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Molecular Pathogenesis of Genetic and Inherited Diseases

Expression of RNA CCUG Repeats Dysregulates Translation and Degradation of Proteins in Myotonic Dystrophy 2 Patients

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Myotonic dystrophy 2 (DM2) is a multisystem skeletal muscle disease caused by an expansion of tetranucleotide CCTG repeats, the transcription of which results in the accumulation of untranslated CCUG RNA. In this study, we report that CCUG repeats both bind to and misregulate the biological functions of cytoplasmic multiprotein complexes. Two CCUG-interacting complexes were subsequently purified and analyzed. A major component of one of the complexes was found to be the 20S catalytic core complex of the proteasome. The second complex was found to contain CUG triplet repeat RNA-binding protein 1 (CUGBP1) and the translation initiation factor eIF2. Consistent with the biological functions of the 20S proteasome and the CUGBP1-eIF2 complexes, the stability of short-lived proteins and the levels of the translational targets of CUGBP1 were shown to be elevated in DM2 myoblasts. We found that the overexpression of CCUG repeats in human myoblasts from unaffected patients, in C2C12 myoblasts, and in a DM2 mouse model alters protein translation and degradation, similar to the alterations observed in DM2 patients. Taken together, these findings show that RNA CCUG repeats misregulate protein turnover on both the levels of translation and proteasome-mediated protein degradation. ***(Am J Pathol 2009, 175:748–762; DOI: 10.2353/ajpath.2009.090047)***

Myotonic dystrophy 2 (DM2) is a muscular disease clinically similar to but distinct from DM1. Both diseases are multisystemic and are characterized by significant clinical heterogeneity.[1,2] Like DM1, DM2 pathology is associated with cataracts, myotonia, and cerebral, endocrine, and cardiac abnormalities.[2] DM1 is caused by untranslated polymorphic CTG triplet repeats in the 3′ untranslated region of the *myotonin protein kinase* gene on chromosome 19q.[3] The mutation causing DM2 is an expansion of an untranslated tetranucleotide CCTG repeat in the first intron of the *ZNF9* gene on chromosome 3q.[4] The identification of untranslated CCTG repeats as the causative mutation in DM2 suggests similarity of clinical symptoms in both diseases. However, there are several important differences. Unlike DM1 patients, DM2 patients show predominantly proximal muscle weakness and only moderate muscle wasting.[2,5] In addition, clinical myotonia in DM2 is usually milder than in DM1. An important distinction of DM2 is the lack of a congenital form of the disease. In DM1, very long CTG repeats (more than 800) lead to the most severe form of disease, congenital DM1, which is associated with significant delay of muscle development, neonatal weakness and mental retardation.[1] Although DM2 patients have much larger expan-

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sions of repeats, there are no known cases of congenital disease.

Examinations of molecular mechanisms of DM1 suggested that untranslated CTG repeats cause the disease through RNA CUG repeats,[6–9] which bind to and misregulate RNA CUG-binding proteins CUGBP1 and MBNL1.[10–12] Studies of mouse models indicated that the main symptoms of DM1 (myotonia and muscular dystrophy) are due to a CUG RNA-mediated mechanism[7–9] associated with an increase of CUGBP1[9,13,14] and with a reduction of MBNL1.[15] CUGBP1 has several functions in RNA metabolism, including translation,[16–18] splicing,[19,20] and regulation of RNA stability[21], whereas MBNL is a splicing regulator.[15,22]

To determine molecular pathways by which RNA CCUG repeats cause DM2 pathology, we have examined the hypothesis that RNA CCUG repeats target large protein-protein complexes in the cytoplasm of DM2 cells. We found that RNA CCUG repeats interact with two cytoplasmic multiprotein complexes and that the amounts of these complexes are increased in the cytoplasm of DM2 myoblasts. One of these complexes is the CUGBP1-eIF2 complex.[17] This complex interacts with CCUGn RNA repeats in the livers of CCTG transgenic mice and in the cytoplasm of C2C12 myoblasts transfected with long CCTG repeats. A major component of a second CCUG-binding complex is the 20S core complex of the proteasome. Consistent with the biological functions of these complexes, protein levels of CUGBP1-regulated translational targets and the stability of short-lived proteins are increased in DM2 muscle cells. Ectopic expression of RNA CCUG repeats in control myoblasts derived from unaffected patients changes protein turnover similar to alterations observed in DM2 myoblasts.

Materials and Methods

Tissue Culture and Western Blot Analysis

C2C12 mouse myoblasts were grown at 5% CO_2 in the growth medium consisting of Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and 100 μg/ml penicillin/streptomycin. Myoblast fusion was induced by switching of growth medium to differentiation medium. Fusion medium contained Dulbecco's modified Eagle's medium, supplemented with 2% horse serum, 0.01 mol/L insulin, 2 mmol/L glutamine, and 2.5 μmol dexamethasone. Cells were maintained in fusion medium for 5 days with daily change of medium and formation of myotubes was monitored microscopically.

Human primary myoblast cultures were established from muscle biopsies of unaffected individuals and DM2 patients. Human myoblasts were grown at 50% density in Ham's F-10 medium containing 15% fetal bovine serum, 5% defined supplemented calf serum, 2 mmol/L L-glutamine, and 100 μg/ml penicillin/streptomycin.

Nuclear and cytoplasmic extracts were prepared as described.[16,18] Western blotting was performed with rabbit polyclonal anti-eIF2α (FL-315), rabbit polyclonal anti-eIF2β (H-203), rabbit polyclonal anti-MEF2A (H-300), rabbit polyclonal anti-C/EBPβ (C19), mouse monoclonal anti-HuR (3A2), rabbit polyclonal anti-p21 (H164), mouse monoclonal anti-CUGBP1 (3B1), mouse monoclonal anti-Hsp70 (3A3), rabbit polyclonal anti-CRT (H-170), rabbit polyclonal anti-c-*myc* (N 262) (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA), and with mouse monoclonal anti-β-actin (AC-15) (Sigma) as recommended by manufacturers. The antibodies to the 20S core of the proteasome were as follows: mouse monoclonal anti-subunit α2 (MCP21), mouse monoclonal anti-α4 (MCP34), mouse monoclonal anti-α6 (MCP106), and mouse monoclonal anti-α7 (MCP72) (all from Affinity Research Products/Biomol International, Devon, United Kingdom, LP). To detect mono- and polyubiquitinylated conjugates, monoclonal antibodies FK-2 (Biomol International, Devon, United Kingdom, LP) were used according to the protocol from the manufacturer.

CCTG Transgenic (TR) Mice and Isolation of Proteins from the Liver

CCTG TR mice expressing non-coding $CCUG_{121}$ repeats in several tissues, including liver, were generated and characterized in the laboratory of Dr. Krahe (M.D. Anderson Cancer Center, Houston, TX). Expression of expanded $CCUG_{121}$ repeats in these mice caused the main symptoms of DM2 (M. Sirito, M. Wojciechowska, L.L. Bachinski, O. Raheem, C. Huichalaf, B.H.G. Schoser, D.R. Mosier, K.A. Sheikh, G. Zhang, P. Mancias, C.S. Van Pelt, L. Timchenko, B. Udd, and R. Krahe, manuscript in preparation). Livers were harvested from 6-month-old wild-type and CCTG TR mice. Cytoplasmic and nuclear extracts were isolated by homogenization of the liver in buffer A containing 20 mmol/L Tris-HCl, pH 7.5, 30 mmol/L KCl, 5 mmol/L dithiothreitol, and 10% glycerol and phosphatase inhibitors. Following centrifugation, the supernatant (cytoplasm) was frozen and stored at −80°C. Nuclear extracts were isolated by incubation of the pellet (nuclei) with high salt buffer B containing 20 mmol/L Tris-HCl, pH 7.5, 0.42 mol/L NaCl, 5 mmol/L dithiothreitol, inhibitors of proteases and phosphatases, and 25% sucrose.

Electrophoretic Mobility Shift Assay (EMSA) and UV Cross-Link Assays

EMSA assay and UV cross-link were performed as described.[16] For EMSA, the sequences of RNA CUG and CCUG probes are shown in Figure 1. The sequences of RNA p21 and C/EBPβ RNA probes were described previously.[16,17] The sequence of control AU-rich c-fos RNA was as follows: 5′-UAUUUAUAUUUUUAUUUUAUUU-3′. RNA probes were labeled by [^{32}P]γATP with T4 kinase and incubated with cytoplasmic proteins (20 μg) from cultured cells (C2C12 myotubes, human primary myoblasts derived from unaffected patients and from patients with DM2), or from mouse liver. The incubation buffer contained 20 mmol/L Tris-HCl, pH 7.5, 100 mmol/L KCl, 5 mmol/L dithiothreitol, 100 ng of tRNA, and phosphatase inhibitors. The binding reactions were resolved by native 5% polyacrylamide gel electrophoresis. The gel was dried and autographed with X-ray film.

