Novel Role for RNA-binding Protein CUGBP2 in Mammalian RNA Editing

CUGBP2 MODULATES C TO U EDITING OF APOLIPOPROTEIN B mRNA BY INTERACTING WITH APOBEC-1 AND ACF, THE APOBEC-1 COMPLEMENTATION FACTOR

Srikant Anant, Jeffrey O. Henderson, Debnath Mukhopadhyay, Naveenan Navaratnam, Susan Kennedy, Jie Min, and Nicholas O. Davidson

From the Departments of Internal Medicine and Pharmacology and Molecular Biology, Washington University Medical School, Saint Louis, Missouri 63110 and the Medical Research Council Molecular Medicine Group, Clinical Sciences Center, Imperial College School of Medicine, Hammersmith Hospital, London W12 0NN, United Kingdom

Mammalian apolipoprotein B (apoB) mRNA editing is mediated by a multicomponent holoenzyme containing apobec-1 and ACF. We have now identified CUGBP2, a 54-kDa RNA-binding protein, as a component of this holoenzyme. CUGBP2 and ACF co-fractionate in bovine liver S-100 extracts, and addition of recombinant apobec-1 leads to assembly of a holoenzyme. Immunodepletion of CUGBP2 co-precipitates ACF, and these proteins co-localize the nucleus of transfected cells, suggesting that CUGBP2 and ACF are bound in vivo. CUGBP2 binds apoB RNA, specifically an AU-rich sequence located immediately upstream of the edited cytidine. ApoB RNA from McA cells, bound to CUGBP2, was more extensively edited than the unbound fraction. However, addition of recombinant CUGBP2 to a reconstituted system demonstrated a dose-dependent inhibition of C to U RNA editing, which was rescued with either apobec-1 or ACF. Antisense CUGBP2 knockout increased endogenous apoB RNA editing, whereas antisense knockout of either apobec-1 or ACF expression eliminated apoB RNA editing, establishing the absolute requirement of these components of the core enzyme. These data suggest that CUGBP2 plays a role in apoB mRNA editing by forming a regulatory complex with the three components of the minimal editing enzyme, apobec-1, ACF, and apoB RNA.

Post-transcriptional C to U RNA editing of apolipoprotein B (apoB) creates an in-frame stop codon in the edited transcript that in turn results in translation of a truncated protein, apoB48 (1–4). ApoB mRNA editing takes place in mammalian small intestine and generates a protein species that participates in dietary lipid absorption yet functions in lipoprotein uptake in a metabolically distinct manner from the full-length protein, apoB100, which is generally secreted by the liver (5). Accordingly, C to U RNA editing of apoB plays an important physiological role in mammalian lipoprotein metabolism.

Efficient deamination of the targeted cytidine requires trans-acting factors whose expression and distribution have been the subject of much interest (6–9). Expression of the catalytic subunit of the editing enzyme, apobec-1, is restricted to intestinal epithelial cells in humans, whereas it is widespread in rodents (10–13). Computer modeling studies, based on structural homology, as well as direct biochemical evidence suggest that apobec-1 is a dimer with the composite active site assembled through the interaction of each monomer (14, 15). In addition, apobec-1 is an RNA-binding protein that binds to the consensus sequence UUUN(A/U)U, located within the terminal loop in apoB RNA immediately downstream of the edited base and spanning the 5’ end of the mooring sequence (16–18). apobec-1 also binds to AU-rich sequence elements in the 3’-untranslated region of several other mRNAs including c-Myc, tumor necrosis factor-α, interleukin-2, and granulocyte-macrophage colony-stimulating factor (18). This apparently broad substrate binding activity, coupled with the observation that overexpression of apobec-1 in the livers of transgenic mice and rabbits results in hepatocellular carcinomas in association with promiscuous editing of other RNAs (19–22), implies that other factors may constrain apobec-1 under physiological conditions to direct its site selection to a single target in apoB mRNA.

apoB-1 is essential but not sufficient for apoB editing activity, there being a requirement for other protein factor(s) (7, 13, 23). Recently, two groups have independently identified a 65-kDa protein (ACF/ASP) that, when added with apobec-1, reconstitutes editing of an apoB RNA template in vitro (24, 25). Data from several laboratories have now confirmed that ACF and apobec-1 together represent the minimal core of the apoB RNA editing enzyme (26, 27). However, it is currently unknown whether other factors can function interchangeably in an editing reaction. Directly coupled to this uncertainty is the lack of conclusive information concerning the functional size of the glutamin; FITC, fluorescein isothiocyanate; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RRM, RNA recognition motif.

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helo-editing enzyme, one that might include both catalytic and regulatory components. Earlier studies demonstrated that apob RNA editing occurs in the context of a large, macromolecular 27 S editing complex or "editosome" that assembles on the apoB mRNA in vitro, suggesting that additional factors may play a role in the editing process (9). In the course of these studies, several proteins were identified, by their ability to bind either apob RNA or apobec-1 (7, 8, 26, 28–33). These include p60 and p40, which cross-link to apob RNA (7, 8, 34); GRY-RBP, ABPP-1, and hhRNPC-C, which interact with apobec-1; and AUX240, which is part of the proposed 27 S editosome complex (26, 28, 29, 31). Some of these factors have been proposed to augment editing activity (29, 31), whereas others appear to be inhibitory (26, 28, 33). Nevertheless, despite extensive examination of editing complexes isolated from both tissue and cell sources, there is no firm consensus concerning the composition of the intact helo-enzyme.

In the present study, we have cloned and identified a 54-kDa protein (CUGBP2) as an apobec-1-binding protein. CUGBP2 has been identified by various groups and demonstrated to play a role in regulating RNA splicing (35–37). We demonstrate that CUGBP2 interacts with apobec-1, ACF, and apob RNA both in vitro and in vivo and co-fractionsate with editing complementing activity in bovine liver S-100 extracts. Addition of GST-APOBEC-1 to bovine liver S-100 extracts resulted in formation of an apob RNA editing holoenzyme, which upon further fractionation was found to contain apobec-1, ACF, and CUGBP2. RNA binding studies demonstrated that CUGBP2 is an apob RNA-binding protein, which binds to an AU-rich sequence upstream of the edited cytidine. Furthermore, immunoprecipitation of CUGBP2 from rat hepatoma cells revealed the presence of co-precipitated apob RNA. In co-transfection experiments, CUGBP2 co-localized with apobec-1 and ACF in the cytoplasmic and nuclear compartments, respectively. Finally, anti-sense-mediated knock-out of CUGBP2 expression in rat hepatoma cells increased apob mRNA editing 3-fold. Taken together, the data suggest that CUGBP2 is a regulatory component of the apoB RNA editing holoenzyme. The evidence suggests that CUGBP2 acts to modulate editing either by binding to apobec-1 in the cytoplasm and restricting apobec-1 shutting to the nucleus and/or by binding to ACF and apob RNA in the nucleus and disrupting their functional interaction.

