Mutagenesis of Apobec-1 Complementation Factor Reveals Distinct Domains That Modulate RNA Binding, Protein-Protein Interaction with Apobec-1, and Complementation of C to U RNA-editing Activity*

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Valerie Blanc‡, Jeffrey O. Henderson‡, Susan Kennedy§, and Nicholas O. Davidson¶¶

From the ‡Departments of Internal Medicine and §Pharmacology and Molecular Biology, Washington University School of Medicine, St. Louis, Missouri 63110

C to U editing of apolipoprotein B (apoB) RNA requires a multicomponent holoenzyme complex in which minimal constituents include apobec-1 and apobec-1 complementation factor (ACF). We have examined the predicted functional domains in ACF in binding apoB RNA, interaction with apobec-1, and complementation of RNA editing. We demonstrate that apoB RNA binding and apobec-1-interacting domains are defined by two partially overlapping regions containing the NH2-terminal RNA recognition motifs of ACF. Both apoB RNA binding and apobec-1 interaction are required for editing complementation activity. ACF is a nuclear protein that upon cotransfection with apobec-1 results in nuclear colocalization and redistribution of apobec-1 from the cytoplasm. ACF constructs with deletions or mutations in the putative nuclear localization signal (NLS) still localize in the nucleus of transfected cells but do not colocalize with apobec-1, the latter remaining predominantly cytoplasmic. These observations suggest that the putative NLS motif in ACF is not responsible for its nucleo-cytoplasmic trafficking. By contrast, protein-protein interaction is important for the nuclear import of apobec-1. Taken together, these data suggest that functional complementation of C to U RNA editing by apobec-1 involves the NH2-terminal 380 residues of ACF.

Posttranscriptional modification of RNA is an important mechanism, whereby the genetic repertoire of the organism may be amplified beyond that encoded in the chromosomal template (reviewed in Ref. 1). RNA editing is one such example, and within this category, there exist two major classes of base modification editing, both of which represent site-specific enzymatic deamination of single nucleotides with important consequences for gene expression in mammals (reviewed in Ref. 1). One example is A to I editing, which while sharing several of the domains present in ACF has been further facilitated by the recent identification of a related gene, GRY-RBP, which while sharing several of the domains present in ACF and described below does not complement apobec-1 in mediating C to U editing of apoB RNA (20, 21).

ACF is a novel 586-residue protein that contains several functional domains involved in the apoB RNA-editing holoenzyme. A comparison of the modular structure of ACF has been further facilitated by the recent identification of a related gene, GRY-RBP, which while sharing several of the domains present in ACF and described below does not complement apobec-1 in mediating C to U editing of apoB RNA (20, 21). ACF cDNA predicts the presence of three RNA recognition motifs (RRMs) that is of interest in view of the observations confirmed in several studies in which ACF binds to apoB RNA (18, 19, 22). Indeed, this characteristic was crucial to its original isolation by Driscoll and colleagues (18) and Greeve and colleagues (19) has facilitated the understanding of the protein components involved and their functional role in the apoB RNA-editing holoenzyme. A comparison of the modular structure of ACF has been further facilitated by the recent identification of a related gene, GRY-RBP, which while sharing several of the domains present in ACF and described below does not complement apobec-1 in mediating C to U editing of apoB RNA (20, 21).

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¶ To whom correspondence should be addressed: Division of Gastroenterology, Washington University Medical School, Box 8124, 660 S. Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-2027; Fax: 314-362-2033; E-mail: NOD@IM.WUSTLE.DU.

1 The abbreviations used are: apoB, apolipoprotein B; ACF, apobec-1 complementation factor; RRM, RNA recognition motif; GRY-RBP, glycine-arginine-tyrosine-rich RNA-binding protein; RG, arginine-glycine; NLS, nuclear localization signal; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; RNP, ribonucleoprotein; Cy, cyanine.
particularly perplexing issue, because apoB-1 itself has RNA binding activity albeit low affinity and yet exhibits an absolute cofactor requirement (24, 25). Accordingly, a central objective of our investigation was to resolve the requirement for RNA binding in the function of ACF and to identify the domains responsible. For example, in addition to three RRMs, ACF contains a region with six RG residues and a COOH-terminal double-stranded RNA binding domain (Fig. 1) (18, 19), each of which has potential significance in relation to apoB RNA binding activity (26, 20).