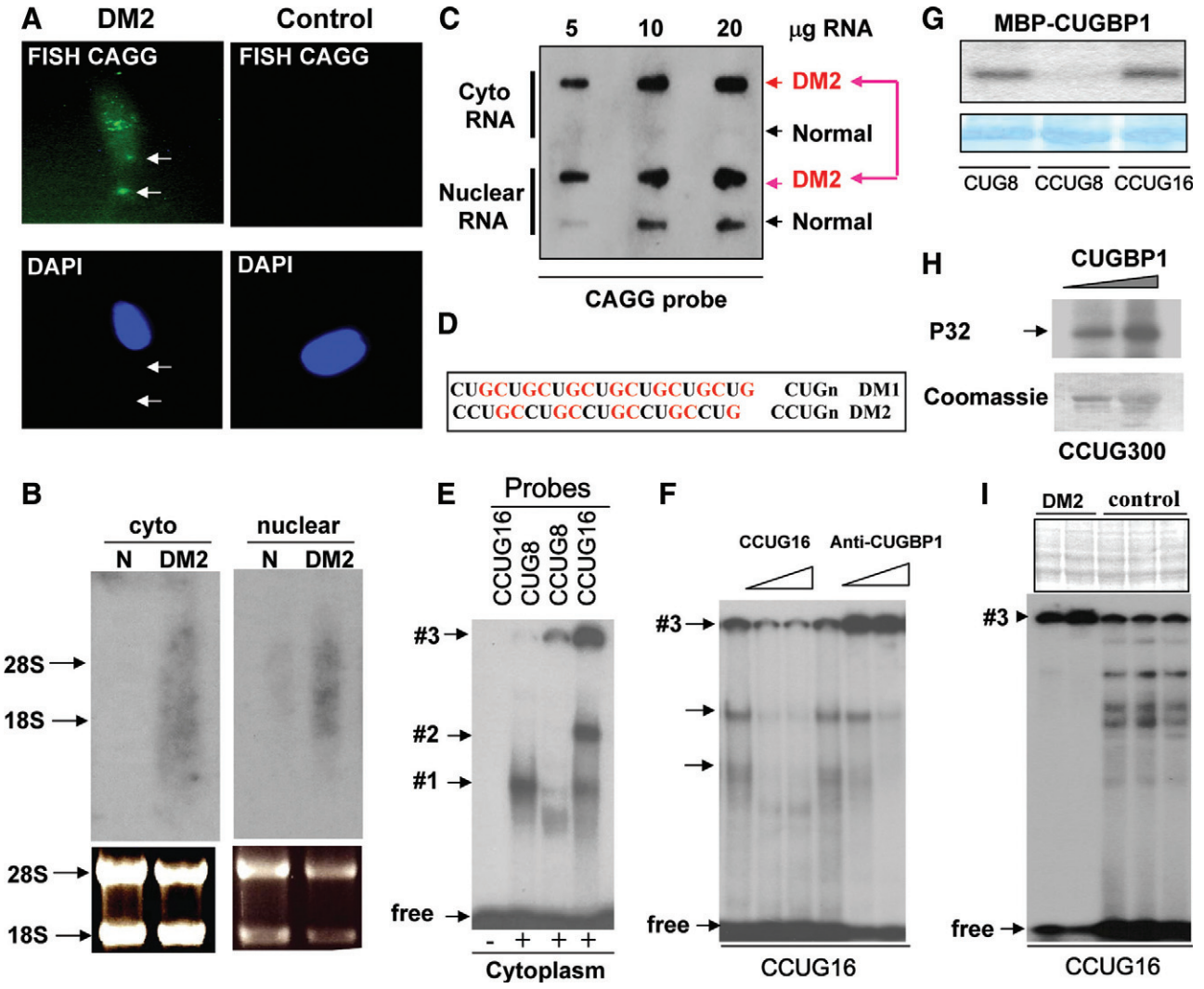

Figure 1. CCUG RNA repeats form RNA-protein complexes in the cytoplasm of myoblasts derived from DM2 patients. **A:** FISH hybridization detects CCUG RNA (green) in cytoplasm and in nuclei. The fluorescence *in situ* hybridization assay was performed with myoblasts from unaffected patients (control) and with myoblasts from DM2 patients. Bottom images show DAPI staining. **Arrows** show cytoplasmic foci generated by the expanded CCUG repeats. **B:** Northern blot analysis of RNA isolated from cytoplasm and from nuclei of normal (N) and DM2 myoblasts with $CAGG_{16}$ probe. Bottom images show ethidium bromide staining of the gels. The 28S and 18S ribosomal RNAs show equal loading and integrity of the used RNAs. **C:** Detection of RNA CCUG repeats in nuclear and cytoplasmic RNAs of DM2 myoblasts by dot-blot hybridization. RNA, 5, 10, and 20 μg, were loaded on the filter and hybridized with $CAGG_{16}$ probe. **D:** CUG and CCUG RNAs contain common elements, GC islands (shown in red). **E:** $CCUG_{16}$ RNA forms three RNA-protein complexes with cytoplasmic extracts from C2C12 myotubes. EMSA was performed with cytoplasmic proteins isolated from C2C12 myotubes with CUG_8, $CCUG_8$, and $CCUG_{16}$ probes, shown on the top of the gel. **Arrows** show three RNA-protein complexes formed with $CCUG_{16}$ RNA. **F:** Complexes 1 and 2 contain CUGBP1. Increasing amounts of non-radioactive $CCUG_{16}$ probe and Abs to CUGBP1 were incorporated in the binding reactions with cytoplasm and [32P]-$CCUG_{16}$ probe. **G:** Homogenous MBP-CUGBP1 binds to CCUG RNA. UV cross-link was performed with CUG_8, $CCUG_8$, and $CCUG_{16}$ probes. The filter was stained with Coomassie blue to verify equal loading of the MBP-CUGBP1. **H:** CUGBP1 binds to the long CCUG RNA. Increasing amounts of CUGBP1 were incubated with the long $CCUG_{300}$ probe, treated with UV, and separated by denaturing gel. Upper image shows radioactive signals; the bottom image shows Coomassie stain of the same membrane. **I:** Amounts of high molecular weight complex 3 are increased in cytoplasm of DM2 patients. Cytoplasmic protein extracts from myoblasts derived from unaffected patients (control) and DM2 patients were examined by EMSA with $CCUG_{16}$ probe. The bottom image shows results of EMSA analysis. Positions of high molecular weight complex 3 and free probe are shown by **arrows**. Upper panel shows a Coomassie stain of the fragment of independent gel with the same loadings of the cytoplasmic proteins as used for the EMSA.

UV Cross-Link Assay

The purified proteins (CUGBP1 fused with maltose-binding protein, complex 3–2, or 20S and 26S proteasomes) or various protein extracts were incubated with RNA probes as described for EMSA assay. Where indicated, specific non-radioactive RNA competitors (100 ng) or specific antibodies (1 μg), were added before radioactive probe addition. After incubation, the binding reactions were treated with UV light and resolved by denaturing 4 to 20% polyacrylamide gel electrophoresis. The proteins were transferred on membrane and exposed to X-ray film. The membranes were stained with Coomassie blue to verify protein loading.

Inhibition of Proteasome Activity

To determine formation of ubiquitin conjugates of total proteins and conjugates of p21, proteasome activity was inhibited by MG132 (Biomol International, Devon, United Kingdom, LP). The cells were treated with 10 μmol/L

MG132 for 4 hours and protein extracts were isolated as described above. Proteins were separated by denaturing 4 to 20% polyacrylamide gel electrophoresis, transferred onto the membrane, and probed with monoclonal antibodies to ubiquitin (clone FK-2, Biomol International, Devon, United Kingdom, LP). To determine p21-ubiquitin conjugates, p21 was immunoprecipitated from these extracts and examined by Western blotting with antibodies to ubiquitin. The membranes were stained with Coomassie blue to verify loading of IgGs.

Examination of Protein Half-Life

Three different sets of experiments were used to determine the half-life of proteins. In the first set of experiments, human primary myoblasts from unaffected patients and from patients with DM2 were maintained in growth medium. In second set, primary human myoblasts derived from unaffected patients were transfected with non-coding synthetic RNAs containing AU repeats (AU_{42}) and CCUG repeats ($CCUG_{16}$) using Amaxa biosystems cell line Nucleofector kit (Amaxa Biosystems, Cologne, Germany). In the third set, C2C12 myoblasts were transfected with AU_{42} and $CCUG_{16}$ RNAs. AU_{42} and $CCUG_{16}$ RNAs were synthesized in Integrated DNA Technologies (Coralville, IA). Myoblasts in all three cases were treated with 10 mmol/L cycloheximide for 30 minutes, 1, 2, and 4 hours. Protein extracts were isolated and examined by Western blotting with antibodies to p21, c-*myc*, C/EBPβ and CUGBP1. In the first set of experiments, 20 μg of proteins from DM2 myoblasts and 50 μg of proteins from control myoblasts were used for Western blotting to adjust for the increase of p21, c-*myc,* and CUGBP1 in DM2 myoblasts. Each membrane was reprobed with β-actin, and the levels of protein were calculated as ratios to β-actin and then as percentages of the 0 time point. A summary of three to four independent experiments is shown.

RNA Dot-Blot Hybridization and Northern Blotting

Cytoplasmic and nuclear extracts were prepared from control and DM2 myoblasts following RNA extraction with guanidine thiocyanate. For dot-blot hybridization increasing amounts of RNA were loaded onto a nylon membrane, treated by UV light, and hybridized with ^{32}P-$(CAGG)_{16}$ probe labeled by [^{32}P]γATP.

Northern Blotting

RNA was loaded on a formaldehyde-agarose gel, transferred onto a nylon membrane, and incubated with $CAGG_{16}$ probe. The pictures of 28S and 18S RNAs were taken from the gels before transfer and used to determine the integrity of the isolated RNAs.

Fluorescence in Situ *Hybridization Assay and IF Analysis*

Control and DM2 primary myoblasts were fixed with 3.7% formaldehyde in 1X phosphate-buffered saline containing 5 mmol/L $MgCl_2$. After washing in 1X phosphate-buffered saline, slides were subjected to prehybridization in a solution of 40% formamide and 2X standard saline citrate for 10 minutes at room temperature. Hybridization solution contained 40% formamide, 2X standard saline citrate, 10% dextran sulfate, 0.2% bovine serum albumin, 2 mmol/L vanadyl adenosine complex, 1 mg/ml tRNA, 200 μg/ml salmon sperm DNA, and 2 μg/ml FITC -labeled $CAGG_{16}$ probe. After hybridization for 2 hours at 37°C, slides were washed with 1X phosphate-buffered saline and incubated with primary antibodies (mouse monoclonal anti-α4 of the 20S proteasome, clone MCP34, Biomol International, Devon, United Kingdom, LP) in 1X phosphate-buffered saline containing 0.2% bovine serum albumin for 1 hour and then with secondary antibodies (tetramethylrhodamine B isothiocyanate-conjugated goat anti-mouse IgGs from Santa Cruz Biotechnologies Inc., Santa Cruz, CA) in 1X phosphate-buffered saline with 0.2% bovine serum albumin. Slides were stained with DAPI and mounted in Vectashield medium.

Purification of Protein-Protein Complexes that Interact with CCUG Repeats

The high molecular weight (MW) CUGBP1-eIF2 and the 20S proteasome complexes were purified by a combination of high-performance liquid chromatography-based chromatography using different ion exchange columns and size exclusion columns (Figure 2A). The location of complex 3 within the fractions was monitored by EMSA with the $CCUG_{16}$ probe. The identity of the CUGBP1-eIF2 and the 20S proteasome complexes was determined by mass spectroscopy analysis in the Protein Chemistry Core Laboratory (Baylor College of Medicine).

Translation of C/EBPβ in a Cell-Free Translation System

Five micrograms of two different preparations of the CUGBP1-eIF2 complex were added to the rabbit reticulocyte system (Promega Corporation, Madison, WI) programmed with C/EBPβ mRNA and supplemented with [^{35}S]methionine. Newly synthesized radioactive C/EBPβ proteins were precipitated with anti-C/EBPβ and the immunoprecipitates were resolved by denaturing 10% polyacrylamide gel electrophoresis. Proteins were transferred to membrane and exposed to X-ray film.