MATERIALS AND METHODS

Cloning and Expression of Recombinant Proteins—apobec-1 was expressed as a GST fusion protein as previously described (16, 38). Full-length CUGBP2 cDNA was cloned into plasmid pGEX-4T3 (Amersham Pharmacia Biotech) at the BamHI and SalI restriction sites, respectively and expressed as a GST fusion protein. ACF cDNA, isolated from HEK293 cells, was expressed as a GST fusion protein. ACF was purified as a GST fusion protein as previously described (16, 38). Full-length CUGBP2 cDNA was cloned into plasmid pCMV-Tag 2B and expressed as an NH2-terminal FLAG epitope-tagged fusion protein. ACF was cloned into the BamHI and XhoI sites of plasmid pCMV-Tag 2B and expressed as an NH2-terminal FLAG epitope-tagged fusion protein. The plasmids were transfected individually using FUGENE-6 transfection reagent (Roche Molecular Biochemicals) into COS-7 cells grown on coverslips. 48 h post-transfection, the cells were fixed in 3.7% formaldehyde and permeabilized with 0.5% Triton X-100. The FLAG and HA epitopes were used to detect the proteins using mouse α-FLAG M2 monoclonal (Stratagene) and rabbit α-HA Y-11 polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), respectively, followed by fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and by Cy3-conjugated mouse anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Apobec-1 and apob RNA immunoprecipitation was carried out using anti-HA-agarose. Gene silencing in HepG2 was performed with a pSilencer RNAi expression vector, which was provided by Dr. Mingwen Jiang (The Ohio State University, Columbus, OH). Western blot analyses were performed as described previously (26, 39).

Immunofluorescence Microscopy—CUGBP2 was cloned into plasmid pHOOK-2 (Invitrogen, Carlsbad, CA) at the HindIII and XhoI restriction sites for expression as a COOH-terminal HA epitope-tagged fusion protein. apobec-1 was cloned into plasmid pCMV-Tag 2B (Stratagene) at the BamHI and XhoI sites for expression as a NH2-terminal FLAG epitope-tagged fusion protein. ACF was cloned into the BamHI and XhoI sites of plasmid pCMV-Tag 2B and expressed as an NH2-terminal FLAG epitope-tagged fusion protein. The plasmids were transfected individually using FUGENE-6 transfection reagent (Roche Molecular Biochemicals) into COS-7 cells grown on coverslips. 48 h post-transfection, the cells were fixed in 3.7% formaldehyde and permeabilized with 0.5% Triton X-100. The FLAG and HA epitopes were used to detect the proteins using mouse α-FLAG M2 monoclonal (Stratagene) and rabbit α-HA Y-11 polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), respectively, followed by fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and by Cy3-conjugated mouse anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Apobec-1 and apob RNA immunoprecipitation was carried out using anti-HA-agarose. Gene silencing in HepG2 was performed with a pSilencer RNAi expression vector, which was provided by Dr. Mingwen Jiang (The Ohio State University, Columbus, OH). Western blot analyses were performed as described previously (26, 39).

Immunodepletion—Anti-CUGBP2 IgG was covalently coupled to N-hydroxysuccinimide-activated Sepharose 4B (Amersham Pharmacia Biotech) as directed by the manufacturer. Brieﬂy, the resin was washed with 15 bed volumes of ice-cold 1 mM HCl and mixed with 200 μg of α-CUGBP2 IgG in coupling buffer (0.2 M NaHCO3, 0.5 M NaCl, pH 8.3) for 4 h at room temperature with gentle rocking. Excess active groups were blocked by incubating with buffer A (0.5 mM ethanolamine, 0.5 mM NaCl, pH 8.3) for 4 h at room temperature with gentle mixing. The antibody-coupled resin was washed extensively and sequentially with buffer A and B (0.1 M sodium acetate, 0.5 M NaCl, pH 4.0) to disrupt any ionic interactions of the antibody with the resin. Subsequently, the resin was washed with buffer D (200 mM Hepes-HCl, pH 7.9, 20% glycerol, 0.1 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM benzamidine) and mixed overnight at 4 °C with 75 μg of Superdex-200 fraction 16 of bovine liver extract. After extensive washes, the proteins bound to the antibody in the column were eluted with 0.1 M glycine, pH 3.0. The eluant was immediately neutralized with 200 mM Tris-Cl, pH 8.0, and dialyzed against buffer D. The starting material, the unbound column flow-through, and the eluant were subjected to Western blot analysis using α-ACF (4–18) antibody (kind gift of Donna Driscoll (25)) and α-CUGBP2 IgG. The unbound column flow-through was also dialyzed against buffer D and assayed for complementation activity in the in vitro editing assay (38).

Western and Far Western Blotting—S–100 extracts or Superdex-200 fraction 16 of the unbound column flow-through was dialyzed against buffer containing 5% nonfat dry milk followed by sequential incubations with either α-CUGBP2 IgG, α-HSP40 (Santa Cruz Biotechnologies, Santa Cruz, CA), or α-ACF (4–18) antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.). The membranes were washed and incubated with 5% nonfat dry milk followed by sequential incubations with either α-CUGBP2 IgG, α-HSP40 (Santa Cruz Biotechnologies, Santa Cruz, CA), or α-ACF (4–18) antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.). The membranes were washed and incubated with 5% nonfat dry milk followed by sequential incubations with either α-CUGBP2 IgG, α-HSP40 (Santa Cruz Biotechnologies, Santa Cruz, CA), or α-ACF (4–18) antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.). The membranes were washed and incubated with 5% nonfat dry milk followed by sequential incubations with either α-CUGBP2 IgG, α-HSP40 (Santa Cruz Biotechnologies, Santa Cruz, CA), or α-ACF (4–18) antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.).
FIG. 1. CUGBP2 co-fractionates with ACF in Superdex-200 fractions of bovine liver S-100 extracts. A, cytoplasmic S-100 extracts were subjected to 30% ammonium sulfate precipitation and fractionated through a Superdex-200 column (Amersham Pharmacia Biotech) to enrich for C to U editing complementation activity. A graph of the protein levels in each fraction, determined by absorbance at 280 nm, is shown along with location of void volume ($V_o$) and peak positions of protein size markers. Fractions 14–16, which demonstrated enriched complementation activity, are shaded. Protein size markers include thyroglobulin ($M_{app}$, 66,900), ferretin ($M_{app}$, 440,000), IgG ($M_{app}$, 160,000), human transferrin ($M_{app}$, 81,000), ovalbumin ($M_{app}$, 43,000), and myoglobin ($M_{app}$, 17,600). B, identification of enriched complementation activity. 1/2H9262 g of each fraction was added to in vitro editing assays containing 250 ng of GST/APOBEC-1 and 20 fmol of 470-nucleotide rat apoB RNA. The RNA was extracted, and the fraction of edited apoB RNA was determined by primer extension analysis. Positive control (+/−) lane contains 10/2H9262 g of bovine liver S-100 extract subjected to 30% ammonium sulfate precipitation. Location of the edited (U), unedited (C), and primer (P) bands are shown to the right, and the percentage of editing is shown below each lane. This is representative of three such experiments. C, characterization of proteins. 5/2H9262 g of each fraction was subjected to size fractionation on a 12% SDS-PAGE gel and silver-stained. The presence of Bio-Rad molecular mass protein markers is shown to the left. D, Distribution of CUGBP2. Top panel, 50 μg of the 30% ammonium sulfate precipitate of bovine liver S-100 extracts (BL), 5 μg of each S200 fraction, and 10 ng recombinant CUGBP2 (C) was size fractionated and transferred to PVDF membrane. The blot was subjected to Western analysis with a rabbit α-CUGBP2 IgG, as described under “Materials and Methods.” CUGBP2 (arrow) runs as a doublet because of the presence of an internal methionine located immediately downstream of the first authentic methionine. The nature of the larger band (arrowhead) is currently unknown. Location of the 43- and 70-kDa protein molecular mass markers is shown to the left. This is representative of three such experiments. E, Distribution of ACF. 100 μg of bovine liver extracts, 25 μg of each fraction, and 10 ng recombinant ACF were subjected to Western blot analysis for presence of ACF with a rabbit α-ACF (4–18) antibody. Location of the ACF is shown by an arrow. The identity of the lower cross-reacting bands (arrowhead) is not known. This is representative of three such experiments. F, Far Western analysis reveals interaction between apobec-1 and CUGBP2. 50 μg of Superdex-200 fraction 16 of bovine liver extracts was separated in a 12% SDS-PAGE gel and blotted on to PVDF membrane. The proteins on the membrane were subjected to 12 cycles of denaturation-renaturation with guanidium hydrochloride and
were then subjected to chemiluminescence detection using luminol according to the manufacturer’s recommendations (Amersham Pharmacia Biotech). For Far Western analyses, the blotted proteins were de- natured in buffer containing 6 M guanidine HCl and renatured by washing 12 times in buffer D containing increasing 2-fold dilutions of guanidine HCl. The membranes were blocked overnight in buffer D containing 5% nonfat dry milk and 5% bovine serum albumin followed by incubation with in vitro translated 35S-CUGBP2 or 35S- apobec-1 at a final concentration of 5 x 10^6 cpm/mg in buffer D containing 2.5 mM MgCl2, 0.5% nonfat dry milk, 2% bovine serum albumin, and 0.1% Tween 20 for 18 h. The membranes were then subjected to Western blot analysis containing 2.5 mM MgCl2, 0.1% Tween 20, dried, and subjected to autoradiography.

