 6 h. Primary cultures of satellite cells were transfected with 2 μg of total plasmid using DreamFect (OZ Biosciences). C2C12 myoblasts were cultured in 36-mm dishes and transfected with 3 μg of total plasmid by using Lipofectamine 2000 (Invitrogen). High level transfection efficiency for C2C12 myoblasts was achieved by using a modified protocol for Lipofectamine 2000. Freshly trypsinized myoblasts were transfected 30 min after plating (300 × 10^3 cells/36-mm dish) with a 2:1 ratio (μl/μg) of Lipofectamine 2000 (10 μl/36-mm dish) to plasmid DNA (5 μg/36-mm dish).

An expression vector coding for the GFP protein was transfected as control of transfection efficacy. GFP expression level in cells was the same. For cycloheximide and MG132 treatments (see Figs. 1A and 2, B and C, and supplemental Fig. S1), cells were transfected with expression vectors as indicated. Twenty-four hours after transfection, cells were trypsinized, pooled, and redistributed in the number of necessary wells for the experiment to homogenize the transfection and protein expression levels for half-life or proteasome stabilization determinations.

Bright-field images of myotubes were randomly taken and analyzed by the Axiovision 4.4 software (Zeiss). The Perfect Image version 5.5 software (Claravision, France) was used to measure diameters of at least 150 myotubes in a region where myonuclei were absent and the diameter was constant.

Immunoprecipitation, Western Blot, and Antibodies—Immunoprecipitation and Western blotting were made essentially as described (7, 17). Analyses of the mobilities of differently phosphorylated forms of 4-EBP1 and phosphorylated ribosomal S6 kinase 1 (S6K1) were made as described previously (18). Following electrophoretic transfer from SDS-PAGE gels to nitrocellulose membranes, they were blocked with 50 ml Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 containing 5% skimmed milk and incubated overnight at 4 °C with primary antibodies: anti α-tubulin (DM1A), anti-myosin heavy chain (My32) (Sigma), anti-HA epitope (12CA5) (Roche Applied Science), anti-FLAG epitope (Sigma), anti-S6K1 (C-18) and anti-Cdk4 (Santa Cruz Biotechnology), anti-GFP (Chemicon), anti-4-EPB1 and anti-Phospho-S6 (Ser-265/236) (Cell Signaling), and anti-eIF3-f (Rockland). The anti-MAFbx antibodies have been described previously (17). Nitrocellulose membranes were then washed and incubated for 1 h with a peroxidase conjugated secondary antibody (Jackson ImmunoResearch Laboratories). The blots were revealed by the enhanced chemiluminescence system kit (Amersham Biosciences) according to
the manufacturer’s instructions. Blots were exposed with Amersham Biosciences Hyperfilm ECL (GE Healthcare) films.

**In Vitro and in Cellulo Ubiquitination Assay—**In vitro ubiquitination assays were determined essentially as described (17). Reactions were carried out at 37 °C and were stopped with Laemmli sample buffer containing β-mercaptoethanol. The products were subject to SDS-PAGE and fluorography. For the in cellulo ubiquitination assay, pCMV-FLAG-eIF3-f and pCDNA-HA-ubiquitin were cotransfected into C2C12 cells in the presence of expression vectors encoding GFP-tagged MAFbx wild type (WT) and/or GFP-MAFbx-ΔF-box. MG132 (30 μM) was added to the culture medium 2 h before harvesting the cells. Cell lysates were prepared in ubiquitination buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% NaDOC, 5 mM EDTA, 0.05% SDS, and protease inhibitors) and were immunoprecipitated with anti-FLAG antibody. Immunoprecipitated proteins were revealed using anti-HA antibody.

**Immunofluorescence Staining—**Cells were cultured on coverslips and fixed for 30 min at room temperature in 2% paraformaldehyde and then permeabilized with 0.25% Triton X-100 for 15 min at room temperature. Cells were treated with 5% normal goat serum and 0.5% bovine serum albumin (Sigma) followed by immunostaining with monoclonal antibody anti-HA and the polyclonal anti-skeletal myosin. The Texas Red-conjugated F(ab′)_2 fragment of donkey anti-mouse IgG was used to visualize the mouse monoclonal antibody, and the fluorescein isothiocyanate-conjugated F(ab′)_2 fragment of goat anti-rabbit IgG was used to visualize the rabbit polyclonal antibody. Cells were rinsed in PBS containing Hoechst, mounted in Citifluor, and viewed and photographed as described above.