EMSA (Gelshift)–Western Assay for the 20S Proteasome Complex

To determine that the 20S proteasome within complex 3–2 binds to CCUG repeats, cytoplasm from myoblasts

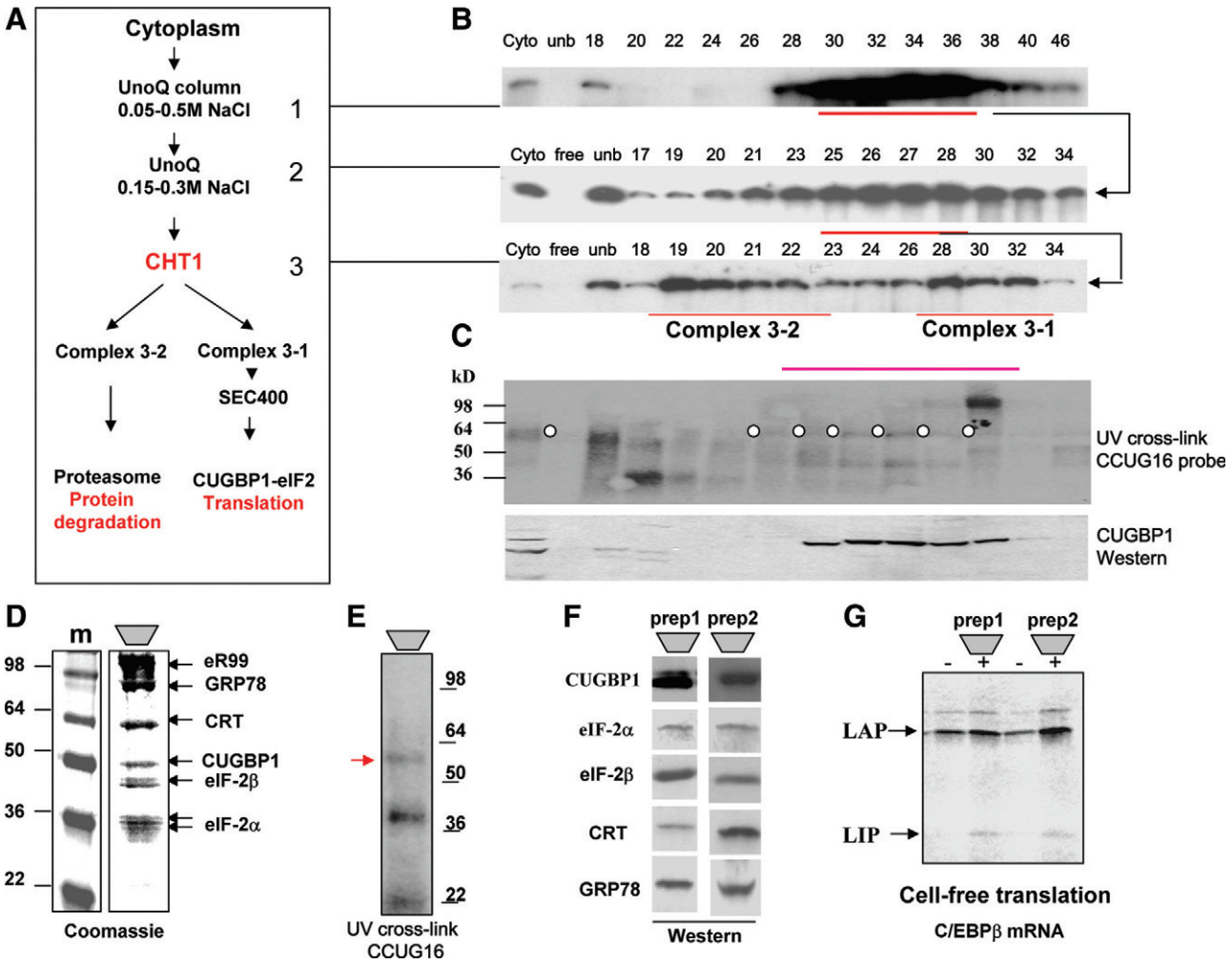

Figure 2. Purification of complex 3. **A:** A diagram showing the strategy for the isolation of the complex. Twenty milligrams of cytoplasmic proteins from livers of wild-type aged mice were used for the isolation of the high molecular weight complexes. **B:** Detection of the complex 3 in each step of purification by EMSA/gel shift assay with $CCUG_{16}$ RNA. The fractions containing complex 3 and complexes 3–1 and 3–2 are underlined. **C:** A representative picture showing the analysis of protein fractions after the second step of purification by UV cross-link with $CCUG_{16}$ RNA and by Western blotting with antibodies to CUGBP1. A major portion of complex 3–1 is located in fractions marked with red line. The position of CUGBP1 in the fractions examined by UV cross-link is shown by open circles. **D:** Coomassie staining of proteins of complex 3–1 after size exclusion chromatography. The identity of these proteins was determined by mass spectroscopy. **E:** UV cross-link analysis of the purified complex. The purified complex was incubated with the $CCUG_{16}$ probe and examined by UV cross-link assay. The **arrow** shows a position of CUGBP1. **F:** The composition of the purified complex 3–1 determined by Western blotting with antibodies shown on the left. Western blotting analysis of two preparations of the complex 3–1 is shown. **G:** The purified complex 3–1 (CUGBP1-eIF2) increases translation of C/EBPβ in a cell-free translation system. Two different preparations of the CUGBP1-eIF2 complex were added in the reticulocyte lysate programmed with C/EBPβ mRNA. Positions of LAP and LIP isoforms of C/EBPβ are shown.

derived from DM2 patients was separated by ion exchange chromatography, and the complex was identified by EMSA with $CCUG_{16}$ probe on native 5% gel. The piece of polyacrylamide gel with the complex was cut out and loaded on a denaturing 5 to 20% gel. Proteins were transferred onto a membrane and probed with antibodies to the components of the 20S proteasome core.

Transient Transfections

$CCTG_{300}$ was synthesized as described[23] and cloned into a pCDNA3.1 vector. $CCTG_{36}$ and AU_{42} repeats containing primers with flanking cloning sites were synthesized by Integrated DNA Technologies, Coralville, IA and cloned into pSUPER expression vector. Plasmids were transfected into control human myoblasts derived from unaffected patients and into mouse C2C12 myoblasts using Amaxa biosynthesis cell line Nucleofector kit. As an alternative approach, synthetic $CCUG_{16}$ and AU_{42} RNAs were transfected into control human and C2C12 myoblasts according to the Amaxa protocol.

Separation of Protein-Protein and RNA-Protein Complexes

Cytoplasmic extracts from control and DM2 myoblasts and tissues from wild-type and CCTG transgenic mice were analyzed as described.[17] Briefly, cytoplasmic proteins were loaded on a size exclusion SEC400 column. The chromatography fractions were divided in portions

and analyzed by dot-blot hybridization, EMSA, and UV cross-link with the $CAGG_{16}$ probe and by Western blotting with antibodies to CUGBP1, eIF2α, CRT, and β-actin.

Results

CCUG-Containing RNA Is Detected in Both the Nucleus and Cytoplasm of DM2 Myoblasts

Differences in DM1 and DM2 phenotypes prompted us to search for specific RNA CCUG-binding proteins that might be affected in DM2 cells. To identify CCUG-binding proteins, we initially analyzed the distribution of RNA CCUG repeats in DM2 myoblasts using several approaches. Fluorescence *in situ* hybridization assays with a $CAGG_{16}$ probe showed that CCUG-positive signals are observed in both the nuclei and the cytoplasm of DM2 myoblasts (Figure 1A). This result was unexpected, since the expanded CCUG repeats are located within the intron of the *ZNF9* gene and should be spliced out before the entry of ZNF9 mRNA into the cytoplasm. Given this unusual distribution of CCUG repeats, we performed additional studies. Nuclear and cytoplasmic RNA was isolated from control and DM2 myoblasts and used for Northern blot hybridization with a $CAGG_{16}$ probe. This analysis showed approximately equal amounts of CCUG-positive signals in the nuclear and cytoplasmic RNAs from DM2 cells. In contrast, in normal cells, a weak signal was observed in the nuclear but not in the cytoplasmic RNA (Figure 1B). Although CCUG-containing RNA appeared as a diffuse signal on the gel, EtBr staining of the same gels showed sharp, distinct bands for ribosomal RNAs 28S and 18S. Since 28S and 18S RNAs were intact, we propose that CCUG-positive RNA in DM2 cells migrated as a "smear" band due to formation of intermediate products of splicing in the nucleus or products of specific cleavage in the cytoplasm. To confirm the cytoplasmic localization of CCUG repeats in DM2 cells, we performed dot-blot hybridization with these RNAs. As can be seen in Figure 1C, DM2 myoblasts contained approximately equal amounts of CCUG-positive RNA in the nuclear and cytoplasmic RNAs, while in control myoblasts from unaffected patients, CCUG repeats were detected only in the nuclear RNA. Increased amounts of RNA CCUG repeats in the cytoplasm of myoblasts derived from DM2 patients were later confirmed by high-performance liquid chromatography-based techniques. Therefore, four independent approaches showed that DM2 myoblasts contain RNA CCUG repeats not only in the nucleus but also in the cytoplasm.

CCUG RNA Interacts with Protein-Protein Complexes, Amounts of Which Are Increased in DM2 Myoblasts

To identify proteins that bind to CCUG repeats in the cytoplasm, we initially analyzed cytoplasmic proteins from mouse C2C12 myotubes by EMSA with three riboprobes: CUG_8, $CCUG_8$, and $CCUG_{16}$. We found that, in mouse myotubes, CUG_8 RNA formed a single specific RNA-protein complex (Figure 1E), which was repressed by non-radioactive CUG_8 and supershifted with antibodies to CUGBP1 (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). On the contrary, $CCUG_{16}$ RNA formed three complexes: complex 1, migrating in the position of CUGBP1-CUG_8, complex 2, and complex 3, observed close to the start of the gel (Figure 1E). The high molecular weight complex 3 interacted specifically with $CCUG_{16}$ RNA, since the CUG probe did not form this complex. To confirm the specificity of the $CCUG_{16}$ interacting complexes, we included $CCUG_{16}$ non-radioactive competitor in the binding reactions. All three CCUG complexes were formed by specific interactions because addition of non-radioactive $CCUG_{16}$ RNA inhibited the formation of these complexes (Figure 1F). Additional competition experiments revealed that the addition of CUG_8 cold competitor abolished formation of complexes 1 and 2, but did not affect the formation of complex 3 (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). These data suggested that complex 3 specifically recognizes CCUG RNA. The addition of antibodies to CUGBP1 to the binding reaction resulted in a supershift of complexes 1 and 2, indicating these complexes contained CUGBP1 (Figure 1F). Because CUGBP1 containing complex 2 did not interact with $CCUG_8$ RNA (Figure 1E), we asked if this lack of the interaction was due to the inability of CUGBP1 to bind to $CCUG_8$ RNA, despite the homology between CUG and CCUG repeats (Figure 1D), or if the interaction was blocked by other proteins within cellular extracts. To address these questions, homogenous CUGBP1 fused with maltose-binding protein (MBP-CUGBP1) was initially examined by UV cross-link assays with CUG_8, $CCUG_8$ and $CCUG_{16}$ probes. Figure 1G shows that MBP-CUGBP1 did not bind to $CCUG_8$ RNA, while the binding to CUG_8 and $CCUG_{16}$ RNAs was strong. To test if purified CUGBP1 binds to long CCUG repeats, we performed UV cross-link with RNA containing 300 CCUG repeats. Figure 1H shows that the purified CUGBP1 binds to the long CCUG repeats. Therefore, these studies suggest that CUGBP1 binds to RNAs containing 16 and more CCUG repeats.