Another issue concerns the topology of C to U editing in relation to the subunits of the holoenzyme. Available evidence suggests that apoB RNA editing is predominantly a nuclear event, although recent work suggests that it may also occur in the cytoplasmic compartment of transfected cells (27–29). Confocal immunolocalization studies suggest that apoB-1 is distributed both in the nucleus and cytoplasm of transfected cells (20, 30, 31). However, following cotransfection with ACF, the distribution of apoB-1 becomes predominantly nuclear, suggesting that the interaction of these two proteins leads to translocation of a complex into the nucleus (20). The functional relevance of a putative bipartite NLS in the NH$_2$-terminus of apoB-1 has been called into question as a result of studies from Smith and colleagues (30), suggesting that two distinct domains are required for nuclear distribution beyond the putative NLS region per se. By contrast, ACF contains a seven-residue stretch (14$^{P}$PKKKRK$^{E256}$) that resembles an SV40-type NLS (18, 19). This result raised the possibility that the determinants of nuclear distribution of the holoenzyme reside not in apoB-1 but perhaps in ACF.

We have undertaken studies to localize specific domains in ACF responsible for its RNA binding activity and for mediating protein-protein interaction with apoB-1. Our findings indicate that these functional activities are distributed in distinct but partially overlapping regions of the protein, and both activities are required for efficient complementation of C to U editing. Epitope-tagged ACF bearing deletions or mutations in the putative NLS revealed a nuclear distribution, suggesting that this domain is not required for nuclear localization. However, the interaction of the NLS mutants with apoB-1 was impaired, and the distribution of apoB-1 in cotransfected cells remained predominantly cytoplasmic compared with the nuclear colocalization found with wild-type ACF. The collective results suggest that the functional domains of ACF involved in C to U editing of apoB RNA are localized in the regions spanning residues 1–380, particularly the second and third RRM.

**MATERIALS AND METHODS**

**Cloning and Expression of Recombinant ACF Mutants**—All mutants were constructed by a two-step PCR method (32). The full-length PCR products were sequenced and subcloned into pTYB1 (New England Biolabs) using the Ndel–SalI sites, which generate a COOH-terminal intein fusion protein as described previously (20). For the expression of GRY-RBP/ACF chimeras, chimera 1 was cloned into pTYB1 using the Nhel–SalI sites, whereas chimeras 2 and 3 were cloned into pTYB2 using the Nhel–Smal sites. Each mutant was also subcloned into pCMV-Tag 2B (Stratagene). Using these two internal restriction sites for ACF, EcoNI, and BstEII, the mutated sequences were digested from the pTYB1-ACF vector and cloned into pCMV-Tag 2B-ACF. This cloning step generated an NH$_2$-terminal FLAG-tagged fusion protein. Recombinant proteins were expressed as intein fusion proteins and purified to homogeneity using chitin affinity chromatography as described previously (20), using ER2566 cells as recommended by the manufacturer. The E. coli culture was resuspended in 50 ml of lysis buffer (20 mM HEPES, pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5 mM phenylmethylsulfonfonyl fluoride). The lysate was loaded on a 10-ml chitin column, and the recombinant protein eluted in the presence of 1 mM NaCl and 50 mM dithiothreitol. Fractions containing the protein of interest were pooled and dialyzed against 20 mM HEPES, pH 8.0, 100 mM KCl, 0.2 mM EDTA, 0.5 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride. Purity was assessed by denaturing SDS–PAGE and determined to be >95% homogeneous in all cases. The secondary structure predictions were obtained using the GORIV method provided by the Network Protein Sequence Analysis web server (npsa-bilc.ibcp.fr).