**Muscle Electrotransfer, Histological Analysis, and Fiber Size Measurement—**In vivo transfection experiments were carried out as described previously (7). Fasted (48 h) and control (fed) C57BL/6 mice were killed by cervical dislocation. Tibialis anterior (TA) muscles were removed, embedded in Cryomatrix (Thermo Shandon, Pittsburgh, PA), and quickly frozen in isopentane cooled with liquid nitrogen. Muscles were then sectioned in a microtome cryostat (Leica). Transversal sections (12 μm) were fixed with methanol for 5 min at −20 °C, rinsed three times with PBS, and permeabilized with 0.1% Triton X-100 and blocking buffer for 1 h (3% bovine serum albumin, 20% normal goat serum in PBS). Rat monoclonal anti-HA antibody (Jackson ImmunoResearch Laboratories, used at 1:100) and rabbit polyclonal anti-laminin antibody (Sigma) were then applied overnight to the treated sections. Bound primary antibodies were detected with cyanine 3-conjugated anti-rat IgG and goat anti-

**FIGURE 1.** eIF3-f degradation requires multiple lysine residues located in the C-terminal domain. A, eIF3-f exhibits six lysines on its C-terminal domain. Point mutations in which lysines were replaced to arginines as indicated in bold. C2C12 cells were transiently transfected with the expression plasmid pCDNA3-3HA-eIF3-f for the indicated substitution mutants. Forty-eight hours after transfection, MG132 (30 μM) was added to the medium for 5 h. eIF3-f and Cdk4 protein levels were determined by immunoblotting analysis using anti-HA and anti-Cdk4 antibodies, respectively, B, quantitative analysis of the data presented in panel A after an incubation period of 5 h in the absence or in the presence of MG132. Quantities are relative to the amount of protein in the absence of MG132 for each eIF3-f mutant protein after normalization with regards to Cdk4. C, eIF3-f WT and mutants were in vitro translated in the presence of [35S]methionine. The labeled eIF3-f proteins were then incubated for 0, 45, and 90 min with the recombinant SCFMAFbx complex in the presence of E1, ATP, ubiquitin, and Cdc34 (E2). Reaction mixtures were separated on SDS-PAGE followed by autoradiography.

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FIGURE 2. Mutation of the C-terminal lysine residues suppresses polyubiquitination and stabilizes eIF3-f. A, C2C12 myoblasts were transiently transfected with expression plasmids encoding HA-ubiquitin, FLAG-eIF3-f wild type, FLAG eIF3-f K5-10R, GFP-MAFbx wild type, and/or GFP-MAFbx ΔF-box. Transfected cells were treated 2 h with MG132 (30 μM) before being harvested. Cell lysates were then diluted in immunoprecipitation (IP) buffer, subjected to immunoprecipitation with anti-FLAG antibody (middle panel), and analyzed by Western blotting (WB) with anti-HA, anti-FLAG, and anti-GFP antibodies (lower panels). B, the cells were treated with CHX 36 h after transfection to block protein synthesis. Cell lysates were prepared at the indicated times and analyzed by immunoblotting (left panel). C, C2C12 cells were cotransfected with the expression vectors encoding FLAG-tagged eIF3-f WT and/or the mutant K5–10R and HA-tagged MAFbx. Cells were treated with CHX as described in panel B and analyzed by immunoblotting. Quantitation of eIF3-f turnover following CHX treatment based on densitometric scanning of three experiments is shown (right panel). The asterisk indicates a nonspecific band.