To determine the amount of CCUG-binding complexes in DM2 myoblasts, we examined cytoplasmic protein extracts from control human myoblasts and from myoblasts derived from DM2 patients by EMSA with the $CCUG_{16}$ riboprobe. As shown in Figure 1I, several CCUG-protein complexes were formed in control myoblasts, including complex 3, similar to that observed in C2C12 extracts. The pattern of RNA binding proteins in DM2 cytoplasm was quite different. The amount of complex 3 was increased in the cytoplasm of DM2 myoblasts compared with normal myoblasts, while complexes of smaller sizes were reduced or undetectable (Figure 1I). Given the increase of complex 3 in the cytoplasm of DM2 myoblasts, we suggested that this complex might be involved in the development of DM2 pathology. Therefore, we performed further isolation and characterization of complex 3.

CCUG-Binding Complex 3 Consists of Two Independent Complexes: 20S Catalytic Core Complex of the Proteasome and the Translational Complex CUGBP1-eIF2

We initially tried to isolate complex 3 from the cytoplasm of C2C12 myotubes, but these experiments were only partially successful. The isolated complex was detectable by EMSA approach; however, amounts of proteins within the complex were not sufficient for mass spectroscopy. To find a better source for the isolation of complex 3, we examined a number of tissues by EMSA with $CCUG_{16}$ probe. We observed that complex 3 was abundant in cytoplasmic extracts from the livers of old mice (data not shown). Therefore, we have purified multiprotein complexes from the livers of old mice using high-performance liquid chromatography-based techniques. The general procedure for the purification of the complexes is shown in Figure 2A. To monitor complex 3 during purification, EMSA/gel shift with a $CCUG_{16}$ probe was applied. A typical picture of gel shift assays at each step of purification is shown in Figure 2B. In the course of these studies, we surprisingly found that CUGBP1 colocalized with complex 3 on the first three steps of the purification, but was separated by chromatography on the CHT1 column. As an example of this colocalization, Figure 2C shows examination of chromatography fractions after the second step of purification on the UnoQ column by UV cross-link and Western blotting analysis. As can be seen, several RNA binding proteins co-purified with complex 3 (Figure 2C, UV cross-link), one of which was CUGBP1 (Figure 2C, Western). However, chromatography on the CHT1 column separated a CUGBP1 containing complex (complex 3–1) and an additional complex (complex 3–2), which was later identified as the complex containing the 20S proteasome.

Further separation of complex 3–1 by size exclusion chromatography and analysis of its protein composition revealed that this complex is identical to one identified in the liver[17] and in human myotubes.[18] Figure 2D shows a Coomassie blue stain of complex 3–1. Identity of the protein components and the RNA binding activity of the purified complex 3–1 were further examined by mass spectroscopy, UV cross-linking, and Western blotting assays. The purified complex 3–1 contained three RNA binding proteins, one of which migrated in the position of CUGBP1 (Figure 2E). Examination of the complex by mass spectroscopy and Western blotting showed that this complex contains CUGBP1, eIF2α, eIF2β, CRT, eR99, and GRP78 (Figure 2, D and F). To determine whether the purified complex 3–1 possesses translational activity similar to the previously identified CUGBP1-eIF2 complex,[17] we examined the effects of the purified complex on the translation of C/EBPβ mRNA in a cell-free translational system. Figure 2G shows that the purified complex 3–1 (subsequently referred to as CUGBP1-eIF2) increased translation of both isoforms of C/EBPβ, LAP, and LIP. Thus, the composition and biological activity of complex 3–1 are identical to those previously described for the CUGBP1-eIF2 complex.

The Activity of the CUGBP1-eIF2 Complex Is Elevated in DM2 Myoblasts

To determine the amount of the CUGBP1-eIF2 complex in DM2 patients, CUGBP1 was immunoprecipitated from control and DM2 myoblasts and the CUGBP1 IPs were probed with antibodies to components of the CUGBP1-eIF2 complex. Figure 3A shows that eIF2α, eIF2β and eR99 are observed in CUGBP1 IPs from DM2 myoblasts, while these proteins are low or not detectable in CUGBP1 IPs from myoblasts derived from unaffected patients. Since CUGBP1 regulates translation of p21 and C/EBPβ mRNAs,[16–18] we also examined if binding of the CUGBP1-eIF2 complex to these mRNAs is elevated in DM2 myoblasts. $CCUG_{16}$ RNA and c-fos (AU-rich RNA probe) were included as positive and negative controls respectively. EMSA assay showed that complex 3, containing CUGBP1-eIF2, strongly interacts with $CCUG_{16}$ and C/EBPβ RNAs and the binding of the complex to these RNAs is significantly increased in DM2 myoblasts (Figure 3B). The interaction of the complex with p21 mRNA is weak and is slightly increased in DM2 myoblasts. The interaction of the complex with tested RNAs was specific, because the CUGBP1-eIF2 complex did not interact with the c-fos RNA probe.

We next examined the levels of CUGBP1 and the levels of its translational targets in cultured myoblasts derived from DM2 patients and in mature skeletal muscle from DM2 patients. Western blotting analysis with cultured myoblasts from DM2 patients showed that CUGBP1 is elevated in the cytoplasm of DM2 myoblasts (Figure 3C). DM2 myoblasts also accumulated low mobility CUGBP1 isoforms migrating in the position of hyperphosphorylated forms of CUGBP1. These alterations are consistent with previous observations, which showed that the interaction of CUGBP1 with eIF2 is mainly regulated by phosphorylation of CUGBP1.[17,18] We have previously shown that CUGBP1 increases translation of MEF2A and C/EBPβ mRNAs.[13,17] Examination of the protein levels of these translational targets of CUGBP1 revealed that amounts of both proteins are increased in cultured myoblasts derived from DM2 patients (Figure 3C). We next examined if these translational targets of CUGBP1 were increased in mature skeletal muscle of DM2 patients. Western blotting showed that, like in cultured myoblasts, protein levels of MEF2A and C/EBPβ are also increased in DM2 muscle biopsies (Figure 3D). These data are consistent with the hypothesis that accumulation of the CUGBP1-eIF2 complex in DM2 patients changes translation of MEF2A and C/EBPβ.

CUGBP1 Is Associated with Cytoplasmic CCUG Repeats in DM2 Cells, in CCTG Transgenic Mice and in Cells Transfected with Vector-Expressing Long CCUG Repeats

The presence of CCUG repeats in the cytoplasm of DM2 myoblasts and the binding of CUGBP1-eIF2 to CCUG RNA prompted us to test if CUGBP1-CCUG complexes

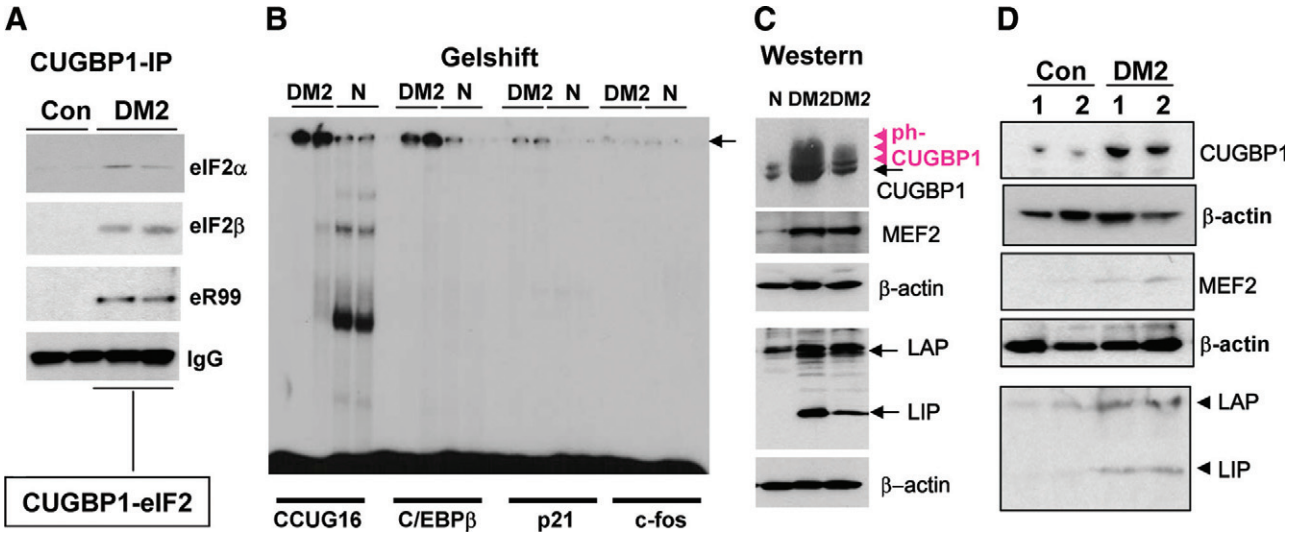

Figure 3. DM2 muscle cells contain high amounts of the CUGBP1-eIF2 complex. **A:** The amount of CUGBP-eIF2 complex is increased in DM2 myoblasts. CUGBP1 was immunoprecipitated from the cytoplasmic extracts of normal control and DM2 myoblasts and examined by Western blotting with antibodies to eIF2α, eIF2β, and eR99. IgG; signals of the heavy chains of IgGs. **B:** The binding of complex 3 to $CCUG_{16}$ and C/EBPβ mRNA is increased in DM2 myoblasts. EMSA was performed with C/EBPβ, p21, $CCUG_{16}$, and c-fos probes with cytoplasmic proteins from myoblasts derived from unaffected patients (normal) and from patients with DM2. The position of the CUGBP1-eIF2 complex is shown by the **arrow**. **C:** Protein levels of CUGBP1 and its translational downstream targets, MEF2A and C/EBPβ, are increased in myoblasts from DM2 patients. Western blotting analyses of myoblasts from unaffected (normal) and DM2 patients with antibodies against CUGBP1, MEF2A, and C/EBPβ are shown. Membranes were re-probed with antibodies against β-actin. Low-mobility CUGBP1 isoforms migrating in the positions of hyperphosphorylated CUGBP1 are shown by **red arrows**. **D:** Protein levels of MEF2A and C/EBPβ are increased in DM2 muscle biopsies. Cytoplasmic (for CUGBP1) and nuclear extracts (for MEF2A and C/EBPβ) were examined by Western blotting with antibodies shown on the right. The membranes were re-probed with anti-β-actin.

exist in the cytoplasm of DM2 myoblasts. Cytoplasmic extracts from control and DM2 myoblasts were separated by high-performance liquid chromatography on SEC400 column and examined by Western blotting with antibodies to CUGBP1. In myoblasts from unaffected patients, the major portion of CUGBP1 migrated as a free protein or as a protein within low MW complexes; however, in DM2 myoblasts, significant amounts of CUGBP1 were translocated into the region containing high MW complexes (Figure 4A). Examination of CCUGn RNA distribution within the fractions showed that CUGBP1 colocalized with CCUGn repeats (Figure 4A). Such co-localization suggests that CUGBP1 is shifted to the high MW complexes due to binding to CCUG repeats. This shift was specific because CCUG repeats did not change the localization of β-actin (Figure 4A).