**UV Cross-linking to ApoB RNA**—A [32P]labeled rat apoB RNA template (50,000 cpm at 4 × 10$^{-5}$ cpm/μg) was incubated with 100 ng of wild-type or mutant ACF for 15 min at room temperature in a binding buffer containing 20 mM HEPES, pH 8.0, 100 mM KCl, 1 mM EDTA, 0.25 mM dithiothreitol, and 2.5% glycerol. The RNA was then treated sequentially with RNase T1 (2 units/μl final concentration) and heparin (2 mg/ml final concentration). The mixture was UV-irradiated on ice in a Stratalinker (Stratagene) at an energy of 250 millijoules/cm$^2$ and analyzed by SDS–10% PAGE under reducing conditions.

**Immunoprecipitation**—Rat-apoB-1 cDNA was cloned into pCMV-Tag 3B and expressed as an NH$_2$-terminal c-Myc-tagged fusion protein. In vitro protein-protein interaction studies were performed using Myc-apoB-1 and FLAG-ACF (wild-type or mutant) expressed in COS-7 cells. Cells were grown to 70% confluence and transiently transfected with the indicated plasmids (wild-type or mutant) expressed in COS-7 cells. Cells were grown to 70% confluence on coverslips. Using 6 μM anti-Myc IgG, Western blotting was performed under native conditions, and 5 μg of wild-type or mutant ACF in buffer containing 20 mM HEPES, pH 8.0, 100 mM KCl, 1 mM EDTA, 0.25 mM dithiothreitol for 3 h at 30 °C. The RNA was reverse-transcribed and cDNA-amplified primer pair was used to transfect cells with DNA plasmid using Lipofectin (Invitrogen). The extended products were fractionated by electrophoresis in an 8% polyacrylamide gel and quantitated by phosphorimaging (Molecular Dynamics, Sunnydale, CA).

**Immunofluorescence and Confocal Microscopy**—COS-7 and McArdle rat hepatoma cells (ATCC, Manassas, VA) were grown to 50–70% confluence on coverslips. Using 6 μl of FUGENE 6 (Roche Molecular Biochemicals) or 10 μl of Superfect reagent (Qiagen), the cells were transfection with 2 μg of pCMV-Tag 2B expressing an NH$_2$-terminal FLAG-tagged ACF (wild-type, ΔRRM2, or NLS/A mutant) and 2 μg of pCMV-Tag 3B expressing an NH$_2$-terminal c-Myc-tagged apoB-1. After 48 h after transfection, the cells were fixed with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100, and stained with anti-Myc IgG and mouse monoclonal anti-FLAG IgG (1 μg/ml) (PA1–984 Affinity BioReagent, Golden) was added. After incubation at 4 °C for 1 h, the cells were washed with cold PBS and then reacted with 1:2000 dilution of rabbit anti-FLAG IgG and mouse monoclonal anti-Myc IgG, followed by Cy3- or rhodamine-conjugated secondary antibodies. The COOH-terminal FLAG-tagged ACF was visualized with a rhodamine-conjugated secondary antibody. Confocal images were taken on a Leica TCS 3D Confocal microscope using a 63× objective. Preparations were imaged using a 63X Zeiss plan apochromat objective and a Bio-Rad MRC 1024 confocal adaptor. A krypton-argon laser was used with epifluorescence filter sets designed for Texas Red (Cy3), fluorescein isothiocyanate, and cyanine (Cy5). The confocal aperture was set at 1.8. Usually 5–12 images at planes separated by 0.5 μm were obtained. This increment allows sectioning of the
entire image giving a range of a signal covering every plane of the cell in that image. Images were processed using Adobe Photoshop 4.0 software. For standard immunofluorescence microscopy, stained cells were mounted with Vectashield, and nuclei were visualized with 4,6-diamidino-2-phenylindole (Vector). Images were obtained with a Zeiss Axioshot 2 MOT microscope equipped with a ×40 plan neofluor objective and a 3-CCD camera (DAGE-MTI, Inc.). A Zeiss Attoarc variable intensity lamp was used with filter sets designed for Cy3, fluorescein isothiocyanate, and 4,6-diamidino-2-phenylindole. Pictures were processed using Adobe Photoshop 4.0 software.