RESULTS

C-terminal Lysine Residues of eIF3-f Control MAFbx-directed Polyubiquitination of eIF3-f—During skeletal muscle atrophy, MAFbx targets the initiation factor eIF3-f for ubiquitination and degradation by the proteasome (7). To determine which region of eIF3-f is involved in the MAFbx-mediated polyubiquitination, we constructed a panel of eIF3-f deletion mutants. The expression constructs were transiently expressed in C2C12 cells, and the stability of eIF3-f protein was assessed after treatment with a proteasome inhibitor (MG132) 48 h later. The expressions of the NH2 domain and the Mov34 motif were not significantly modified after MG132 treatment and were stable with regard to eIF3-f WT. In contrast, the C-terminal domain of eIF3-f (amino acids 192–361) was accumulated, suggesting that it is involved in the destabilization of the protein (supplemental Fig. S1). Because the conserved eIF3-f protein contains six lysines in the C terminus, point mutations in which lysines are sequentially replaced by arginine (Lys → Arg) starting by lysine 218 (K5) to lysine 357 (K10) were used in in vitro and in cellulo experiments. The ability of these eIF3-f mutants to accumulate in the presence of MG132 was tested in cellulo. The addition of MG132 for 5 h demonstrated that all the eIF3-f mutants proteins retained sensitivity to proteasome degradation (Fig. 1A). Independent mutation of the six lysines did not confer resistance to proteasome-mediated eIF3-f degradation. Although some lysines such as Lys-242, Lys-258, and Lys-357 seem to be more implicated in the polyubiquitination of eIF3-f, Fig. 1B illustrates that none of these residues is the preferential ubiquitination site. In contrast, the mutation of the six lysines in arginines (mutant K5-10R) abolished the sensitivity to MG132 (Fig. 1A). Altogether, these data suggest that all six lysine residues are involved in the proteasome-mediated degradation of eIF3-f. In addition, in an in vitro ubiquitination assay, SCFMAFbx purified from recombinant baculoviruses produced in Sf9 cells (17) catalyzed the polyubiquitination of each of the different eIF3-f mutants (Fig. 1C). As starvation increases the expression of MAFbx, leading to rapid myotube atrophy (5, 7), the effects of atrophic lysates were tested on eIF3-f ubiquitination. The
polyubiquitination of each of the six elf3-f mutants was dramatically increased after incubation in extracts from atrophic myotubes when compared with extracts of normal C2C12 myotubes (Fig. 1D). Because of the functionality of the six lysines, mutant (K5–10R) was examined for MAFbx-mediated polyubiquitination of elf3-f. The elf3-f mutant K5–10R showed a dramatically reduced polyubiquitination by the SCFMAFbx purified complex (Fig. 1C) and was no longer polyubiquitinated when incubated with extracts from atrophic myotubes (Fig. 1D). Furthermore, MAFbx-mediated polyubiquitination of elf3-f mutant K5–10R was tested in cellulo. Expression vectors encoding either MAFbx WT or the mutant MAFbx ΔF-box (deletion of the F-box domain required for Skp1 interaction) were cotransfected with FLAG-tagged elf3-f WT or mutant elf3-f K5–10R and HA-ubiquitin, into C2C12 cells. elf3-f was first immunoprecipitated with anti-FLAG antibody and then probed with anti-HA to detect ubiquitinated elf3-f protein. elf3-f WT showed increasing amounts of polyubiquitination only in the presence of MAFbx WT but not in the presence of MAFbx ΔF-box mutant (7), whereas MAFbx WT and/or the ΔF-box mutant did not promote the ubiquitination of the elf3-f mutant K5–10R (Fig. 2A). To exclude the possibility that the observed resistance of mutant K5–10R to MAFbx-mediated polyubiquitination resulted from a failure to bind MAFbx, this interaction was analyzed in cellulo. In transfected C2C12 cells, HA-MAFbx coimmunoprecipitated with FLAG-elf3-f WT and/or FLAG-elf3-f mutant K5–10R (supplemental Fig. S2). Finally, to assess the consequence of the C-terminal lysine polyubiquitination on elf3-f stability, we compared the half-life of transiently expressed FLAG-elf3-f WT and mutant K5–10R in C2C12 cells. As shown in Fig. 2B, substitution mutagenesis of the C terminus lysines was sufficient to markedly stabilize elf3-f (half-life >240 min). To test whether MAFbx could influence the turnover of the mutant elf3-f K5–10R in cellulo, we cotransfected HA-MAFbx with FLAG-elf3-f WT or the mutant K5–10R into C2C12 cells and then monitored elf3-f protein stability after CHX treatment. As expected, overexpression of MAFbx did not affect elf3-f K5–10R stability (half-life was >240 min) but enhanced destabilization of elf3-f WT by decreasing its half-life to ~80 min (Fig. 2C). A sequence comparison revealed that all six lysines and the surrounding residues are well conserved in elf3-f homologs from chicken to human (supplemental Fig. S3). Our results suggest that the MAFbx-mediated polyubiquitination of elf3-f and degradation by the proteasome require each of six lysine residues (K5 to K10) located in the C-terminal domain. Thus, mutation of C-terminal lysine residues prevents the polyubiquitination and stabilizes elf3-f.