We performed additional studies, using tissue culture and a CCTG TR mouse model (see *Materials and Methods*) to characterize interactions of CUGBP1 complexes with the CCUG repeats. To this end, we used two systems: C2C12 myoblasts transfected with plasmid expressing 300 CCUG repeats, and the livers from TR mice expressing non-coding $CCUG_{121}$ RNA. Cytoplasmic extracts from C2C12 myoblasts transfected with an empty vector or with long CCTG repeats were separated by size exclusion chromatography, and CUGBP1 and eIF2α were examined in the chromatography fractions using UV cross-link assay (for CUGBP1) and Western blotting (for CUGBP1 and eIF2α). As shown in Figure 4B, in C2C12 myoblasts transfected with an empty vector, CUGBP1 and eIF2α were located in the regions containing free proteins and low MW complexes. However, in C2C12 myoblasts expressing RNA $CCUG_{300}$, significant amounts of CUGBP1 and eIF2α were shifted to the fractions containing high molecular weight complexes. CUGBP1 RNA-binding activity was also shifted to the fractions containing high MW complexes (Figure 4B, UV cross-link). eIF2α and CRT (data for CRT not shown) co-localized with CUGBP1 in C2C12 myoblasts, transfected with an empty vector, and they were shifted together with CUGBP1 into fractions containing high MW complexes in CCUG-expressing C2C12 myoblasts (Figure 4B, Western). Blot hybridization of RNA isolated from the chromatography fractions with a $CAGG_{16}$ probe showed that CUGBP1-eIF2 complexes are located in the fractions containing CCUG RNA (Figure 4B, hybridization). Thus, ectopically expressed mutant CCUG RNA binds to the CUGBP1-eIF2 complex.

We next studied the effects of CCUG repeats on CUGBP1 in the cytoplasm of CCTG TR mice. Since expression of CUGBP1 is increased in DM2 (Figure 3C), we first examined protein levels of CUGBP1 in CCTG TR mice and found a threefold elevation of CUGBP1 in the livers of these mice (Figure 4C). Because DM2 myoblasts have increased amounts of complex 3 (Figure 1I), we examined this complex by size exclusion chromatography in wild-type and in CCTG TR mice. EMSA analysis of the chromatography fractions demonstrated that wild-type livers have limited amounts of complex 3. However, the complex was abundant in the cytoplasm of CCTG TR mice (Figure 4D, top). Examination of the gel filtration fractions by Western blotting showed that CUGBP1 and eIF2α in wild-type livers were located in fractions containing free proteins and low MW complexes. Quite a different distribution of these proteins was observed in CCTG TR mice. We found that more than 50% of CUGBP1 and

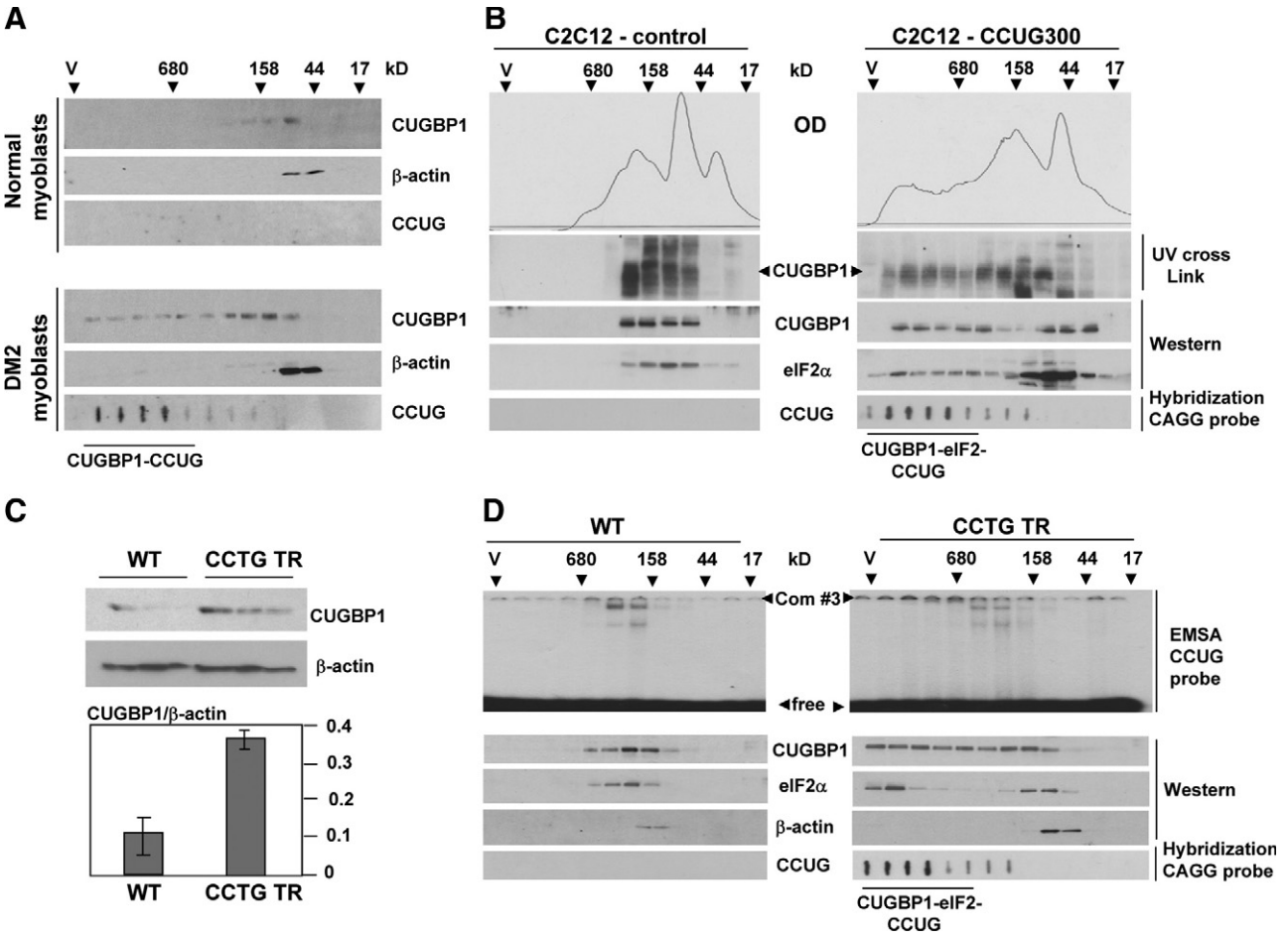

Figure 4. CUGBP1 is associated with the expanded CCUG repeats in the cytoplasm of DM2 myoblasts and in cytoplasm of livers of CCTG TR mice. **A:** CUGBP1 is bound to the mutant CCUG RNA in cytoplasm of DM2 myoblasts. Cytoplasmic proteins from myoblasts derived from unaffected (normal) patients and patients with DM2 were fractionated by gel filtration. Western blot analysis of gel filtration fractions was performed with antibodies to CUGBP1 and β-actin. RNA from the same fractions was examined by blot hybridization with $CAGG_{16}$ probe. **B:** Ectopically expressed $CCUG_{300}$ RNA binds to the CUGBP1-eIF2 complex. Cytoplasmic proteins from C2C12 myoblasts transfected with an empty vector (control) and from cells transfected with a plasmid expressing $CCUG_{300}$ were fractionated on size exclusion column SEC400. Optical density (OD_{280}) profiles of gel filtrations are shown on the top. Gel filtration fractions were examined by UV cross-link with $CCUG_{16}$ probe, by Western blotting with CUGBP1 and eIF2α antibodies, and by blot hybridization with $CAGG_{16}$ probe (CCUG). The position of the CUGBP1-eIF2 complex associated with the CCUG repeats is shown below. **C:** CUGBP1 levels are increased in the livers of CCTG-transgenic mice. Western blot analyses of cytoplasmic liver extracts from three wild-type (WT) and three CCTG TR mice were performed with antibodies to CUGBP1 and β-actin. Bar graphs show the levels of CUGBP1 as ratios to β-actin. **D:** Complex 3 is abundant in the livers of CCTG-transgenic mice. Cytoplasmic proteins from wild-type and CCTG TR mice were fractionated on SEC400 column. **Top** image shows EMSA with $CCUG_{16}$ probe, which located complex 3 within gel filtration fractions. The positions of complex 3 and free probe are shown by **arrowheads**. Western: Gel filtration fractions were analyzed by Western blotting with antibodies to CUGBP1, eIF2α and β-actin (control). **Bottom** image shows results of blot hybridization of the fractions with $CAGG_{16}$ probe.

eIF2α were shifted to fractions containing very high MW complexes. Blot hybridization of RNA from the chromatography fractions showed that the CUGBP1-eIF2 complex co-localized with CCUGn RNA in the livers of transgenic mice. Examination of β-actin in the fractions shows that the translocation of CUGBP1 and eIF2α was specific, since the location of β-actin in size exclusion fractions was not changed on expression of CCUGn RNA. Thus, these studies demonstrated that the mutant CCUG repeats target complex 3, which consists of CUGBP1-eIF2 and 20S proteasome (see below).