**Oligonucleotides—**The oligonucleotides used in this study are listed below. The underlined nucleotides represent restriction sites introduced for subsequent cloning into bacterial (pPCR-Script) and pTYB vectors and mammalian expression vectors (pCMV2B). The boldface nucleotides represent the mutated bases. For each mutant, the first set of PCR was performed using pTYB1-ACF plasmid as template, two external primers, 5′ primer TT 5′-ATAACAGCTCACATAAGG-3′ and 3′ primer ACF-R 5′-GGTGGTTGCTTCCCCGAGAAGGTTGCTC-3′ (SapI site), and two internal primers surrounding the region to be deleted. The full-length product was obtained using the primers ACF-F 5′-GGTGGTGCTCATATGGGATTACAAATACACAAATTCGG-3′ (NdeI site) and ACF-R. The internal primers used in the first set of are

**Fig. 2. Functional analysis of natural mutants of ACF.** A, an analysis of ACF mRNA reveals multiple isoforms in human liver. These include wild-type ACF (wt-ACF), an isof orm lacking 55 amino acid residues 203–257 (∆55), an isof orm missing the first 84 NH2-terminal residues (∆N84), and a splice form containing an 8-amino acid insertion (381–388) (∆ 8aa). The deleted regions are indicated by interrupted lines. The insertion is highlighted by a black box. B, 2 μg of recombinant protein were separated on SDS-10% PAGE and stained with Coomassie Blue. C, C to U editing assays were conducted with 250 ng of recombinant glutathione 6-transferase-apobec-1 and 2 ng of wild-type ACF (lane 1) or ACF mutants (lanes 2–4). The RNA was extracted, and C to U editing was analyzed by primer extension assay. The data from three independent assays were averaged (mean ± S.D.) and are represented as a percent U. A representative experiment is shown. The relative mobility of the primer (P), edited U, and unedited C products are indicated. D, RNA binding activity of ACF mutants. UV cross-linking was performed by incubating 100 ng of recombinant protein with a 32P-labeled 105-nucleotide apoB RNA (RB105), spanning the edited base. After treatment with RNase T1 and UV irradiation, the products were analyzed by SDS-10% PAGE. The molecular weight markers are shown at the left of the gel. Lane 1, wild-type ACF; lanes 2–4, ACF mutants. This is representative of three independent assays. E, communoprecipitation of wild-type and mutant ACF with apobec-1 in transfected COS-7 cells. COS-7 cells were transfected simultaneously with DNA encoding c-Myc-tagged apobec-1 and FLAG-tagged ACF (lanes 2–5). After 48 h, cell lysates were prepared, and extracts were analyzed by Western blot with anti-FLAG (upper panel) and anti-Myc IgG (middle panel). Lower panel, the extracts from transfected COS cells were immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were separated by SDS-10% PAGE and analyzed by Western blotting using anti-Myc IgG. As a control, an extract from nontransfected COS cells was analyzed by Western blot and immunoprecipitation (lane 1). The data from panels C–E indicate that the NH2-terminal half of ACF is required for the apobec-1 complementing activity. This is representative of three independent assays.
AAATCCCCACTGATGGAGG-3. The mutant ΔN84 was generated by PCR using the primers 5'-GGTGGTCATATGAGAATGATGATG-3' (Nde I site) and ACF-R. The full-length product was sequenced and cloned into pTYB1 (Nde I-SapI site).