The elf3-f Mutant K5–10R Delays Starvation-induced Atrophy in Primary Mouse Skeletal Muscle Myotubes—Among the myofibrillar proteins, the myosin heavy chain (MyHC) is a preferred target of multiple pro-cachectic factors inducing muscle wasting in cellulo and in vivo (19). This prompted us to test whether the elf3-f mutant K5–10R was able to prevent the loss of MyHC in muscle cells. Primary myoblasts from satellite cells were transfected with expression vectors encoding HA-tagged elf3-f WT and/or the mutant elf3-f K5–10R under the control of the MCK promoter. After 72 h in differentiation medium, myotubes were starved for 6 h in PBS. Western blot analysis revealed an increase in MAFbx expression in atrophy, and...
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A

|                  | Vector          | eIF3-f wt       | eIF3-f K5-10R |
|------------------|-----------------|-----------------|---------------|
| **control**      | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| **fasted**       | ![Image](image4) | ![Image](image5) | ![Image](image6) |

α-HA, Laminin, DAPI, Merge.

B

Fed  | fasted
---|---
vector | ![Graph](image7) | ![Graph](image8) | ![Graph](image9)
eIF3-f wt | ![Graph](image10) | ![Graph](image11) | ![Graph](image12)
eIF3-f K5-10R | ![Graph](image13) | ![Graph](image14) | ![Graph](image15)

C

CONTROL

| Fiber cross-sectional area (µm²) | Vector | HA-eIF3f wt | HA-eIF3f K5-10R |
|----------------------------------|--------|-------------|-----------------|
| 0-250                           | 640±55 | 835±70      | 930±75          |
| 250-500                          |        |             |                 |
| 500-750                          |        |             |                 |
| 750-1000                         |        |             |                 |
| 1000-1250                        |        |             |                 |
| 1250-1500                        |        |             |                 |
| 1500-1750                        |        |             |                 |
| 1750-2000                        |        |             |                 |
| 2000-2250                        |        |             |                 |
| 2250-2500                        |        |             |                 |
| 2500-2750                        |        |             |                 |
| >2750                            |        |             |                 |