The 20S Catalytic Core Complex of the Proteasome Interacts with CCUG Repeats

Chromatography on the CHT1 column separated the CUGBP1-eIF2 complex (3–1) from complex 3–2 (Figure 2). CUGBP1 and eIF2α were no longer detectable within complex 3–2 suggesting that this complex is a new CCUG-binding complex. The purified complex 3–2 was subjected to mass spectroscopy, which identified five subunits of the 20S catalytic core complex of the proteasome within this complex (Figure 5A). To confirm the identity of the proteins, the purified complex was tested by Western blotting with antibodies to subunits of the 20S proteasome. We found that α2, α4, and α7 subunits were detected within the complex, while HuR (control) was not observed in the complex (Figure 5B). In addition to 20S proteasome, mass spectroscopy analysis identified BiP, Hsp70, Disulfide Isomerase, isovaleryl coenzyme A dehydrogenase and delta aminolevulinate dehydrogenase (data not shown). To examine if Hsp70 is associated with the proteasome *in vivo*, we immunoprecipitated the proteasome from the cytoplasmic extracts of livers from old

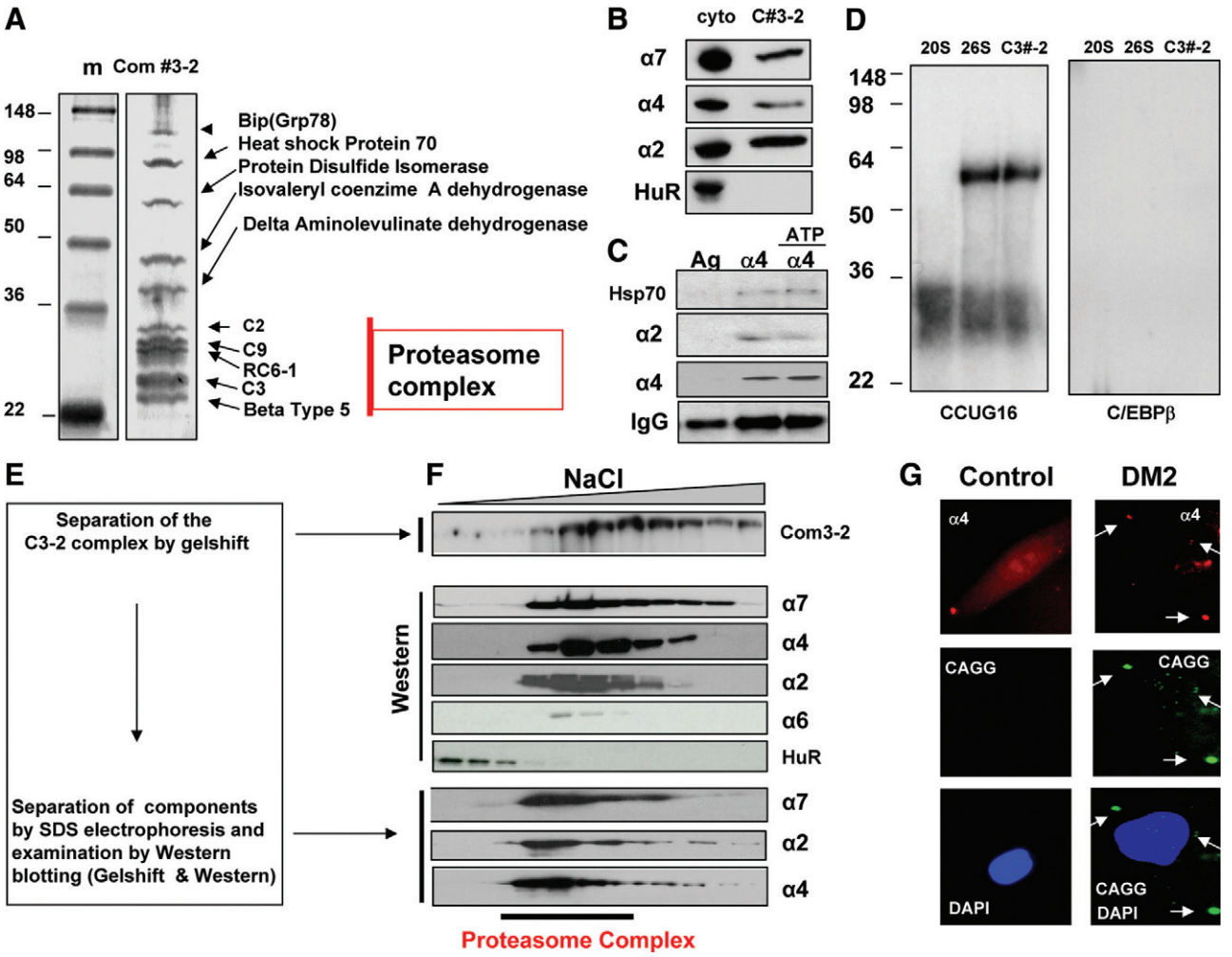

Figure 5. Complex 3–2 contains the 20S catalytic core of the proteasome. **A:** Coomassie staining of the complex 3–2 after CHT1 column. Identity of proteins within the complex was determined by mass spectroscopy. Positions of protein markers are shown on left. **B:** The purified CCUG-binding complex 3–2 contains 20S proteasome. The purified complex was tested by Western blot assay with antibodies against subunits α2, α4 and α7 of the 20S proteasome. Cytoplasmic protein extract from C2C12 myotubes (cyto) and anti-HuR were used as controls. **C:** Hsp70 protein is associated with the proteasome complex. Proteasome complex was immunoprecipitated from the cytoplasm of old mouse livers by Abs to the α4 subunit of the 20S proteasome and Hsp70 was examined in this IP by Western blotting. Third lane shows Hsp70 in the α4-IP from cytoplasmic liver extract treated with ATP. IgG; heavy chains of IgGs used for IP. The filter was re-probed with Abs to α2 and α4 subunits of the 20S proteasome. **D:** Complex 3–2 directly binds to RNA CCUG repeats. Left image: UV cross-link assay of the 20S and 26S proteasomes (BioMol International, LP) and the complex 3–2 with RNA $CCUG_{16}$. Right image shows the UV cross-link of these complexes with C/EBPβ RNA probe (negative control). **E** and **F:** Complex 3–2 contains proteins of 20S proteasome. Proteins were fractionated by ion exchange chromatography and fractions were examined by gel shift assay with $CCUG_{16}$ probe (upper) and by Western blot with antibodies against α2, α4, α6, and α7 subunits of the 20S proteasome (middle). The fragment of gel containing the complex 3–2 was cut out, loaded onto a 4 to 20% polyacrylamide gel and examined by Western blotting with antibodies against α2, α4 and α7 subunits of the 20S proteasome. **G:** RNA CCUG repeats bind with the 20S core complex in DM2. FISH hybridization with $CAGG_{16}$ probe (green) was performed using myoblasts from DM2 patients and normal control myoblasts following immunofluorescent analyses (top) with antibodies against the α4 subunit of the 20S proteasome. The positions of the proteasome associated with CCUG repeats in the cytoplasm are shown by **arrows**.

mice with antibodies to the α4 subunit, and probed these α4 IPs with antibodies to α2 and Hsp70. Figure 5C shows that Hsp70 is associated with the subunits α2 and α4 of the proteasome. It has been shown that Hsp70 might be associated with the proteasome through misfolded proteins, a process that is regulated by ATP.[24] Therefore, we examined if the addition of ATP releases Hsp70 from the proteasome complex. ATP was added to the protein extracts, the proteasome was precipitated with antibodies to α4, and the presence of Hsp70 was determined by Western blot. As seen in Figure 5C, ATP did not release Hsp70 from the complex. Thus, Hsp70 seems to be associated with the proteasome via specific interactions, which do not involve unfolded proteins. It is currently unclear whether the remaining proteins are associated with the 20S proteasome, or whether they represent an additional complex interacting with RNA $CCUG_{16}$. Thus, the major component of the purified CCUG-binding protein complex 3–2 is the 20S proteasome.

To further determine relationships of the complex 3–2 with CCUG repeats, we asked if complex 3–2 directly interacts with RNA CCUG repeats. Since the 20S proteasome is part of the larger 26S complex, we have also analyzed commercially available human 20S and 26S proteasomes (Biomol International, LP) by UV cross-link with RNA $CCUG_{16}$ riboprobe. The results of these experiments showed strong binding of several proteins located in the position of the purified 20S proteasome (Figure

5D). Additional CCUG RNA binding protein was observed in the position of 60 kd in both the 26S proteasome (BioMol International, LP) and the purified CCUG-binding complex 3–2. The interaction of the proteasome complex with CCUG repeats was specific, since a similar UV cross-link assay with the C/EBPβ riboprobe showed no detectable interactions (Figure 5D, right image). Thus, these data show that purified complex 3–2 and commercially available 20S and 26S proteasomes directly interact with RNA CCUG repeats.

To obtain additional evidence that the 20S proteasome within complex 3–2 binds to CCUG repeats, complex 3–2 was separated by ion exchange chromatography and localized within fractions by EMSA assay with $CCUG_{16}$ probe (Figure 5E). The localization of individual components of the 20S proteasome was determined by Western blotting with antibodies against the 20S core complex. As can be seen (Figure 5F), fractions possessing CCUG-binding activity contain α2, α4, α6, and α7 subunits of the 20S catalytic core of the proteasome. This co-localization suggests that the 20S proteasome binds to CCUG repeats. To verify this co-localization, the region of the gel containing complex 3–2, was cut out, proteins were resolved by denaturing gel, transferred on the membrane and probed with antibodies to components of the 20S proteasome (Figure 5F; Gelshift & Western). As seen, the subunits α2, α7, and α4 are the components of complex 3–2.

To investigate interactions of the 20S complex with RNA CCUG repeats in DM2 myoblasts, we have applied a combination of FISH assay with CAGG probe and immunofluorescence with antibodies against the α4 subunits of the 20S core proteasome complex. Results of these experiments revealed that the 20S proteasome is associated with RNA CCUG repeats in the cytoplasm of DM2 myoblasts (Figure 5G). Taken together, these studies demonstrated that RNA CCUG repeats interact with the 20S proteasome and that expanded CCUG repeats are associated with the 20S proteasome in cytoplasm of DM2 myoblasts.

Inhibition of Protein Degradation in DM2 Myoblasts

Given the association of the 20S proteasome complex with CCUG repeats in DM2 myoblasts (Figure 5G), we suggested that this association might affect the amounts and activities of the 20S proteasome in DM2 myoblasts. To test this suggestion, we examined the 20S proteasome in DM2 myoblasts and determined the stability of proteins that are degraded by the proteasome. Because Hsp70 is a part of the 20S proteasome complex that binds to the CCUG repeats, the proteasome was immunoprecipitated with antibodies to α4 from cytoplasmic extracts of control and DM2 myoblasts, and the IPs were probed with antibodies to Hsp70. Figure 6A shows that Hsp70 was observed in the proteasome complex in DM2 myoblasts, while normal myoblasts do not have detectable amounts of Hsp70 in the proteasome. As mentioned above, the association of Hsp70 with the proteasome might be mediated through misfolded proteins and this type of association might be disrupted by ATP.[24] Therefore, ATP was added to the protein extracts before precipitation of the proteasome. We found that the addition of ATP to the protein extracts slightly reduced the association of Hsp70 with the proteasome; however, a significant portion of Hsp70 remains in the complex. Therefore, a significant portion of Hsp70 is associated specifically with the 20S proteasome, but not through misfolded proteins. Given the association of the 20S proteasome complex with CCUG repeats in DM2 (Figure 5G), we examined levels of short-lived proteins such as p21, c-*myc* and CUGBP1 by Western blotting. These studies showed that these three proteins are elevated in myoblasts derived from DM2 patients (Figure 6B). To examine if alterations in the activity of the proteasome in DM2 myoblasts are involved in the elevation of the short-lived proteins, we have used the proteasome inhibitor MG132. In control myoblasts derived from unaffected patients, inhibition of the proteasome by MG132 significantly increased protein levels of c-*myc* and p21, while the effect of MG132 on CUGBP1 levels was minor, perhaps, due to the relatively longer half-life of CUGBP1 (3.5 hours). On the contrary, incubation of DM2 myoblasts with MG132 did not lead to an elevation of these proteins (Figure 6C). We suggest that the lack of MG132-dependent stabilization of these proteins in DM2 myoblasts reflects the fact that the biological functions of the proteasome are already inhibited by titration to the expanded CCUG repeats.

