Biochemical and Biophysical Research Communications 398 (2010) 38–43

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

APOBEC-1 Complementation Factor (ACF) forms RNA-dependent multimers

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ARTICLE INFO

Article history:
Received 25 May 2010
Available online 10 June 2010

Keywords:
Apolipoprotein B
mRNA editing
APOBEC-1
APOBEC-1 complementation factor
ACF
Editosome

ABSTRACT

Limited proteolysis of APOBEC-1 complementation factor (ACF) and computational secondary structure modeling were used to guide the construction of a well-folded, truncation protein spanning residues 1–320 and containing three RNA recognition motifs (RRMs). ACF320 bound preferentially to *apoB* mRNA and supported APOBEC-1 dependent editing at 40% of the activity of full length ACF. Live cell FRET and immunoprecipitation assays revealed that ACF320 formed homomultimers *in situ* that were bridged by RNA. Our study predicted that the C to U editosome may be assembled on the mooring sequence of *apoB* mRNA as a dimer of ACF bound to a dimer of APOBEC-1.

1. Introduction

ACF was discovered as an RNA-binding protein that facilitated site-specific targeting of *apoB* mRNA for C to U editing [1,2]. The basis for this requirement has been proposed to be the maintenance of *apoB* mRNA in a single-stranded conformation that enables recruitment of the cytidine deaminase, APOBEC-1, for site-selective cytidine deamination of nucleotide 6666 [3,4]. ACF has three consecutive RNA recognition motifs (RRMs). RRMs are a well-described structural fold consisting of 4 antiparallel β-strands packed by two alpha helices in a $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ topology (reviewed [3,5]). One RRM was sufficient to bind to a minimum of 2 nucleotides, demonstrated by CBP20 [6,7] and Nucleolin RRM2 [8], so their appearance in multiple copies is thought to represent a way to increase specificity and/or affinity for their substrate.

ACF RRM1 starts at amino acid (a.a.) 58, but the C-terminal boundary of the third RRM (RRM3) ends at either a.a. 293 [9] or 303 [10]. The minimal portion of ACF that retains RNA-binding activity remains to be determined and includes a.a. up to either 304, 380 or 391 [3,9,10]. Deletion mapping of ACF showed that the most significant loss in RNA-binding activity occurred when RRM2 was deleted [10] but point mutations of conserved residues with RRM1 also inhibited ACF RNA binding [9].

ACF truncated to a.a. 304 bound RNA but was not evaluated for APOBEC-1 binding or complementation of editing activity [3]. So far ACF binding to APOBEC-1 only has been demonstrated for ACF truncations 1–380 and 1–391 [9,10]. *In vitro apoB* mRNA editing activity was reduced by truncating the C-terminus of ACF beyond a.a. 380 and was lost when any one of the three RRMs had been deleted [3,9]. Portions of RRMs 2 and 3 are implicated in ACF interaction with APOBEC-1, specifically a.a. 144–257 [9,10]. Phosphorylation of serine 154 within RRM2 enhanced ACF binding to APOBEC-1 and ACF complementation of RNA editing [11,12]. Residues C-terminal to RRM3 may also be required for APOBEC-1 interaction as ACF truncated to less than a.a. 1–377 fail to bind APOBEC-1 [9]. All three RRMs may be required for complementation of editing activity in living cells [9,10,13,14] and that the C-terminal portion of ACF may modulate their function.

We show in this communication that the N-terminal 320 amino acids of ACF retained RNA-binding selectivity and supported complementation of editing activity and in addition demonstrate a novel RNA-bridged multimerization of ACF. The data support a model for the C to U editosome in which ACF dimers are bridged by the mooring sequence.

2. Materials and methods

2.1. Recombinant ACF cloning, expression, and purification

Rat ACF64 was cloned and expressed as previously described [15]. ACF was dialyzed into 50 mM Tris pH 8.0 with 1 mM $CaCl_2$ and digested with 3.5 U of TPKC-trypsin (Worthington Biochemicals, Lakewood, NJ) for 20 min at room temperature and resolved by SDS–PAGE and either stained with Coomassie blue or western blotted. Identities of tryptic peptides were confirmed by western blot analysis using peptide-specific, rabbit polyclonal antibody raised against the N- or C-terminus of ACF [12] and by comparison to the predicted trypsin cleavage sites using Peptide Cutter (http://www.expasy.ch/tools/peptidecutter/). Secondary structure boundaries were predicted using PHYRE (www.sbg.bio.ic.ac.uk/). A cDNA fragment encoding amino acids 1–320 was PCR amplified ACF320,

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doi:10.1016/j.bbrc.2010.06.021

cloned into pet28a mod and sequence verified [15]. The F59A point mutant of ACF320 was created using a mutagenesis kit from Stratagene. Ni^{2+} affinity purification of *Escherichia coli* expressed protein was conducted as described [15]. For HPLC ACF320 was concentrated and chromatographed on an sephacryl S-100 column (GE Healthcare, Waukesha, WI).