ATROPHY

| Fiber cross-sectional area (µm²) | Vector | HA-eIF3f wt | HA-eIF3f K5-10R |
|----------------------------------|--------|-------------|-----------------|
| 0-250                           | 517±32 | 809±67      | 906±54          |
| 250-500                          |        |             |                 |
| 500-750                          |        |             |                 |
| 750-1000                         |        |             |                 |
| 1000-1250                        |        |             |                 |
| 1250-1500                        |        |             |                 |
| 1500-1750                        |        |             |                 |
| 1750-2000                        |        |             |                 |
| 2000-2250                        |        |             |                 |
| 2250-2500                        |        |             |                 |
| 2500-2750                        |        |             |                 |
| >2750                            |        |             |                 |
expression of the eIF3-f mutant K_{5-10}R not only increased the levels of the MyHC in control conditions but efficiently prevented MyHC loss in myotubes undergoing atrophy (Fig. 3A). In addition, overexpression of the mutant K_{5-10}R efficiently prevented the starvation-induced atrophy by blocking the decrease of mean myotube diameter in satellite cells (Fig. 3, B and C). As expected, in control cells, overexpression of eIF3-f WT showed an increase in myotube diameter when compared with empty vector. Interestingly, eIF3-f K_{5-10}R showed a higher enhanced hypertrophic effect than eIF3-f WT as observed by the increased myotubes diameter (Fig. 3, B and C). Muscle hypertrophy is associated with increased protein synthesis (20). This process is mediated by the activation of the PI3K/Akt/mTOR pathway (21). mammalian target of rapamycin (mTOR) regulates the translational machinery by phosphorylation of its known targets, the S6K1 (22) and the translational repressor protein 4E-BP1 (eukaryotic initiation factor 4E-binding protein) (23). Moreover, eIF3-f interacts directly with mTOR and S6K1 to coordinate the assembly of the translation preinitiation complex (24, 25). This prompted us to examine whether the eIF3-f-mediated hypertrophy in cellulo is accompanied with phosphorylation of downstream targets of mTOR by using an approach described previously (18). Briefly, differentially phosphorylated forms of S6K1 and 4E-BP1 differed in their mobilities with the more highly phosphorylated ones migrating more slowly. As judged by this criterion, we found an increased phosphorylation of S6K1 in primary myotubes overexpressing eIF3-f WT and higher in those overexpressing the mutant eIF3-f K_{5-10}R when compared with empty vector. Furthermore, the phosphorylated forms of 4E-BP1 (γ and β) were also observed. Under atrophic conditions, phosphorylation of S6K1 in myotubes overexpressing eIF3-f WT and mutant K_{5-10}R remained higher, and phosphorylated γ and β forms of 4E-BP1 were still observed, whereas the γ form was not found in cells transfected with the empty vector. In addition, we assessed the phosphorylation of the direct target of S6K1, the ribosomal protein S6 (rpS6). As expected, in control cells, overexpression of eIF3-f WT showed an increased phosphorylation of rpS6 in Ser-235/236, when compared with empty vector, and even higher in cells overexpressing the mutant eIF3-f K_{5-10}R. Moreover, in myotubes undergoing atrophy, phosphorylation of the rpS6 was not longer observed in cells transfected with an empty vector, but it was present in myotubes overexpressing eIF3-f WT and at a highest level with the mutant K_{5-10}R, suggesting that the translational machinery was still functional during starvation-mediated atrophy (Fig. 3A).

**Electroporation of eIF3-f K_{5-10}R Expression Vector in Mice**

*Not Only Protects against Muscle Atrophy but Also Induces Hypertrophy—*To extend our findings in vivo, we electroporated expression vectors encoding HA-tagged eIF3-f WT and/or the mutant eIF3-f K_{5-10}R transfection (under the control of the MCK promoter) into the TA muscle of the right hind leg and the empty vector into TA muscle of the left hind leg as control. Fourteen days after electroporation, mice were either fasted for 48 h (atrophy) or allowed free access to food (control). Fiber size was measured and compared with the cross-sectional area of fiber control. Starvation reduced the cross-sectional area by 20% in fibers electroporated with empty vector or not (Fig. 4, A–C). Similarly to what observed in primary culture cells, the analysis of cross-sectional area showed reduced atrophy in fibers electroporated with eIF3-f WT and were completely resistant to food privation in those fibers expressing eIF3-f K_{5-10}R mutant, confirming a key role for eIF3-f in disuse of muscle atrophy.

Moreover, genetic activation of eIF3-f has been recently shown to induce hypertrophy in myotubes and in mouse skeletal muscle (7). Because Lys-to-Arg mutation of the C terminus prevents polyubiquitination and degradation of eIF3-f during muscle atrophy but also induced increasing myotube diameters, we set out to determine whether these mutations would also affect eIF3-f function in normal skeletal muscle. We evaluated the role of eIF3-f K_{5-10}R in adult mouse muscles. Muscle fibers overexpressing eIF3-f WT were bigger than control fibers, but interestingly, fibers overexpressing eIF3-f mutant K_{5-10}R showed the same enhanced hypertrophic pattern as that observed in cultured cells (Fig. 4, A–C).