An important step of proteasome-mediated degradation of proteins is the formation of ubiquitin conjugates of the degraded proteins.[25] Therefore, we looked at ubiquitin conjugates in DM2 myoblasts. Control and DM2 myoblasts were treated with MG132 for 4 hours and proteins were examined by Western blotting with antibodies to ubiquitin. Figure 6D shows that treatment of control myoblasts with MG132 leads to increased amounts of high MW ubiquitin-protein conjugates. In contrast to normal myoblasts, untreated DM2 myoblasts already contained large amounts of ubiquitin-protein conjugates and the treatment with MG132 only slightly increased amounts of these conjugates. Free ubiquitin was not detectable in human myoblasts under sensitivity of our assay. To determine whether DM2 myoblasts accumulate ubiquitin conjugates of specific proteins, p21 was immununoprecipitated from untreated and treated control and DM2 myoblasts, and p21 IPs were probed with antibodies to ubiquitin. In control myoblasts treated with MG132, a mono-ubiquitin form of p21, migrating in the position of 30 kd, was dramatically increased. In contrast, untreated and MG132 treated DM2 myoblasts contained mono- and polyubiquitinated forms of p21 (Figure 6E). These data show that proteasome function is inhibited in DM2 myoblasts and that this inhibition leads to the accumulation of ubiquitin-protein conjugates.

We next compared the half-life of c-*myc*, p21 and CUGBP1 in control and in DM2 myoblasts. Protein

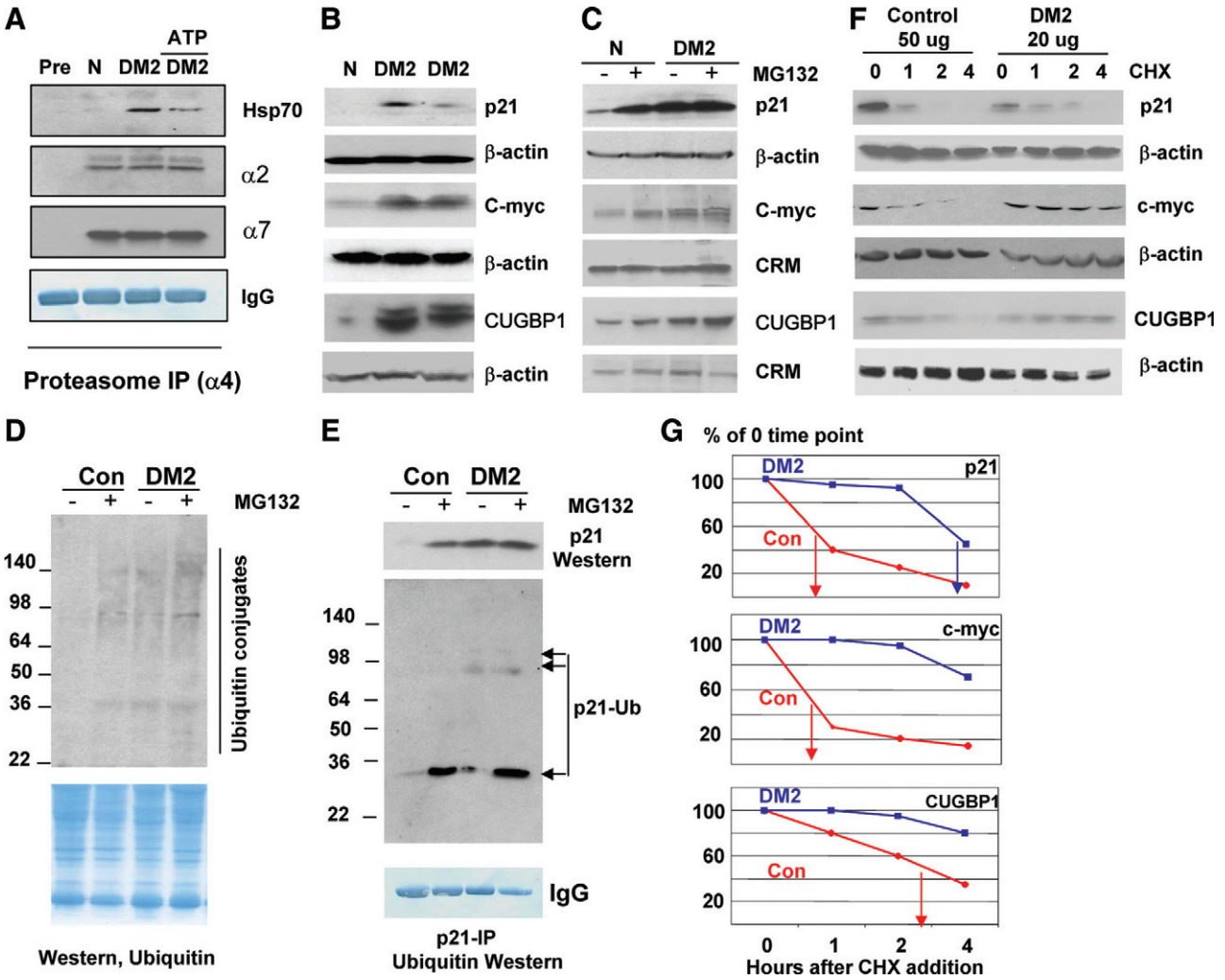

Figure 6. Proteasome activity is inhibited in DM2 myoblasts leading to the increased stability of short-lived proteins in DM2. **A:** Hsp70–20S proteasome complex is increased in DM2 myoblasts. Proteasome was immunoprecipitated from myoblasts derived from unaffected (N) and DM2 patients and probed with Abs to Hsp70 (upper panel). Two middle panels show examination of α2 and α7 subunits of the 20S proteasome within α4 IPs. Bottom panel shows Coomassie staining of heavy chains of immunoglobulins (IgGs) used for IP. First lane in all panels is a control with preimmune serum. Fourth lane in all panels shows the results of IP-Western with cytoplasmic extract from DM2 myoblasts containing ATP. **B:** Western blot analyses of control (N) and DM2 myoblasts with antibodies to p21, c-*myc* and CUGBP1. Protein loading was verified by Western blotting with antibodies against β-actin. **C:** The activity of proteasome is inhibited in DM2 myoblasts. Control and DM2 myoblasts were treated with MG132 and protein levels of p21, c-*myc* and CUGBP1 were examined. β-actin or cross-reactive molecule (CRM) signals show equal protein loading. **D:** Amounts of ubiquitin-protein conjugates are increased in DM2 myoblasts. Total protein extracts from untreated control and DM2 myoblasts and myoblasts treated with MG132 were examined by Western blotting with antibodies to ubiquitin. **Bottom** image shows a Coomassie staining of the filter. **E:** Amounts of p21-Ub conjugates are increased in DM2 myoblasts. Upper image shows levels of p21 in extracts used for detection of ubiquitin-p21 conjugates. Middle image: p21 was immunoprecipitated from control myoblasts derived from unaffected patients and from DM2 myoblasts and p21 IPs were probed with antibodies to ubiquitin. **Bottom** image shows Coomassie stain of heavy chains of immunoglobulins (IgG) after analysis of p21-IPs. **F:** Stability of p21, c-*myc* and CUGBP1 proteins is increased in DM2 myoblasts. Control and DM2 myoblasts were treated with cycloheximide to inhibit protein translation. Proteins were analyzed by Western blotting assay with antibodies against p21, c-*myc* and CUGBP1. 20 μg of proteins from DM2 myoblasts and 50 μg of proteins from control myoblasts were used to compensate for the increase of p21, c-*myc* and CUGBP1 in DM2. Membranes were re-probed with anti-β-actin to verify protein loading. **G:** Protein levels of p21, c-*myc*, and CUGBP1 were calculated as ratios to β-actin in control (red) and DM2 (blue) myoblasts. The picture shows percent (*y* axis) of each protein at 1, 2, and 4 hours (*x* axis) after addition of cycloheximide relative to 0 time point.

synthesis was blocked by cycloheximide and protein levels of c-*myc*, p21 and CUGBP1 were analyzed at different time points after addition of cycloheximide. As shown in Figure 6, F and G, stability of the examined proteins was significantly higher in DM2 myoblasts than in control myoblasts derived from unaffected patients. Thus, the stability of short-lived proteins is increased in DM2 myoblasts. These data are consistent with the hypothesis that CCUG-mediated sequestration of the 20S proteasome leads to impaired turnover of the proteins in DM2 myoblasts.

Expression of CCUG Repeats in Normal Myoblasts Alters Translation and Degradation of Proteins Similar to Alterations Observed in DM2 Myoblasts

Given the observations that DM2 myoblasts have altered translation of CUGBP1 targets and that stability of short-lived proteins is increased in DM2 myoblasts, we examined if ectopic expression of RNA CCUG repeats in C2C12 myoblasts and in human myoblasts from unaf-