In Vivo Association of CUGBP2 with apoB RNA—S-100 extracts from McArdle cells were prepared as previously described (38). 1 mg of extract was incubated with 5 μg of α-CUGBP2 IgG or preimmune serum and 200 units RNAsin (Promega, Madison, WI) at 4 °C for 60 min with agitation. The immune complex was precipitated by addition of 50 μl of protein A-agarose and washed twice with NET buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40), and the RNA was extracted with 250 μl of Trizol (Life Technologies, Inc.). The RNA was resuspended in water and used for first strand cDNA synthesis with random hexanucleotides and Moloney murine leukemia virus reverse transcriptase followed by PCR for apoB and, as control, GAPDH. The PCR primers used for these PCR reactions have been previously published with either 2.5 mM MgCl2, 0.5% nonfat dry milk, 2% bovine serum albumin, and 0.1% Tween 20 for 18 h. The membranes were subsequently washed in buffer containing 2.5 mM MgCl2, 0.1% Tween 20, dried, and subjected to autoradiography.

**RESULTS**

**Molecular Cloning of CUGBP2 as an apoB-1-interacting Protein**—To identify apoB-1-interacting factor(s), a yeast two-hybrid screen was performed upon a chicken intestinal cDNA library with apoB-1 as bait. Chicken intestinal cells were selected for library construction for the following reasons: (i) they have been previously identified to express robust complementation activity (42), and (ii) they lack apoB-1 (42, 43). This latter property is important because apoB-1 is known to homodimerize efficiently in the yeast two-hybrid system (15). A library containing 2 x 10^6 independent clones was screened and yielded three specific clones. Two of these clones yielded ACF and a homolog of ACF, GRY-RBP (26). 5’ and 3’ rapid amplification of cDNA ends was performed to isolate full-length cDNA for the third clone, which was determined to be 1,527 base pairs in length, encoding a 490-amino acid protein with a predicted molecular mass of 54.3 kDa (data not shown). This cDNA encoded CUGBP2, previously identified as a CUGRNA-binding protein whose expression was demonstrated in heart, muscle, and nervous system (44, 45). This protein has been identified in other publications as Brunol-3, ETR-3, and napor-2 (35, 37, 46). Based on the nomenclature suggested in the UniGene data base of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/UniGene), we refer to this protein as CUGBP2. We have subsequently isolated the human and murine forms of CUGBP2 cDNA, which show ~99% sequence identity between the three species (data not shown). The human cDNA was used for all the experiments reported in this manuscript. Examination of the UniGene data base (Hs. 211610) suggests that the transcript is ubiquitously expressed and predicts the cytogenetic position of the gene to be on chromosome 10p15–13 between intervals D10S189 and D10S191. Based on a Prosit search for protein domains, we determined that the protein has a similar structure to members of the elav family of RNA-binding proteins (47, 48), in particular, the presence of RNA recognition motifs (RRMs). CUGBP2 contains three RRMs and a linker region that separates the first two RRMs from the third RRM (44, 45).

**CUGBP2 Co-purifies with Complementation Activity**—To determine whether CUGBP2 is detectable in a tissue extract containing editing complementation activity, we turned to preparations of bovine liver S-100 extracts because we determined that this is a plentiful source of complementation activity. These liver extracts were subjected to 30% ammonium sulfate precipitation and subsequently fractionated by fast phase liquid chromatography over a Superdex-200 column (Fig. 1A). Each fraction was supplemented with GST/APOBEC-1 and assayed for complementation activity in an *in vitro* apoB RNA editing assay. Editing enrichment was observed in fractions 14–16 (Fig. 1, A and B). Based on the fractionation of protein standards, this complementation activity was calcu-
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lated to be in the broad size range of 45–120 kDa (Fig. 1A) (6, 8). Size fractionation on a 10% SDS-PAGE coupled with silver staining demonstrated the presence of proteins in the size range of 30–100 kDa in fractions 14–16 (Fig. 1C). To determine whether CUGBP2 was present in fractions containing complementation activity, Western blot analysis was performed using α-CUGBP2 IgG. The data suggest that CUGBP2 was present in fractions 13–16, with the highest levels observed in fractions 15 and 16 (Fig. 1D, top row). A doublet for CUGBP2 was observed in the Western blot that we speculate is accounted for by the presence of an internal AUG in CUGBP2 cDNA (data not shown). A similar doublet was observed when CUGBP2 cDNA was subjected to a coupled transcription/translation reaction in an in vitro system (data not shown). An additional band of ~60 kDa was also recognized in fractions 14 and 15, whose identity is currently unknown (Fig. 1D). We considered the possibility that these reactive bands may indicate the presence of homologs of CUGBP2 such as CUGBP, CELF-3, CELF-4, and CELF-5 (36, 37). However, RT-PCR using primers to the known gene products failed to reveal a product in bovine liver (data not shown), but the possibility exists that these may represent a currently unidentified homolog of this protein.

Given the importance of ACF in the apoB RNA editing enzyme, Western blot analysis was performed with the enriched complementation fractions using an α-ACF antibody (25). The starting material used for size fractionation, namely the 30% ammonium sulfate precipitate of bovine liver S-100 extracts, demonstrated a band (Fig. 1E) that appeared to be enriched in the starting material used for size fractionation, namely the 30% ammonium sulfate precipitation. Location of the primer (P), unedited (U), and edited (H) bands are shown to the left. This is representative of three such experiments.