2.2. Live cell quenched FRET (FqRET)

EGFP acts as a fluorescence donor and REACh2, a variant of YFP, is an acceptor that quenches EGFP fluorescence when in close proximity [16]. EGFP-V5-ACF320 was transfected into HEK 293T cells alone or together with either REACh2-HA-ACF320 or HA-ACF320-REACh2 chimeras at the indicated ratios. Empty pIRES vector (Clonetech, Mountain View, CA) was used to maintain equivalent input of transfected DNA. Twenty-four hours following transfection EGFP fluorescence was imaged through a 20× objective with an Olympus IX 70 inverted fluorescence microscope and the gray value for each cell was determined with ImageJ software (National Institutes of Health). The average gray value was calculated by counting all of the cells in 5 random fields after subtracting the average gray value of REACh2 constructs as the background. The statistical significance of EGFP quenching was determined using an unpaired Student's *t*-test with $n = 109–115$.

2.3. UV cross-linking

Increasing amounts of ACF320 and ACF64 (0.3–40 pmol) were reacted at 30 °C for 1 h with a fixed amount of *in vitro* transcribed, ^{32}P ATP and CTP labeled *apoB* RNA (150 fmol) containing the editing site at nucleotide 6666 (448 nt long) [17]. For the RNA excess competition studies, non-specific, unlabeled, *xef* competitor RNA (Ambion, Austin, TX) was transcribed using mMessage mMachine (Ambion) and added to the assembly reactions in 100-fold molar excess to *apoB* RNA. UV cross-linked reactions were digested with RNase A and T1 (Roche, Indianapolis, IN) for 1 h and then precipitated with acetone and resolved by SDS–PAGE. Cross-linking was quantified by PhosphorImager analysis of the PAGE gels as described previously [18].

2.4. Immunoprecipitation assays

Whole cell extracts from HEK 293T cells co-transfected with ACF320-V5-EGFP and ACF320-HA-EGFP were prepared 48 h following transfection using Reporter Lysis Buffer (Promega) per manufacture's protocol supplemented with EDTA-free complete protease inhibitor cocktail (Roche). Equivalent microgram amounts of protein from each extract were resolved by SDS–PAGE to evaluate the expression of each ACF construct. Immunoprecipitation was conducted overnight at 4 °C using 1 μg of αV5 (Invitrogen) on protein A agarose (Roche) with precleared extract that had or had not been digested with 25 μg of RNase A (Sigma–Aldrich). Bound protein was eluted from 500 mM NaCl washed beads using SDS–PAGE sample treatment buffer at 37 °C. Eluants were acetone precipitated, resolved by SDS–PAGE and then western blotted with either HA or V5 epitope reactive antibodies. For co-immunoprecipitations, V5 epitope tagged *acf64* and *acf320* cDNA cloned into pcDNA3 were co-transfected into HEK 293T cells with pcDNA3 HA-tagged *apobec-1* cDNA [12]. Whole cell extracts were prepared 48 h following transfection. Immunoprecipitations and protein detection were conducted as described above.

2.5. ApoB RNA editing assays

HEK 293T cells were co-transfected with APOBEC-1 and human *apoB* RNA editing reporter alone or in combination with ACF64 or ACF320 and a using FuGene6 (Roche). Following transfection (48 h), 50% of each culture was harvested for cell extract preparation and western blotting analysis and the rest was placed into TRI-Reagent (MRC, Cincinnati, OH) for RNA extraction. *ApoB* RNA was amplified from 2 μg of total cellular RNA [19] and purified PCR products were primer extended with a ^{32}P radio-labeled DD3 primer in a poison-primer extension assay and the relative proportion of edited RNA quantified by PhosphorImager scanning densitometry [19].

2.6. Analytical ultracentrifugation

Ultracentrifugation was conducted at the University of Connecticut on S-100 column purified ACF320 in 20 mM Hepes, 10 mM NaCl, 1 mM DTT, pH 8.0. Sedimentation velocity analysis was conducted at 20 °C and 55,000 RPM using interference optics with a Beckman-Coulter XL-I analytical ultracentrifuge. Interference scans were acquired at 60 s intervals for 5½ h. Data were collected at four protein concentrations. Normalized $g(s^*)$ distribution plots were created using DcDt+ version 2.1.0 [20,21] and $c(s)$ distribution plots were created using Sedfit, version 11.71 [22].