RESULTS AND DISCUSSION

Deletional Analysis of ACF: Functional Implications from Natural Mutants of ACF—We constructed two deletion mutants (ΔN84 and ΔS55) and a full-length ACF clone with an 8-amino acid insertion (Δ8aa) both based upon earlier (19, 20) and more recent findings (44) of alternatively spliced ACF transcripts in human liver and small intestine (Fig. 1). The dominant transcript produced in vivo, however, encodes the wild-type ACF, analogous to that used in the current construction. Each splice variant of ACF was expressed as recombinant protein (Fig. 2B) and used in an in vitro RNA-editing assay with apobec-1, and the extent of C to U editing was determined. As shown in Fig. 2C, lane 1, wild-type ACF demonstrates >80% C to U editing activity, similar to the value observed with the
Δ8aa alternatively spliced form. However, two other splice mutant forms of ACF, Δ55 and ΔN84, failed to demonstrate complementation activity (Fig. 2C, lanes 2 and 3). ApoB RNA binding was virtually eliminated with the Δ55 and ΔN84 forms (Fig. 2D, lanes 2 and 3), and none of the isoforms demonstrated coimmunoprecipitation of apobec-1 (Fig. 2E, lanes 3 and 4). These results together point to the NH2-terminal half of ACF as containing crucial functional domains.

Deletion Analysis of ACF: Role of RRMs and Other Potential RNA Binding Domains—The amino acid coordinates for the next set of deletions are shown in Fig. 3A. We expressed mutants lacking either RRM1 or RRM2 but were unable to express an RRM3 deletion in isolation. However, as noted above (Fig. 2B), the Δ55 mutant eliminates a portion of both RRM2 and RRM3. Deletion Δ331–385 removes a region of 54 residues that is unique to ACF, no comparable region being expressed in GRY-RBP (Fig. 3A). As allowed to findings noted earlier, GRY-RBP lacks complementation activity in C to U RNA-editing assays, and we speculated that this particular region in ACF may be of relevance in this regard (20). Deletion Δ331–385 and a finer deletion Δ380–402 were also used to examine the role of the RG domain in ACF, because RG domains have been demonstrated in other proteins to exhibit RNA binding activity (33, 34). Finally, Δ438–515 was constructed to eliminate the putative double-stranded RNA binding domain from the COOH terminus of ACF (33). Three of these mutants retained detectable C to U editing complementation activity, suggesting that the loss of the corresponding domain is tolerated at least partially. These mutants include ΔRRM1, the Δ380–402, and Δ438–515 mutants (Fig. 3C, lanes 2, 5, and 6). The Δ438–515 mutant in particular demonstrated close to wild-type levels of C to U editing activity (Fig. 3C, lane 6). The results suggest that the double-stranded RNA binding domain is unlikely to play a major role in the complementation function of ACF, despite predictions from folding algorithms that apob RNA in the region of the edited base adopts a conformation that resembles double-stranded RNA (35–37). This said, apob RNA binding activity was reduced with both the Δ380–402 and Δ438–515 mutants (Fig. 3D).

Two mutants revealed no C to U editing complementation activity. These include the ΔRRM2 and the Δ331–385 mutants (Fig. 3C, lanes 3 and 4). These findings were replicated in assays using up to 100 ng of recombinant protein (data not shown). Neither protein exhibited RNA binding activity as inferred from the UV cross-linking analysis (Fig. 3D). However, Δ331–385 revealed wild-type levels of apobec-1 interaction (Fig. 3E, lane 6), whereas ΔRRM2 failed to coimmunoprecipitate with apobec-1, suggesting that protein-protein interaction involves a region NH2-terminal to residue 330.

Taken together, the findings from this series of studies indicate that diminished RNA binding activity with preservation of apobec-1 interaction is tolerated but diminishes complementation activity. However, mutations that abolish RNA binding,