**DISCUSSION**

We recently provided biological and biochemical evidence that in response to various treatments inducing muscle atrophy, the Ub ligase MAFbx physically interacts with eIF3-f and contributes to its ubiquitination and its degradation by the proteasome. We demonstrated that eIF3-f could act as a “translational enhancer” that increases the efficiency of the structural muscle protein synthesis, leading to muscle hypertrophy in cellulo and in vivo. These data strongly suggest that eIF3-f is a substrate of MAFbx during skeletal muscle atrophy, and it is a key target that accounts for MAFbx function in atrophy by controlling translation of proteins important for homeostasis. Degradation by the proteasome is the main mechanism that abrogates all function of eIF3-f, and this is involved by the ubiquitin-proteasome pathway (2, 7). To identify lysine residue(s), which are the sites of modification by ubiquitin, a series of deletion mutants of the eIF3-f protein was created, and their proteasome-mediated degradation was assessed. We show that the C-terminal domain, which is highly conserved in the eIF3-f protein family, is the target for proteasomal degradation of eIF3-f. The C-terminal domain contains 6 of the 10 lysine residues found in eIF3-f. The identification of the ubiquitination site(s) involved in the lysine-dependent degradation was undertaken by progressively replacing the lysine residues to arginine in the mouse eIF3-f protein. We show that no single Lys to Arg substitution of eIF3-f K_{5-10}R overexpression reduces muscle atrophy and displays enhanced hypertrophic activities in vivo. A, adult TA muscles were electroporated with either HA-eIF3-f WT or HA-eIF3-f mutant K_{5-10}R (under the control of the MCK promoter, right leg) or the empty vector (left leg) and mice (control or fasted by 2 days) were sacrificed 14 days later. Expression of HA-eIF3-f was detected in transverse sections stained with rat monoclonal anti-HA antibody. Fiber membranes were stained with polyclonal anti-laminin antibody. Scale bar, 20 μm. DAPI, 4′-6-diamidino-2-phenylindole. B, mean cross-sectional area of TA fibers in control mice (Fed) and fasted mice (fasted). *p < 0.05 between control and eIF3-f WT and eIF3-f K_{5-10}R electrophoresed fibers. #, p < 0.05 between eIF3-f WT and eIF3-f K_{5-10}R. C, the histogram shows the fiber size distribution of TA from four mice per expression vector (in control, fed, and atrophy/fasted mice). Black bars, empty vector; gray bars, HA-eIF3-f WT; and white bars, mutant eIF3-f K_{5-10}R.
MAFbx-mediated Polyubiquitination of eIF3-f

MAFbx affects protein degradation, suggesting that none of the lysines is essential for substrate ubiquitination that could represent a potential preferential ubiquitination site (Fig. 1). The six C-terminal lysines serve as ubiquitination sites, and their mutations do not suppress the MAFbx-eIF3-f interaction nor its activity. This finding excludes that the reduced ubiquitination of eIF3-f K5–10R may reflect its decreased affinity for MAFbx. Furthermore, it is interesting to note that the physical interaction of this stable eIF3-f mutant is able to stabilize MAFbx as suggested by Fig. 2B and supplemental Fig. S2. It is tempting to speculate that this interaction down-regulates MAFbx function toward other substrates such as MyoD. This mechanism is under investigation.

eIF3-f-targeting and degradation may be related to the decreased protein synthesis observed in several models of skeletal muscle atrophy by induction of the ubiquitin-proteasome pathway (6). The role of eIF3-f in the mammalian eIF3 translation complex as well as its potential role in translation specific pool of mRNAs would suggest that muscle atrophy hallmark result in part from a specific translation inhibition of key muscle proteins expression. As first evidence, we recently showed that in an inducible protein expression system, genetic activation of eIF3-f in differentiated myotubes induced a massive hypertrophy associated with an increase of sarcromeric proteins (7). Our observation that eIF3-f can prevent muscle atrophy caused by starvation is consistent with in cellulo data showing that antisense RNA-mediated loss of eIF3-f in myotubes is sufficient to induce atrophy (7). In such a model, muscle atrophy occurs without MAFbx induction. Importantly, we have found that when levels of eIF3-f are maintained by electroporation of the eIF3-f K5–10R mutant into adult fibers, muscles are protected to a large extent from atrophy induced by fasting. Mutations of lysines K5 to K10, not only influence the rate of eIF3-f degradation but also are likely to be responsible for the hypertrophic activity detected both in primary mouse myotubes and in the tibialis anterior in mice. Our findings also strongly suggest that in the absence of a capacity for C-terminal lysine modifications, eIF3-f K5–10R is more active than eIF3-f WT. Therefore these data suggest that these conserved C-terminal lysines have evolved to enable a fine regulation of eIF3-f activity in skeletal muscle.