To determine whether apobec-1 interacts with CUGBP2 in these enriched S-100 extracts, we performed a Far-Western analysis using radiolabeled apobec-1 as probe (Fig. 1F). Aliquots of fraction 16 were size-fractionated through a 12% SDS-PAGE gel and transferred to PVDF membranes. After 12 rounds of denaturation-renaturation, the membrane was probed with 35S-labeled apobec-1 followed by autoradiography. Four bands, ranging in size from 45 to 75 kDa were detected in the Far Western analysis, of which the 54-kDa CUGBP2 band was the most dominant (Fig. 1F). This was identified as CUGBP2 by Western blot analysis. apobec-1 also hybridized to identification of RNA editing. 1 µg of each fraction was added to the in vitro editing assay as mentioned above in Fig. 1, without the addition of any GST APOBEC-1. Positive control (lane +) contains 250 ng of GST/APOBEC-1 and 10 µg of bovine liver S-100 extract subjected to 30% ammonium sulfate precipitation. Location of the primer (P), unedited (U), and edited (H) bands are shown to the right, and the percentage of editing (%U) is shown below. This is representative of three experiments. C, distribution of GST/APOBEC-1. 250 ng of GST/APOBEC-1 (C) and 5 µg of each fraction was subjected to size fractionation and transferred to PVDF membrane. The blot was subjected to Western analysis with a rabbit α-apobec-1 antibody, as previously described (18). Location of GST/APOBEC-1 band is shown by an arrow. This is representative of three such experiments. D, distribution of CUGBP2. 5 µg of each S200 fraction and 10 ng of recombinant CUGBP2 (lane C) was size-fractionated and transferred to PVDF membrane. The blot was subjected to Western analysis with a rabbit α-CUGBP2 IgG. Location of CUGBP2 (arrow) and the larger unknown band (arrowhead) are shown to the right. The locations of the protein molecular mass markers are shown to the left. This is representative of three such experiments. E, distribution of ACF. The membrane used to detect CUGBP2 in D was stripped and subsequently subjected to Western blot analysis for presence of ACF with a rabbit α-ACF (4–18) antibody. Location of the ACF is shown by an arrow, and the molecular mass markers are shown to the left. This is also representative of three such experiments.

Fig. 2. CUGBP2 co-fractionates with a large apoB RNA editing complex. A, GST/APOBEC-1 (0.5 mg) was added to 30% ammonium sulfate precipitate of cytoplasmic S-100 extracts (5 mg) and fractionated through a Superdex-200 column as mentioned for Fig. 1 above. B,
a band of 65 kDa that was immunologically reactive with α-ACF antiserum, as well as an anti-ACF immunoreactive 45-kDa band (Fig. 1F) (25). The identity of the 45- and ~75-kDa proteins is currently unknown.

To further evaluate whether CUGBP2 and ACF can bind and form heteromers in the S-100 extracts, Far Western analysis was performed with 35S-labeled CUGBP2. Recombinant Histagged ACF and Superdex-200 fraction 16 were subjected to size fractionation in a 12% SDS-PAGE gel, transferred to PVDF membranes, and probed with 35S-labeled CUGBP2. Autoradiography showed a dominant band that corresponds to a band of 65 kDa that was immunologically reactive with α-ACF antibody (Fig. 1G), which was further confirmed by Western analysis of the blot with α-ACF antibody (Fig. 1G). Taken together, the data from Fig. 1 (F and G) demonstrate that CUGBP2 can interact with apobec-1 and ACF independent of the presence of apoB RNA.

Previous studies have demonstrated the formation of a large macromolecular apoB RNA editing complex, referred to by Smith and colleagues as an editosome (9, 28). To examine the composition and assembly of an intact holoenzyme in vitro, we added recombinant GST/APOBE-1 to a 30% ammonium sulfate precipitate of bovine liver S-100 extracts and performed molecular exclusion fractionation through Superdex-200. Each fraction was assayed for apoB RNA editing activity. The results of this fractionation experiment (Fig. 2, A and B) should be contrasted with the data in Fig. 1 (A and B). Upon supplementation of S-100 extracts with apobec-1 and fractionation through Superdex-200, apoB RNA editing activity was now observed in fractions 9–12 (Fig. 2, A and B). Based on the fractionation of protein standards, this editing activity was calculated to be in the size range of ~250–669-kDa (Fig. 2, A and B). Western blot analysis of these fractions demonstrated enrichment of GST/APOBE-1, CUGBP2, and ACF in these fractions (Fig. 2, C–E). Apobec-1 immunoreactivity was virtually confined to these editing competent fractions (Fig. 2C). The distribution further revealed a dramatic shift in ACF and, to a lesser extent, CUGBP2 to these fractions, consistent with their incorporation into a larger complex. It bears emphasis that in the absence of apobec-1, no CUGBP2 immunoreactivity was demonstrated in fractions 9–12 (Fig. 1D). Taken together, the data suggest that CUGBP2 may be an integral member of the native apoB mRNA editing enzyme complex.

**CUGBP2 Co-localizes with apobec-1 and ACF**—To pursue the interaction between CUGBP2 and either apobec-1 or ACF in *vivo*, we performed immunofluorescence studies in transfected cells. We chose to examine this question in transfected cells, because the abundance of these proteins in rat hepatoma cells or even tissues containing editing activity is below the level of detection by available antibodies (25, 27). CUGBP2 was expressed as a fusion protein tagged with the HA epitope, whereas apobec-1 and ACF were tagged with the FLAG epitope. First, the intracellular localization of CUGBP2, apobec-1, and ACF, when introduced alone, was determined in COS-7 cells. Indirect immunofluorescence staining for the epitope tag revealed both a nuclear and a cytoplasmic localization for both CUGBP2 (Fig. 3A) and apobec-1 (Fig. 3E), whereas ACF staining was predominantly nuclear (Fig. 3C). This distribution pattern was also observed following transfection of individual expression constructs into HepG2 or McArdle cells (data not shown).

To further evaluate whether CUGBP2 co-localizes with ACF or apobec-1, HA-tagged CUGBP2 was transiently overexpressed with either FLAG-tagged apobec-1 (Fig. 4) or with FLAG-tagged ACF (Fig. 5) in COS-7, HepG2, and McArdle cells. These three cell lines were selected because of informative differences in the expression of apoB mRNA, apobec-1, and ACF. For instance, COS-7 cells express neither apoB mRNA nor the editing factors apobec-1 and ACF (25, 26). HepG2 cells express apoB RNA and ACF but not apobec-1 and thus fail to edit endogenous apoB mRNA (26). McArdle cells are competent to edit endogenous apoB RNA and express low levels of both ACF and apobec-1 (26, 27, 30). Co-transfection of CUGBP2 and apobec-1 demonstrated a predominantly cytoplasmic staining pattern for CUGBP2 in all three cell lines (Fig. 4, A, E, and I). This pattern is different from the mixed nuclear-cytoplasmic distribution noted above (Fig. 3) and suggests that the cellular localization of CUGBP2 changes in the presence of apobec-1 to a more cytoplasmic distribution. apobec-1 also showed a predominantly cytoplasmic staining pattern when co-expressed with CUGBP2 (Fig. 4, B, F, and J), with confocal merged images demonstrating co-localization of the two proteins (Fig. 4, C, G, and K). An important caveat to the conclusions, however, is emphasized below.

When co-transfected with ACF, CUGBP2 demonstrated predominantly nuclear staining, particularly evident in COS-7 and McArdle cells (Fig. 5, A and I). This pattern contrasts with that noted in the single transfection of CUGBP2 noted above (Fig. 3), which revealed both nuclear and cytoplasmic staining. The distribution of ACF, however, was consistently found to be nuclear in all three cell lines (Fig. 5, B, F, and J). Finally, confocal images of all three cell lines indicated co-localization of ACF and CUGBP2 in the nuclei (Fig. 5, C, G, and K). Taken together, the data in Figs. 3–5 suggest that the distribution of the core component apoB RNA editing factors, apobec-1 and ACF, may modulate the distribution of other proteins that interact in the context of the apoB holo-enzyme.