**Fig. 4.** Mutations in RRM2 and the region-spanning residues 331–380 reduce apoB RNA editing. A, top panel, schematic representation of ACF protein. Two specific pairs of residues were mutated to alanine residues. The resulting mutants are identified as L359A, L368A (L359+368A) and R371A, R375A (R371+375A). Middle panel, sequence alignment of the RRM2 domains of hnRNPC1/C2 and ACF. The conserved amino acid residues are in gray boxes. The point-mutated residues of ACF are highlighted in bold. The point mutations were constructed by changing the conserved Gly-141 and the basic KKKR amino acids to alanine residues. The mutants are identified as G141A (G141/A) and NLS/A. The corresponding secondary structure of the RNA binding domain, predicted from the GORIV algorithm, is shown below. The RNA binding domain contains four β-sheets interrupted by two α-helices. B, an analysis of 2 μg of recombinant mutants by SDS-10% PAGE and Coomassie Blue staining. C, 2 ng of wild-type ACF (lane 1) or mutants (lanes 2–5) were added to 250 ng of glutathione S-transferase-apobec-1 in a C to U editing assay. The data represent the mean ± S.D. from three independent assays. A representative assay is shown. D, RNA binding activity of ACF mutants. RB105 was in vitro transcribed with [32P]UTP and incubated with 100 ng of wild-type ACF (lane 1) or mutants (lanes 2–5) for 15 min. After UV irradiation, the samples were analyzed by SDS-10% PAGE. The molecular weight markers are indicated at the left of the gel. This is representative of three independent assays. E, coimmunoprecipitation with apobec-1 in transfected COS-7 cells. COS-7 cells were transiently transfected with plasmids encoding apobec-1 and wild-type ACF or mutant ACF. The expression of the mutants and apobec-1 was analyzed by Western blot using the indicated antibodies (upper and middle panels). The extracts from transfected cells were immunoprecipitated with anti-FLAG IgG, and the immunoprecipitates were analyzed for the presence of Myc-apobec-1 by Western blot using anti-Myc IgG (lower panel). This is representative of three independent assays.
Table I
Summary of activities associated with mutants of ACF

| Activity                | ACF     | ΔN84    | Δ55     | Δ RRMM1 | ΔRRM2 | NLS/A | G141A | Δ(331–385) | L359A/L368A | R371A/R375A | Δ(380–402) | Δ(438–515) |
|------------------------|---------|---------|---------|---------|--------|-------|-------|------------|-------------|-------------|------------|------------|
| C to U editing         | 100     | 1       | 1       | 23      | 1      | 9     | 35    | 1          | 12          | 35          | 93         | 93         |
| apoB RNA binding       | + +     | + / −   | −       | +       | −      | −     | +    | +          | +           | +           | +          | +          |
| apobec-1 interaction   | +       | + / −   | −       | +       | −      | −     | +    | +          | +           | +           | +          | +          |

Fig. 6. Schematic representation of the apobec-1 and apoB RNA interaction domains of ACF. The RRM motifs are indicated by gray boxes. The deletions and mutations generated for this study are indicated. ACF interacts with apobec-1 through domain spanning residues 144–257, including regions of RRMM2 and RRMM3 (light gray box). The apoB RNA binding domain partially overlaps the apobec-1 interaction domain and extends to residue 380 (dark gray box).

Fig. 5. Immunofluorescence microscopy of transfected mutants lacking the putative NLS. A, COS-7 cells were grown on coverslips and transiently transfected with plasmids expressing FLAG-tagged wild-type or mutant ACF constructs as described under "Materials and Methods." Upper panel, wild-type ACF; middle panel, ΔRRM2 mutant; lower panel, NLS/A mutant. Nuclear counterstaining was performed with 4,6-diamidino-2-phenylindole (right panels). These images are representative of three independent transfections and reveal a nuclear localization of both ACF mutants despite the deletion of the putative NLS. B, McArdle rat hepatoma cells were cotransfected with Myc-apobec-1 and FLAG-ACF (A–D), Myc-apobec-1 and FLAG-ΔRRM2 (E–H), or Myc-apobec-1 and FLAG-NLS/A (I–L). The confocal images were merged (C, G, and K) as detailed under "Materials and Methods." This is representative of three independent assays.