In skeletal muscle, insulin growth factor 1 (IGF-1) or insulin signaling is mediated via two major signaling pathways: the Ras-Raf-MEK-ERK (mitogen-activated protein kinases (MAPKs)) pathway and the PI3K/Akt/mTOR pathway. The IGF-1/PI3K/Akt/mTOR pathway is the major signal that regulates postnatal growth of skeletal muscle and promotes protein synthesis by activation of the translational machinery but also by repressing protein degradation as well as the expression of various autogenes (26–28). Activation of the PI3K/Akt pathway induces hypertrophy by stimulating translation via regulation of the kinase mTOR. mTOR increases protein synthesis via activation of two important translational regulators, S6K1 and the eukaryotic initiation factor 4E (eIF4E), and inhibition of the translation regulator 4E-BP1. More important is the finding that mTOR and S6K1 can both interact directly with eIF3-f to mediate the assembly of the translation preinitiation complex through a coordinated process involving protein-protein interactions and phosphorylation events (24, 25). Indeed overexpression of eIF3-f WT and more yet eIF3-f K5–10R increases phosphorylation of 4-EBP1 and S6K1 and its substrate rpS6, confirming the stimulation of the translation and the induction of the hypertrophy. It evidences as well the scaffolding function of eIF3-f in translation initiation as the control of protein synthesis. These provide important insight into the mechanisms of mTOR and S6K1 signaling and the control of protein synthesis.

Muscle mass uptake results from three different processes: hyperplasia, which results in an increase in fiber number; hypertrophy consecutive to myoblast fusion; and hypertrophy provoked by cytoplasmic volume increase. mTOR initiates S6K1 activation in response to cellular energy status, nutrient levels, and mitogens. Fully sustained S6K1 activation requires additional PI3K-dependent and -independent inputs and requires multiples phosphorylation events. Our recent data show that genetic activation of eIF3-f induces hypertrophy, whereas repression of eIF3-f in differentiated skeletal muscle induces atrophy and down-regulates muscle differentiation. These different observations lead us to envision the involvement of eIF3-f in the regulation of S6K1 and mTOR activation in the assembling of a preinitiation complex specific to mRNA encoding proteins involved in muscle hypertrophy. Preliminary results indicate that the inactive hypophosphorylated form of S6K1 physically associates with the Maf34 domain of eIF3-f. Interestingly, the same Mov34 domain is necessary to MAFbx binding and ubiquitination of eIF3-f. Moreover, muscles undergoing atrophy accumulate the inactive hypophosphorylated form of S6K1, suggesting that the MAFbx-mediated degradation of eIF3-f participates in S6K1 inactivation during atrophy and raises the question of whether MAFbx interacts with free eIF3-f molecules or with molecules bound to S6K1.

Acknowledgments—We thank Dr. C. Bertrand-Gaday from the Transgenic Technology Facility (INRA Montpellier) and Dr. Julie Lagirand-Cantaloube for valuable technical help.

REFERENCES

1. Tisdale, M. J. (2005) J. Support. Oncol. 3, 209–217
2. Jagoe, R. T., and Goldberg, A. L. (2001) Curr. Opin. Clin. Nutr. Metab. Care 4, 183–190
3. Ciechanover, A., and Schwartz, A. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2727–2730
4. Gomes, M. D., Lecker, S. H., Jagoe, R. T., Navon, A., and Goldberg, A. L. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 14440–14445
5. Bodine, S. C., Latres, E., Baumbueter, S., Lai, V. K., Nunez, L., Clarke, B. A., Poueymirou, W. T., Panaro, F. J., Na, E., Dharmarajan, K., Pan, Z. Q., Valenzuela, D. M., DeChiara, T. M., Stitt, T. N., Yancopoulos, G. D., and Glass, D. J. (2001) Science 294, 1704–1708
6. Sachek, J. M., Hyatt, J. P., Raffaello, A., Jagoe, R. T., Roy, R. R., Edgerton, V. R., Lecker, S. H., and Goldberg, A. L. (2007) FASEB J. 21, 140–155
7. Lagirand-Cantaloube, J., Offner, N., Csibi, A., Leibovitch, M.-P., Batonnet-Pichon, S., Tintignac, L. A., Segura, C. T., and Leibovitch, S. A. (2008) EMBO J. 27, 1266–1276
8. Fraser, C. S., Lee, J. Y., Mayeur, G. L., Bushell, M., Doudna, J. A., and Hershey, J. W. (2004) J. Biol. Chem. 279, 8946–8956
9. Lampheare, B. J., Kirchweiger, R., Skern, T., and Rhoads, R. E. (1995) J. Biol. Chem. 270, 21975–21983