It must be emphasized, however, that an underlying assumption in the studies detailed in Figs. 3–5 is that the composition and stoichiometry of the holoenzyme, following transfection of cDNAs for the individual component subunits, is indeed preserved. In view of the fact that these are low abundance proteins and because robust methodology to determine
their absolute concentration is not presently available, these assumptions remain open to challenge.

**CUGBP2 Is an apoB RNA-binding Protein**—The presence of three RRMs suggests that CUGBP2 may be an RNA-binding protein, a suggestion consistent with previous demonstrations that CUGBP2 binds AU-rich RNAs (35). Electrophoretic mobility shift assay was performed with recombinant GST/CUGBP2 and a 105-nucleotide $^{32}$P-labeled rat apoB RNA (16), revealing a single shifted band (Fig. 6A). The intensity of this band increased in a dose-dependent manner with increasing quantities of recombinant GST/CUGBP2. Moreover, addition of affinity purified α-CUGBP2 IgG to the binding reaction resulted in a supershift (Fig. 6A). To determine the specificity of this interaction, we performed UV cross-linking studies with different RNA templates. These included AU-rich templates, because apoB RNA surrounding the edited base is ~70% AU-rich (16, 18). Strong cross-linking of CUGBP2 was observed with the rat (RB) and human apoB (HB), a mutant form of human apoB (AUCAGU → uagcu; Fig. 6C), NAT-1, and a transcript containing three tandem repeats of an AUUA sequence (Fig. 6B). Weak cross-linking was not observed with NF1 RNA (Fig. 6B).

To further refine the binding site of CUGBP2, UV cross-linking was performed with radiolabeled human apoB RNA in the presence of cold apoB transcripts of the same size into which various scrambled 6-nucleotide mutations have been introduced (Fig. 6C) (39). Data from these studies suggested that CUGBP2 binds preferentially to a sequence motif located immediately upstream of the edited C, as evidenced by the loss of inhibition by mutant C (AUGAUA) (Fig. 6C). This sequence, along with the 5′-flanking sequence (UAUAUGAUA) in apoB RNA is very similar to the Bruno response element (UGUAUG(A/U)U(A/U)) previously demonstrated to bind CUGBP2 by Good and colleagues (35). Furthermore, CUGBP2 may bind to sequence motifs D (AAUUUG) and F (AUAUUA) located downstream of the edited base, albeit with lower affinity, as inferred from the decrease in binding following the addition of the mutant templates (Fig. 6C). The cumulative evidence from these experiments strongly suggests that CUGBP2 is an apoB RNA-specific binding protein.

To determine whether CUGBP2 binds apoB RNA in vivo, we prepared cytosolic S-100 extracts from McArdle hepatoma cells, a known source of apoB RNA editing activity. The extracts were immunoprecipitated with either α-CUGBP2 IgG or a nonspecific rabbit IgG. Total RNA was extracted from the
FIG. 6. CUGBP2 is an apoB RNA-binding protein. A, increasing concentrations (50–500 ng) of GST/CUGBP2 were incubated with 32P-labeled rat apoB cRNA (RB105, nucleotides 6639–6743), and the complex was analyzed by nondenaturing 5% PAGE. The presence of a CUGBP2:apoB RNA complex is shown by an arrowhead (lanes 2–5). The addition of rabbit α-CUGBP2 IgG to the reaction resulted in a supershift of the CUGBP2:apoB RNA complex (arrow, lanes 6–9). This is representative of experiments performed in duplicate. B, UV cross-linking of CUGBP2. 250 ng of GST/CUGBP2 was added to 32P-labeled cRNA and incubated for 20 min. After treatment with RNase T1 and UV cross-linking, the cross-linked products were analyzed on a 10% SDS-PAGE. Molecular mass markers are shown to the right. RNA templates used in the assay are three tandem copies of AUUUA sequence (3-AU), rat apoB (RB), human apoB (HB), NAT-1 (NT1), NF1 (NF1), human apoB with a scrambled mutation in lane E (see C below), and β-actin (act). C, competition with mutant apoB RNAs. UV cross-linking to a 55-nucleotide human apoB RNA (nucleotides 6645–6703) was carried out in the absence (−) or presence of competitor RNA. Competitors include the wild type (WT), β-actin (act), and scanning mutants of apoB RNA (lanes B–F), representing six nucleotide changes either upstream (lanes B and C) or downstream (lanes D–F) of the edited base as shown in the lower panel. D, co-precipitation of apoB RNA in the immunoprecipitation of CUGBP2 from S-100 extracts. A cytosolic S-100 extract from McArdle cells was prepared and subjected to immunoprecipitation with α-CUGBP2 IgG. As control, immunoprecipitation was performed with normal rabbit IgG (IgG). Total RNA from the S-100 extracts as well as the pellet and supernatants of the immunoprecipitations were isolated and subjected to RT-PCR (RT +) for GAPDH and apoB mRNAs. PCRs were performed with the RNA without reverse transcription (RT −) as control. Water controls (Ctrl) are also shown (G, GAPDH PCR; A, apoB PCR). Migration of the GAPDH and apoB PCR products and DNA molecular mass standards are shown to the right and left, respectively. E, co-immunoprecipitation of apoB RNA with CUGBP2. 32P-Labeled 470-nucleotide rat apoB RNA (3000 or 30,000 cpm) was incubated with 500 ng of cytosolic S-100 extracts of McArdle cells, followed by immunoprecipitation with either α-CUGBP2 IgG or normal rabbit IgG (NRS). The pellet (P) and supernatant (S) were collected, and the counts were determined. The counts in each fraction was plotted as a percentage of total counts in each condition. F, PCR products from the S-100 extracts (Total) and from the α-CUGBP2 IgG immunoprecipitation (IP) were subjected to primer extension analysis, and the products were resolved by denaturing PAGE. As control for primer extension analysis (Ctrl), an apoB cDNA flanking the edited base was used as template. The percentage of edited RNA was quantified by phosphorimaging and expressed as the percentage of editing (%U). Location of the primer (P), unedited (C), and edited (U) bands are indicated to the right.
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CUGBP2 results in the loss of C to U RNA editing complementation (Fig. 6E). At two different input amounts of apoB RNA, almost all the radioactivity was present in the immunoprecipitated fraction, suggesting that CUGBP2 binds apoB RNA with high avidity (Fig. 6E). Returning to the observation that CUGBP2 functions as an apoB RNA-binding protein in vivo, primer extension analyses, performed with the amplified apoB PCR products from endogenous cellular sources, revealed that the α-CUGBP2-bound apoB RNA was edited to a greater extent (−30%) compared with apoB RNA in starting S-100 extracts (−12%) (Fig. 6F). The apparent enrichment with edited apoB RNA is intriguing because the RT-PCR reaction in Fig. 6D implies that virtually all the apoB mRNA is bound by CUGBP2. We speculate that the remaining unbound apoB mRNA is either lost in the subsequent purification process or is immunoprecipitated (α-CUGBP2 bound fraction) and the supernatant (α-CUGBP2 unbound fraction), and each was examined by RT-PCR for the presence of apoB and GAPDH mRNAs. No products were obtained when RT-PCR was performed either in the absence of RNA (Fig. 6D, Ctrl, lanes 1 and 2) or when the RNA was used in the PCR reaction without prior RT (Fig. 6D, RT −). RT-PCR of total RNA from S-100 extracts demonstrated the presence of both GAPDH and apoB (Fig. 6B, lanes 4 and 6). Following immunoprecipitation, apoB mRNA was found only in the pellet (α-CUGBP2 bound fraction; Fig. 6D, lane 16) but not in the supernatant (α-CUGBP2 unbound fraction; Fig. 6D, lane 12). Conversely, GAPDH was found in the α-CUGBP2 supernatant but not the pellet (Fig. 6D, compare lanes 10 and 14). To confirm the suspicion that CUGBP2 binds almost quantitatively to apoB transcripts in these S-100 extracts, we added 32P-labeled apoB cRNA and undertook immunoprecipitation (Fig. 6E). At two different input amounts of apoB RNA, almost all the radioactivity was present in the immunoprecipitated fraction, suggesting that CUGBP2 binds apoB RNA with high avidity (Fig. 6E). Returning to the observation that CUGBP2 functions as an apoB RNA-binding protein in vivo, primer extension analyses, performed with the amplified apoB PCR products from endogenous cellular sources, revealed that the α-CUGBP2-bound apoB RNA was edited to a greater extent (−30%) compared with apoB RNA in starting S-100 extracts (−12%) (Fig. 6F). The apparent enrichment with edited apoB RNA is intriguing because the RT-PCR reaction in Fig. 6D implies that virtually all the apoB mRNA is bound by CUGBP2. We speculate that the remaining unbound apoB mRNA is either lost in the subsequent purification process or is