(RNP) sequences, RNP1 and RNP2 (38, 39), separated by conserved hydrophobic residues (40, 41). Based on recent studies demonstrating the importance of the conserved glycine residue in RNP2 in mediating the binding of hnRNPC1 to a poly(U) substrate (42), we introduced a single point mutation into ACF at the corresponding position (G141A, Fig. 4A). This RNP2 domain is in immediate proximity to the putative NLS in ACF (18, 19), which contains a cluster of basic residues (Fig. 4A). Clusters of basic residues, particularly arginine and lysine, are a feature of some RNA-binding protein domains, notably the Rev peptide (43), suggesting to us that mutagenesis of these residues in RRMM2 might disrupt RNA binding. Accordingly, we generated the ACF mutant NLS/A (Fig. 4A). In addition, we also attempted to localize the residues between 331 and 385 that contribute to the loss of function, particularly apoB RNA binding, observed with the deletion mutant Δ(331–385). We selected two pairs of residues, (Leu-359 + Leu-368) and (Arg-371 + Arg-375), each of which was mutated to alanine (Fig. 4A). These leucine residues were selected following the recent report of Dreyfuss and colleagues (42) stating that specific leucine residues at the COOH terminus of the RNA binding domain of hnRNPC1 influence the RNA binding activity of the protein. Arginine residues were selected following the prediction that these residues exert electrostatic interactions with RNA in the Rev-RRE complex (43).

Recombinant proteins (Fig. 4B) were examined for C to U complementation activity. The results demonstrate a 90% re-

despite preserving protein-protein interaction, fail to complement C to U editing.

Point Mutagenesis within RRMM2 and the Region-spanning Residues 330–380—The observation, in which a mutant ACF protein lacking RRMM2 failed to bind apoB RNA and also failed to coimmunoprecipitate with apobec-1, pointed to this region as containing crucial functional domains that are in turn required for C to U editing complementation activity. As illustrated in Fig. 4A, RRMM2 contains two consensus ribonucleoprotein
duction with the NLS mutant (NLS/A) and a 60% reduction in the point mutant (G141A), disrupting the RNP2 domain (Fig. 4C, lanes 1–3). C to U complementation activity was completely abolished in the L359A,L368A mutant and reduced by almost 90% in the R371A,R375A mutant, suggesting that these residues are important determinants of ACF function. These mutant proteins were further examined in relation to apoB RNA binding and apobec-1 interaction. The NLS/A, G141A, and R371A,R375A mutants produced a modest decrease in apoB RNA binding (Fig. 4D, lanes 2, 3, and 5), as inferred from visual inspection of the UV cross-linking assay. The role of specific residues cannot be determined with certainty from this mutagenesis study, and further work will be required to examine whether, for instance, other basic residues would function interchangeably. In addition, confirmation of the UV cross-linking assays will need to be made through quantitative analysis of RNA binding. With these reservations notwithstanding, the L359A,L368A mutant demonstrated no apoB RNA cross-linking activity (Fig. 4D, lane 4), implicating these residues as crucial to the RNA binding function of ACF. Apobec-1 interaction was disrupted in the NLS/A mutant (Fig. 4E) but preserved in the other mutants, consistent with the prediction from the initial experiments described above suggesting that the protein-protein interaction domain is located in a region proximal to residue 257.

An important consideration in our interpretation of the mechanisms underlying the effects of these mutations on ACF function concerns the structural conformation of the protein. Using the GORIV algorithm, there was no overall change predicted with the G141A, NLS/A, or L359A,L368A mutants. However, the R371A,R375A mutant is predicted to change to a coiled structure into an α-helix downstream of the mutation. Thus, the observed reduction in C to U editing complementation activity, without obvious disruption in either RNA binding or apobec-1 interaction, may reflect this potential conformational change in the R371A,R375A mutant. However, further analysis will be required to confirm this suggestion.

Subcellular Distribution of ACF: Role of Putative NLS—A further series of experiments was undertaken to examine the role of the putative NLS in directing the expression of epitope-tagged ACF to the nucleus of COS-7 and McArdle hepatoma cells. COS-7 cells were selected, because they barely express detectable levels of endogenous ACF and do not express apobec-1 or apoB RNA (20). McArdle rat hepatoma cells were selected, because they express all the required valuable discussions with our colleagues, S. Anant and D. Mukhopadhyay.

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