4 Csibi, A., unpublished results.
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10. Hoffmann, K., and Bucher, P. (1998) Trends Biochem. Sci 23, 204–205
11. Zhou, C., Arslan, F., Wee, S., Krishnan, S., Ivanov, A. R., Oliva, A., Leath-erwood, J., and Wolf, D. A. (2005) BMC Biol. 3, 14
12. Xiao, H., Xu, L. H., Yamada, Y., and Liu, D. X. (2008) PLoS ONE 3, e1494
13. Doldan, A., Chandramouli, A., Shananas, R., Bhattacharyya, A., Cunning-ham, J. T., Nelson, M. A., and Shi, J. (2008) Mol. Carcinog. 47, 235–244
14. Doldan, A., Chandramouli, A., Shananas, R., Bhattacharyya, A., Leong, S. P., Nelson, M. A., and Shi, J. (2008) Mol. Carcinog. 47, 806–813
15. Shi, J., Kahle, A., Hershey, J. W., Honchak, B. M., Warneke, J. A., Leong, S. P., and Nelson, M. A. (2006) Oncogene 25, 4923–4936
16. Csibi, A., Tintignac, L. A., Leibovitch, M.-P., and Leibovitch, S. A. (2008) Cell Cycle, 7, 1698–1701
17. Tintignac, L. A., Lagirand, J., Batonnet, S., Sirri, V., Leibovitch, M.-P., and Leibovitch, S. A. (2005) J. Biol. Chem. 280, 2847–2856
18. Beugnet, A., Tee, A., Taylor, P. M., and Proud, C. (2003) Biochem. J. 372, 555–566
19. Acharyya, S., Ladner, K. J., Nelson, L. L., Damrauer, J., Reiser, P. J., Swoap, S., and Guttridge, D. C. (2004) J. Clin. Invest. 114, 370–378
20. Goldspink, D. F., Garlick, P. J., and McNurlan, M. A. (1983) Biochem. J. 210, 89–98
21. Bodine, S. C., Stitt, T. N., Gonzalez, M., Kline, W. O., Stover, G. L., Bauer-lein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J. C., Glass, D. J., and Yancopoulos, G. D. (2001) Nat. Cell Biol. 3, 1014–1019
22. Avruch, J., Belham, C., Weng, Q., Har, K., and Yonezawa, K. (2001) Prog. Mol. Subcell. Biol. 26, 115–154
23. Gingras, A. C., Raught, B., and Sonenberg, N. (2001) Genes Dev. 15, 807–826
24. Holz, M. K., Ballif, B. A., Gygi, S. P., and Blenis, J. (2005) Cell 123, 569–580
25. Harris, T. E., Chi, A., Shabanowitz, J., Hunt, D. F., Rhoads, R. E., and Lawrence, J. C., Jr. (2006) EMBO J. 25, 1659–1668
26. Sandri, M., Sandri, C., Gilbert, A., Skurk, C., Calabria, E., Picard, A., Walsh, K., Schiaffino, S., Lecker, S. H., and Goldberg, A. L. (2004) Cell 117, 399–412
27. Stitt, T. N., Drujan, D., Clarke, B. A., Panaro, F., Timofeyva, Y., Kline, W. O., Gonzalez, M., Yancopoulos, G. D., and Glass, D. J. (2004) Mol. Cell 14, 395–403
28. Latres, E., Amini, A. R., Amini, A. A., Griffiths, J., Martin, F. J., Wei, Y., Lin, H. C., Yancopoulos, G. D., and Glass, D. J. (2005) J. Biol. Chem., 280, 2737–2744