FIG. 7. CUGBP2 and ACF interact as a complex in bovine liver S-100 extracts. A, determination of the specificity of α-CUGBP2 and α-ACF antibodies. Top panel, 100 ng each of recombinant CUGBP2 and recombinant ACF were size-fractionated and transferred to PVDF membrane. The blot was first subjected to Western analysis with a rabbit α-CUGBP2 IgG, after which it was stripped and subsequently subjected to Western blot analysis for the presence of ACF with a rabbit α-ACF (4–18) antibody. The locations of the protein molecular mass markers are shown to the left. This is representative of three such experiments. Bottom panel, COS-7 cells were transfected with DNA containing HA-tagged CUGBP2 or FLAG-tagged ACF. After 48 h, cell lysates were prepared, and the extracts analyzed by Western blotting with anti-HA or anti-FLAG IgG (data not shown). The lysates were mixed with either anti-CUGBP2 IgG (α-C) or anti-ACF antibody (α-A) followed by precipitation by protein A-Sepharose. The immunoprecipitates were resolved in a 10% SDS-PAGE and Western blotted with anti-HA (α-HA) and anti-FLAG (α-FLAG) IgG. B, immunodepletion of CUGBP2 results in the loss of C to U RNA editing complementation activity. Fraction 16 (Fr16) was generated by size fractionating a 30% ammonium sulfate precipitate of bovine liver extract in the Superdex-200 column by fast phase liquid chromatography (Fig. 2A). Proteins in fraction 16 were either immunodepleted with normal rabbit IgG (IgG, lane 3) or with α-CUGBP2 IgG (α-CUGBP2, lanes 4–6) and tested for presence of complementation activity in the in vitro apoB RNA editing assay. Depletion with α-CUGBP2 resulted in complete loss of editing complementation activity (labeled C). To reconstitute the complementation activity recombinant GST/CUGBP2 (lane 5) or recombinant ACF (lane 6) was added to immunodepleted fraction 16, and editing assays were performed. Only recombinant ACF, but not GST/CUGBP2, reconstituted editing activity. The edited RNAs were quantified by phosphorimaging and shown as the percentage of editing (%U). The presence of primer (P) unedited (U), and edited (E) RNA is shown to the right. This is representative of three experiments. C, ACF co-precipitates with CUGBP2 in the α-CUGBP2 immunoprecipitation. Proteins in fraction 16 (Fr16) as well as the supernatant (Sup) and immunoprecipitate (Bead Eluant) of α-CUGBP2 IgG were subjected to Western blot analysis for ACF (top panel) and CUGBP2 (bottom panel). Antibodies used for the analysis were rabbit α-ACF (4–18) antibody and rabbit α-CUGBP2 IgG, respectively. Recombinant ACF (top panel) and GST/CUGBP2 (bottom panel) were used as positive control for the analyses. Mobility of the ACF (top panel) and CUGBP2 (bottom panel) bands are shown by arrows. The identity of a second nonspecific band in the top panel (arrowhead) is not known.
below the limits of detection by our RT-PCR reaction.

Immunodepletion of Editing Activity by α-CUGBP2 Antibody—Co-fractionation of CUGBP2 with editing competent fractions of bovine liver S-100 extracts and its interaction with apobec-1, ACF, and apoB RNA strongly suggests that CUGBP2 is a component of the apoB RNA editing holoenzyme. Accordingly, we examined the possibility that removal of CUGBP2 from the extracts might affect editing complementation activity. Western blot analysis with recombinant proteins established specificity for both α-CUGBP2 and α-ACF antibodies (Fig. 7A, top panel). In addition, coupled immunoprecipitation and Western blotting of COS-7 cell extracts, singly transfected with either HA-tagged CUGBP2 or FLAG-tagged ACF, demonstrated that α-CUGBP2 IgG and α-ACF antibody recognized only HA-tagged CUGBP2 and FLAG-tagged ACF, respectively (Fig. 7A, bottom panel).

Having established the specificity of the α-CUGBP2 IgG, the enriched fraction (Fig. 1B, fraction 16) from bovine liver S-100 extracts was immunodepleted with either α-CUGBP2 IgG, or a nonspecific IgG, and used in in vitro apoB RNA editing assays. The supernatants from the α-CUGBP2 immunoprecipitation contained no complementation activity (Fig. 7B, compare lanes 3 and 4), and a nonspecific IgG was without effect. The addition of recombinant GST/CUGBP2 to the immunodepleted extracts did not restore editing, suggesting that CUGBP2 alone is not sufficient to rescue the enzymatic activity (Fig. 7B, lane 5). Add-back of bead eluant from the immunoprecipitated material or direct addition of the beads, however, also failed to restore editing activity (data not shown). Conversely, addition of 50 ng recombinant His-ACF restored in vitro apoB RNA editing activity to wild-type levels (Fig. 7B, lane 6). These data suggest that CUGBP2 binds ACF in the S-100 extracts, resulting in ACF sequestration and depletion upon immunodepletion with α-CUGBP2 IgG. Western blots of the immunodepleted extracts demonstrated the presence of ACF and CUGBP2 in the α-CUGBP2 immunoprecipitate (Fig. 7C, Bead Eluant) but not in the supernatant (Fig. 7C). Taken together, the data strongly suggest that CUGBP2 and ACF interact in a complex whose function is critical to apoB RNA editing.

Regulation of C to U RNA Editing by CUGBP2: Interaction with apobec-1 and ACF Modulates Editing Activity—The minimal components of the core editing enzyme consist of two proteins, apobec-1 and ACF. We have used a reconstituted system to determine the effects of CUGBP2 on C to U RNA editing activity. Assays containing apobec-1 and up to 1000 ng of recombinant CUGBP2 did not demonstrate RNA editing activity, suggesting that CUGBP2 itself does not act as a complementation factor (Fig. 8A, top panel, lanes 8 and 9).