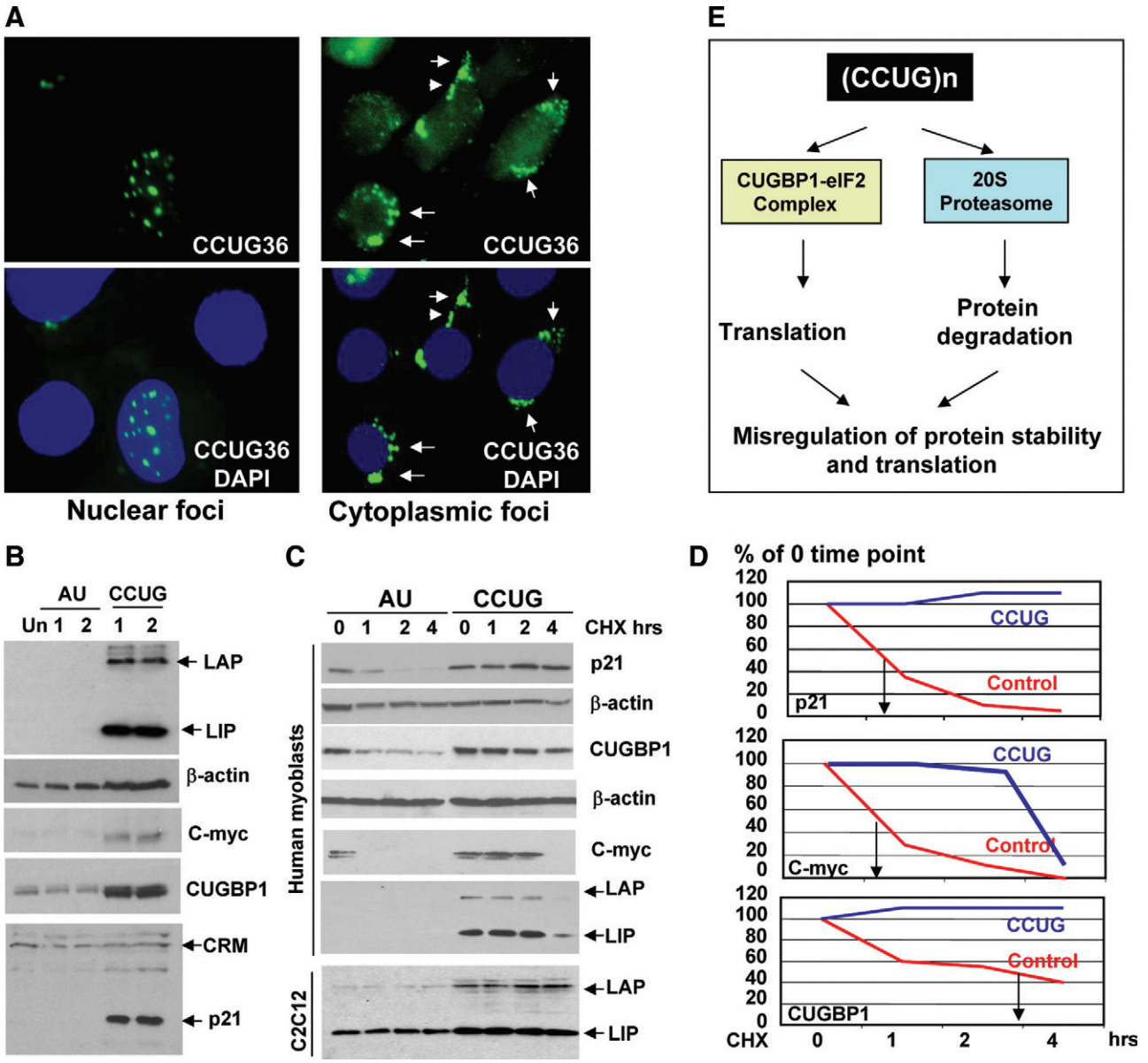

Figure 7. Expression of CCUG RNA repeats in control myoblasts from unaffected patients and from C2C12 myoblasts increases the levels of C/EBPβ isoforms and stabilizes c-*myc*, p21 and CUGBP1 proteins. **A:** Transfected CCUG RNA is located in nuclei and in cytoplasm. Upper. C2C12 myoblasts were transfected with a plasmid expressing $CCUG_{36}$ repeats. FISH hybridization with $CAGG_{16}$ probe (green) was performed. Cytoplasmic CCUG RNA is shown by **arrows**. Bottom images show a merge of nuclei stained with DAPI (blue) and CCUG RNA. **B:** Protein levels of C/EBPβ, p21, c-*myc* and CUGBP1 are increased in human primary myoblasts derived from unaffected patients transfected with RNA CCUG repeats. Western blotting was performed with total protein extracts isolated from control human myoblasts transfected with RNA $CCUG_{16}$ and with control AU_{42} RNA. Un; protein extracts from un-transfected cells. **C:** CCUG repeats increase the half-life of p21, c-*myc* and CUGBP1. Control human myoblasts were transfected with control AU-rich RNA (AU_{42}) and $CCUG_{16}$ RNA, protein synthesis was blocked by cycloheximide and protein levels of p21, c-*myc*, CUGBP1 and C/EBPβ were determined. The bottom part shows a similar experiment in mouse C2C12 myoblasts containing higher levels of C/EBPβ isoforms LAP and LIP. **D:** Bar graphs show the half-life of the proteins calculated as a percentage of the 0 time point. **E:** A hypothetic model suggesting the role of RNA CCUG repeats in the disruption of protein synthesis and protein degradation in DM2 muscle.

fected patients would cause similar alterations. Examination of CCUG repeats in C2C12 myoblasts transfected with $CCUG_{36}$ by FISH assay showed that, similar to the distribution of the CCUG repeats in DM2 myoblasts (Figure 1), CCUG repeats were detected in the cytoplasm and in the nucleus (Figure 7A).

We next examined if the expression of CCUG repeats in myoblasts from unaffected patients increases the translational activity of CUGBP1 toward a known target, C/EBPβ. A single C/EBPβ mRNA produces mainly two isoforms, liver enriched activator protein (LAP) and liver enriched inhibitory protein (LIP), and the CUGBP1-eIF2 complex increased translation of both these isoforms in an *in vitro* translation system (Figure 2G). Western blotting experiments showed that the levels of LAP and LIP isoforms were undetectable in human normal myoblasts, transfected with control AU_{42} RNA, while normal myoblasts transfected with CCUG RNA had increased levels of LAP and LIP (Figure 7B). Because LIP can be produced only by alternative translation from the third AUG codon,[26] these data show that the induction of C/EBPβ isoforms by

CCUG RNA is mediated through translational mechanisms. Analysis of C/EBPβ stability in the presence of CCUG repeats confirmed this conclusion (Figure 7C).

We next determined the levels of short-lived proteins in human myoblasts from unaffected patients transfected with CCUG repeats. We found that the transient expression of CCUG repeats increased protein levels of c-*myc*, CUGBP1 and p21 in normal myoblasts (Figure 7B). Examination of the half-lives of c-*myc*, p21 and CUGBP1 in normal myoblasts transfected with AU_{42} RNA and in cells expressing CCUG repeats revealed that CCUG repeats stabilized these proteins (Figure 7, C and D). To verify if the CCUG-mediated induction of C/EBPβ isoforms (LAP and LIP) is mediated through a translational mechanism, we tested whether the inhibition of proteasome activity contributes to the increase of C/EBPβ isoforms. We found that myoblasts from unaffected patients contain very low amounts of LAP and LIP, while LAP and LIP levels were increased in the presence of CCUG repeats. Our data show that, in human myoblasts, LAP and LIP are relatively stable proteins, which were reduced at 4 hours after inhibition of protein synthesis (Figure 7C). Since control human myoblasts express low amounts of LAP and do not express LIP, we also examined the stability of C/EBPβ isoforms in C2C12 myoblasts that express both LAP and LIP. We found that, in C2C12 myoblasts, LAP and LIP were more stable and were not affected by inhibition of protein synthesis within 4 hours. Similar to human myoblasts from unaffected patients, expression of CCUG repeats in C2C12 myoblasts increased amounts of both isoforms of C/EBPβ but did not affect the half life of these isoforms. Taken together, these data show that accumulation of CCUG repeats in normal myoblasts leads to alterations in the translation of CUGBP1 targets and in stabilization of proteins that are degraded by proteasome. These alterations mimic changes observed in DM2 myoblasts and confirmed that the expansion of CCUG repeats in DM2 myoblasts is responsible for the alterations in protein turnover (Figure 7E).

Discussion

CCUG Repeats Inhibit Biological Activities of the 20S Proteasome Complex

A number of observations suggest that pathological alterations in DM1 and DM2 are caused by accumulation of RNA CUG (DM1) and CCUG (DM2) repeats. In DM1, soluble CUG repeats (outside of intranuclear foci) increase the protein levels of CUGBP1, affecting cytoplasmic (translation) and nuclear (splicing) functions of CUGBP1. Here we present evidence showing that, in DM2 patients, CCUG repeats alter protein degradation and protein translation through binding to cytoplasmic multiprotein complexes. We have identified a translational CUGBP1-eIF2 complex and a complex containing the 20S catalytic core of the proteasome as direct targets of the CCUG repeats. Identification of the 20S proteasome complex as a complex that interacts with RNA CCUG repeats was quite surprising and prompted us to examine the ability of commercially available proteasome to interact with RNA containing CCUG repeats. We found that the interaction of the purified proteasome complex and commercially available proteasomes with RNA CCUG repeats is specific since these complexes do not interact with control RNA (Figure 5D). Although precise mechanisms of the interactions of the proteasome with CCUG repeats are not known, our UV cross-link study suggests that this interaction might be direct. Among several additional proteins within the proteasome complex, we have observed a molecular chaperone Hsp70. We found that a major portion of Hsp70 is associated with the 20S proteasome complex independent of binding of unfolded proteins. Intranuclear inclusions containing proteasome are found in polyglutamine disorders[27]; however, in polyglutamine diseases the proteasome complexes interact with misfolded glutamine-containing proteins. HSP40, ubiquitin and the 20S core complex of the proteasome have also been identified within the inclusions of the brains of mice expressing RNA CGG repeats.[28] Data presented in this manuscript suggest that RNA CCUG repeats directly bind to the proteasome in DM2 myoblasts and inhibit proteasome function.

CCUG Repeats Interact with the CUGBP1-eIF2 Complex and Affect Translational Targets of the Complex

Another complex interacting with CCUG repeats is the translational CUGBP1-eIF2 complex. CUGBP1 promotes cap-dependent translation through interaction with the eukaryotic initiation complex eIF2.[17] We have found that the CUGBP1-eIF2 complex binds to the mutant CCUG repeats in the cytoplasm of DM2 myoblasts, in the livers of CCTG transgenic mice and in C2C12 myoblasts that ectopically express $CCUG_{300}$. Consistent with the translational activity of the CUGBP1-eIF2 complex, the levels of CUGBP1-dependent translational targets, such as MEF2A and C/EBPβ, are increased in primary myoblasts and in mature muscle biopsies derived from patients with DM2. This result suggests that the increase of CUGBP1 in the cytoplasm of DM2 muscle cells might misregulate translation of additional unknown mRNAs, regulated by CUGBP1.

Cytoplasmic CCUG Repeats Cause Alterations in the Protein Turnover

RNA CCUG repeats in DM2 are located within intron 1 of ZNF9 pre-mRNA, which should be spliced out before export of ZNF9 mRNA from the nucleus to the cytoplasm. Surprisingly, we have observed significant amounts of CCUG repeats in the cytoplasm of DM2 myoblasts. Since splicing of ZNF9 is not affected in DM2 patients,[29] we hypothesize that CCUG repeats in the cytoplasm of DM2 myoblasts might be the products of the degradation of the CCUG expansion. Consistent with cytoplasmic localization of the CCUG repeats, CCUG-binding multiprotein complexes were detected in the cytoplasm of DM2 myo-

blasts. Our experiments with induced expression of CCUG repeats in myoblasts from unaffected patients and in mouse C2C12 myoblasts demonstrated the accumulation of CCUG repeats in the cytoplasm and in the nuclei. We also observed mutant CCUGn in the cytoplasm of the livers of CCTG transgenic mice. The accumulation of CCUG repeats alters the translation of the targets of the CUGBP1-eIF2 complex and increases the stability of c-*myc* and p21, which are targets of proteasome. In conclusion, our data suggest that expression of expanded CCUG repeats and their accumulation in the cytoplasm of DM2 muscle cells alter the biological functions of the CUGBP1-eIF2 and 20S core complexes, affecting global protein turnover.

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