However, the addition of increasing amounts of recombinant CUGBP2 (25–500 ng) to an editing assay containing 250 ng of GST/APOBEC-1 and 2 ng of ACF demonstrated a dose-dependent inhibition of C to U RNA editing, with complete abrogation at 500 ng of CUGBP2 (Fig. 8A). A ~50% reduction in editing activity was observed when 50 ng of GST/CUGBP2 was used in the assay (Fig. 8A, lane 4). To determine the mechanism of this inhibition, we performed in vitro RNA editing assays containing 50 ng of GST/CUGBP2 and increasing amounts of either GST/APOBEC-1 or ACF. These studies demonstrate that the addition of either apobec-1 or ACF rescues the editing activity (Fig. 8, B and C). Conversely, addition of increasing amounts of apoB RNA did not rescue the CUGBP2-mediated inhibition of apoB RNA editing (data not shown). These data suggest that CUGBP2 most plausibly exerts its inhibitory effects by inhibiting the interaction between apobec-1 and ACF. A more formal evaluation of these interactions (CUGBP2-apobec-1, CUGBP2-ACF, and apobec-1-ACF) will be necessary to conclude the nature of this inhibition and its implications for enzyme kinetics.

Antisense Inhibition of CUGBP2, apobec-1, and ACF: Effects on C to U RNA Editing—The data to this point suggest the possibility that CUGBP2 may interact in the apoB RNA editing enzyme to inhibit C to U RNA editing. To examine more directly its potential role in vivo, we undertook antisense oligonucleotide transfection experiments to knock out CUGBP2 expression and determine the effects on C to U editing of endogenous apoB mRNA. We again turned to McArdle cells to perform this experiment because of their capacity to edit endogenous apoB mRNA. First, we performed Western blot and immunofluorescence analyses to confirm that a significant reduction of CUGBP2 protein expression had indeed occurred in cells transfected with the antisense oligonucleotide (Fig. 9, A and B). Second, we isolated RNA from the transfected cells and performed RT-PCR analysis to amplify apoB and GAPDH transcripts. As inferred from RT-PCR analysis, transfection of the antisense oligonucleotides did not grossly affect expression of apoB mRNA (Fig. 9C). Based on primer extension analysis of the RT-PCR products, wild type, untransfected cells, and cells transfected with a scrambled oligonucleotide demonstrated ~15% C to U editing of endogenous apoB RNA (Fig. 9D, lanes 4–6). This value is similar to that demonstrated previously (38). By contrast, cells transfected with an antisense oligonucleotide to CUGBP2 demonstrated a significant, 3-fold increase in endogenous apoB RNA editing (>40%, p < 0.001, Fig. 9D, lanes 7–9). These data strongly suggest that abrogation of CUGBP2 expression results in an increase in apoB RNA editing and strongly implies a negative regulatory role for CUGBP2 in the holo-enzyme. As a positive control for this experiment, antisense oligonucleotide inhibition of apobec-1 expression demonstrated the anticipated elimination of editing activity (Fig. 9D, lanes 13–15), findings consistent with the results of gene targeting in mice (12, 49, 50). We have extended this paradigm with the demonstration that antisense oligonucleotide inhibition of ACF also results in loss of editing activity (Fig. 9D, lanes 10–12), suggesting that ACF is also essential for apoB RNA editing in McArdle cells. Taken together, these data provide strong evidence that apobec-1 and ACF are indispen-

sible to apoB RNA editing and suggest that CUGBP2 may participate in the holo-enzyme complex as a negative regulator.

DISCUSSION

A detailed characterization of the protein components of the apoB mRNA editing machinery has been the focus of considerable investigation over the last several years. With the recent cloning of ACF and, along with apobec-1, its demonstration as being necessary and sufficient for in vitro editing activity (24–26), it is reasonable to ask whether yet other factors might participate in the apoB editing enzyme and, if so, what might their role be?

Earlier studies from the laboratories of Smith et al. (9) and Greeve et al. (28) demonstrated that large macromolecular complexes containing apoB RNA along with the requisite editing factors could be isolated by glycerol gradient centrifugation of rat liver S-100 extracts as 11–60 S complexes. Furthermore, these complexes were shown to recapitulate editing activity on a synthetic apoB RNA template (9, 28). These findings strongly imply that the holo-enzyme, or “editosome,” may contain multiple proteins whose stoichiometry and organization with respect to the dimeric catalytic subunit, apobec-1, may regulate C to U RNA editing activity. In regard to the composition of these large, 11–60 S complexes, proteins thus far identified include 45-, 55-, and 240-kDa species, a group represented in a complex precipitated by monoclonal antiserum raised against the 27 S complex and referred to as AUX 240 (31). These observations
FIG. 8. CUGBP2 inhibits *in vitro* apoB RNA editing through interactions with *apobec-1* and ACF. A, inhibition of editing by CUGBP2. *In vitro* apoB RNA editing assays were performed with increasing amounts of GST/CUGBP2 (25–1000 ng) and 250 ng of GST/APOBEC-1, either in the presence (+) or absence (−) of 2 ng of ACF. A representative of experiments performed in triplicate is shown (top panel). ApoB RNA editing
add support to the concept that apoB RNA editing occurs in the context of a large multicomponent structure that includes the nuclear transcript and potentially many proteins. These proteins could include those that bind to apoB RNA, those that bind to apobec-1 and/or ACF, and those that have the capacity to bind all the above. This last category would include CUGBP2.

In considering the importance of apoB RNA-binding proteins in the regulation of apoB RNA editing, it bears emphasis that one of the strategies used to clone ACF involved apoB RNA affinity chromatography, a procedure that yielded several proteins from an active S-100 extract, including ACF itself (25). Using a similar strategy, we have identified the presence of ACF as well as a related homolog, GRY-RBP, in an enriched fraction of chicken intestinal S-100 extracts (26). Greeve and co-workers (24) used a different affinity matrix to obtain ACF and was able to co-purify another RNA-binding protein, KSRP. The cumulative implications of these various approaches indicate that there may be a number of proteins that bind either to apoB RNA and/or to members of the minimal editing core enzyme (apobec-1 and ACF). This is not altogether surprising in light of the fact that apoB RNA is over 14 kilobases in length and that RNA editing may occur temporally and perhaps physically in proximity to splicing and polyadenylation (52, 53), two other post-transcriptional processes known to require the presence of multiple protein complexes. Thus, notwithstanding the obvious importance of apobec-1 and ACF in the in vitro RNA editing reaction, there is precedent for the involvement of other proteins in modulating editing activity as well as conferring site specificity and preventing promiscuous editing of other RNA sequences.

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Fig. 9. Antisense oligonucleotide knockdown of CUGBP2, ACF, or apobec-1 in rat hepatoma cells. A. McArdle cells were transfected with 5 μM morpholino antisense oligonucleotides, directed against CUGBP2 (CUG AS, lane 3) or a scrambled nonspecific oligonucleotide (Scr, lane 2). 72 h after transfection, total cell lysates were prepared and subjected to Western blot analysis with α-CUGBP2 IgG. The blots were subsequently subjected to Western blot analysis with α-HSP40 IgG (Santa Cruz). Migration of molecular mass standards is shown to the left. The locations of CUGBP2 and HSP40 are shown to the right. B, McArdle cells were transfected with HA-tagged CUGBP2 and subsequently with α-CUGBP2 (CUGBP2 + AS) or scrambled morpholino oligonucleotides. 48 h after transfection, the presence of CUGBP2 was visualized by immunofluorescence. C, McArdle cells were transfected with morpholino antisense oligonucleotides, directed against CUGBP2 (CUGBP2 AS, lanes 5–7), ACF (ACF AS, lanes 8–10), apobec-1 (Apobec-1 AS, lanes 11–13), or a scrambled nonspecific oligonucleotide (lanes 2–4). 72 h after transfection, total RNA was isolated from the cells and subjected to RT-PCR analysis to estimate the levels of apoB and GAPDH mRNA. Migration of the 1-kilobase ladder DNA molecular mass standards (Life Technologies, Inc.) is shown to the right. D, endogenous apoB mRNA editing was determined by primer extension analysis. U, edited apoB RNA; C, unedited apoB RNA; P, primer. The gels were subjected to phosphorimaging, and the percentage of editing was determined. Transfection of antisense CUGBP2 oligonucleotide demonstrated a significant increase in editing (p < 0.001). In contrast, transfection of either anti-ACF or anti-apobec-1 oligonucleotides resulted in complete abrogation of editing. The results shown are representative of four different experiments.

was determined by phosphorimaging and depicted as the percentage of C to U conversion (lower panel). B, rescue of CUGBP2 inhibition with apobec-1. C to U RNA editing was performed with 2 ng of ACF, 50 ng of GST/CUGBP2, and increasing concentrations of GST/APOBEC-1 (250–1000 ng). Editing activity was restored with 750 ng of GST/APOBEC-1. A representative gel of experiments performed in triplicate is shown. C, rescue of CUGBP2 inhibition with ACF. C to U RNA editing was performed in the presence of 250 ng of GST/APOBEC-1, 50 ng of GST/CUGBP2, and increasing amounts of ACF (2–20 ng). Editing activity was restored upon addition of 4 ng of ACF. A representative of three independent experiments is shown.
targets. The importance of this latter consideration is evidenced by the cancer phenotype associated with unconstrained editing in the setting of transgenic overexpression of apobec-1 in the livers of mice and rabbits (20–22). These observations in turn emphasize the importance of apobec-1-binding proteins and particularly those that also bind apoB RNA, in the process by which site selection and editing activity is so tightly constrained in vivo.

In regard to apobec-1-interacting proteins, several candidates have been identified through yeast two-hybrid screens, including hnRNPC-1, ABP1, GRY-RBP, and, in this report, CUGBP2 26, 28, 29). ABP1 is an alternatively spliced variant of the hnRN-A/B protein, which, like CUGBP2, was identified as an apobec-1-binding protein in a yeast two-hybrid screen and is an apoB RNA-binding protein 29). Immuno-depletion of ABP1 decreased in vitro apoB RNA editing and transfection of an antisense construct reduced endogenous apoB RNA editing in HepG2 cells stably transfected with apobec-1 29). However, studies with recombinant ABP1 were not performed to examine complementation activity, and its distribution in relation to the other components of the core editing enzyme is unknown. HnRNPC-C was identified by Grieve et al. 28 as an apobec-1-binding protein that binds apoB RNA. Similar to CUGBP2, recombinant hnRNPC-C inhibited in vitro apoB RNA editing 28). However, unlike CUGBP2, which co-factoriates with ACF in bovine liver S-100 extracts, hnRNPC-C fractionated quite separately from editing activity, and its physiological role is currently unknown 28).

What distinguishing features of CUGBP2 suggest that it is an authentic component of the apoB RNA editing holoenzyme? First, we demonstrate that CUGBP2 co-factoriates with ACF in bovine liver S-100 extracts and that its distribution in the most enriched fractions closely matches that of ACF. Second, CUGBP2 was observed to be associated with a reconstituted apoB RNA editing holoenzyme that fractionated in the broad size range of ~250–660-kDa. This holoenzyme was assembled upon the addition of recombinant apobec-1 to bovine liver S100 extracts. Further analysis demonstrated that apobec-1 and ACF were both present in these editing-competent fractions, along with a fraction of CUGBP2. The observation that a substantial proportion of CUGBP2 continues to elute in fractions 14–16 even after supplementation with apobec-1 suggests that not all of the CUGBP2 is present in the apoB RNA editing holoenzyme. Third, immuno-depletion of CUGBP2 from bovine liver extracts results in loss of editing activity. We have further determined that this loss in complementation activity was accounted for by co-depletion of ACF (Fig. 7). The failure to restore complementation activity with add-back of either the bead eluant or the beads themselves from the immuno-depletion reaction, however, is unexplained. Moreover, these findings differ from the results of similar experiments performed by Driscoll and co-workers 25 with ACF, where the add-back of beads from the immunoprecipitation restored low levels of editing activity. One might speculate that under these conditions, CUGBP2 and ACF co-precipitate in a complex that is incapable of interaction with apoB and/or apobec-1. The rescue of CUGBP2-mediated inhibition with apobec-1 supplementation indeed suggests that ACF may still be able to interact with apobec-1 in the presence of CUGBP2 but with lower affinity, perhaps the result of steric hindrance at the apobec-1-binding site(s) of ACF. Further study of the interaction of ACF and apobec-1 and resolution of the domains involved in this interaction will be necessary before a formal conclusion can be brought to this speculation, however. Fourth, we demonstrate by confocal microscopy that upon co-transfection into a variety of cell lines, ACF and CUGBP2 co-localize in the nucleus, whereas CUGBP2 and apobec-1 co-localize predominantly in the cytoplasm. These studies lend indirect support to the concept that the compartmentalized distribution of ACF and apobec-1 may be regulated through their interactions with other protein components of the apoB RNA editing machinery. Because apoB RNA editing is presumed to occur in the nucleus (52, 53), the findings from confocal microscopy, showing predominantly cytoplasmic staining of apobec-1, raise the possibility that alterations in the nuclear import of apobec-1 may be an important restriction point in the regulation of C to U RNA editing. This possibility will require clarification, however, and such studies are currently in progress.

Two additional features of CUGBP2 are worthy of emphasis. First, we demonstrate that CUGBP2 is an RNA-binding protein with activity toward apoB RNA. These findings are consistent with earlier studies that CUGBP2 binds CUG triplet repeats and exhibits homology to members of the Bruno family of Drosophila proteins, bruno and bruno2 35, 44, 45). Bruno family members bind to a AU- rich RNA sequence referred to as a bruno response element in the 3′-untranslated region of oskar mRNA and inhibit translation 54–57). Thus, the ability of CUGBP2 to bind to the AU-rich apoB RNA is consistent with these earlier results.

Finally, as evidence of the role of CUGBP2 in the regulation of apoB RNA editing in vivo, we undertook antisense oligonucleotide knockout of its expression in McArule rat hepatoma cells. These results demonstrate that a decrease in CUGBP2 expression was associated with an increase in editing efficiency, as predicted from the inhibition studies. In addition, by way of a positive control, we demonstrate that antisense knock-out of ACF eliminates C to U editing of apoB RNA, thereby establishing a proof of principle that this protein is likely essential to apoB RNA editing in vivo. Of course, this conclusion will require formal proof in gene-targeted mice.

Taken together, the results from this report establish a novel function for an RNA-binding protein, namely as a regulatory factor in the apoB RNA editing holoenzyme. CUGBP2 mRNA has itself been shown to undergo multiple alternative splicing reactions, resulting in alternatively spliced CUGBP2 mRNAs, some with distinct 5′-untranslated regions that each encode different protein isoforms 44, 51). Further studies are currently underway to determine whether these protein variants have distinct functions in relation to apoB RNA editing.

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Novel Role for RNA-binding Protein CUGBP2 in Mammalian RNA Editing: CUGBP2 MODULATES C TO U EDITING OF APOLIPOPROTEIN B mRNA BY INTERACTING WITH APOBEC-1 AND ACF, THE APOBEC-1 COMPLEMENTATION FACTOR

Shrikant Anant, Jeffrey O. Henderson, Debnath Mukhopadhyay, Naveenan Navaratnam, Susan Kennedy, Jing Min and Nicholas O. Davidson

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