3. Results

3.1. ACF64 RRMs reside within an trypsin-resistant domain

Recombinant ACF64 expressed in *E. coli* was purified and digested with limiting amounts of trypsin. Coomassie stained SDS–PAGE showed three highly abundant proteolytic cleavage products with estimated molecular masses of approximately 40, 34 and 27 kDa (denoted by arrowheads in Fig. 1A) (Fig. 1A). Western blotting with peptide-specific polyclonal antibodies that recognized epitopes within either the extreme C- (a.a. 508–522) or N-terminus (a.a. 1–14) of ACF [12,23] indicated that the 40 kDa species corresponded to a C-terminal deletion while the smaller species were N-terminal deletions (Fig. 1B). As the minimal amino terminal portion of ACF that contains all three RRMs was predicted to have a maximum molecular weight of ~34 kDa [9,10], the 40 kDa tryptic fragment must have contained all three RRMs. Peptide Cutter software predicted that the C-terminal tryptic cleavage of ACF to be at K364. PHYRE (Protein Homology/analog Y Recognition Engine) secondary structure homology modeling of ACF took into consideration the domain boundaries from the crystal structures of HuD [24], polyA binding protein [25] and sex lethal [26]. K364 was predicted to lie within a 40–50 amino acid disordered region adjacent to an α-helix ending with a.a. 320 (www.sbg.bio.ic.ac.uk/).

3.2. RNA-depleted ACF320 is a monomeric protein

An ACF fragment lacking the disorder region and consisting of a.a. 1–320 (ACF320) was cloned and expressed in *E. coli* with an N-terminal 4 His tag. ACF320 was purified using Ni^{2+} affinity resin binding and resolved by SDS–PAGE with an apparent molecular mass of 36.7 kDa (Fig. 1C). Fractions were pooled and analyzed by size exclusion chromatography. ACF320 eluted at its predicted monomeric molecular mass (Fig. 1D). The predominant monomeric nature of ACF320 also was suggested by dynamic light scatter (DLS) where samples appeared mono-dispersed with a hydrodynamic radius of 2.7–2.9 nm, consistent with a globular protein of 33–39 kDa.

Chromatographically purified, RNA-depleted ACF320 was evaluated by analytical ultracentrifugation (AU) over a broad range of ACF320 concentrations. An overlay of the normalized $g(s^*)$, which is the sedimentation coefficient distribution derived using the time derivative of the concentration distribution, over four

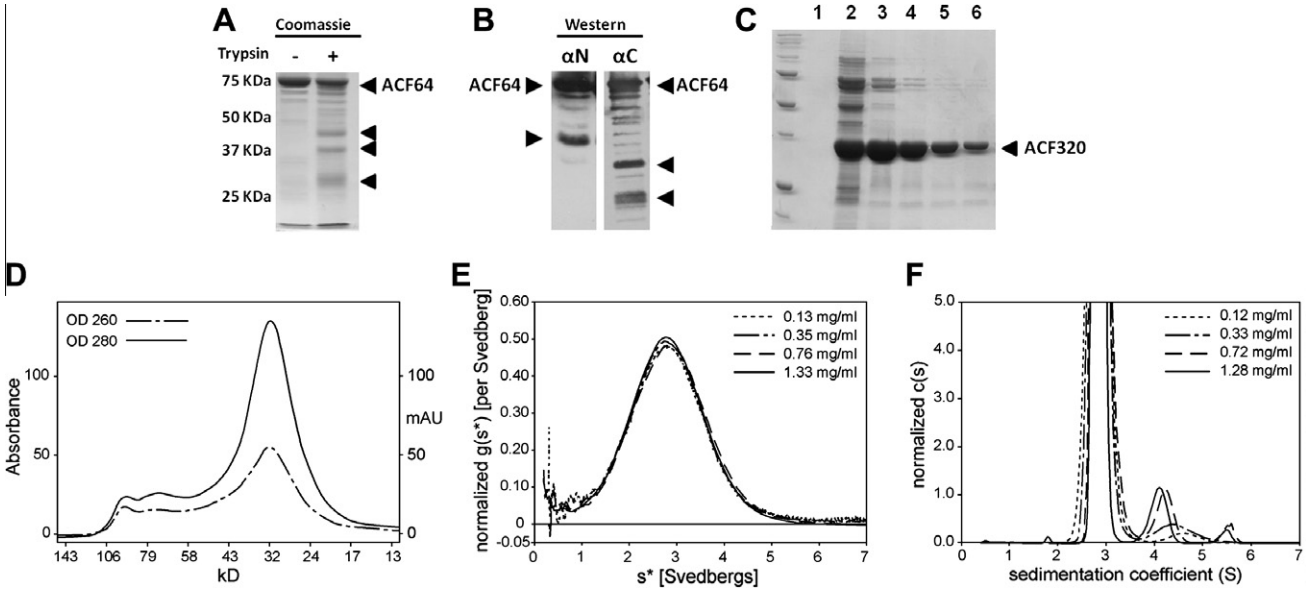

Fig. 1. Expression of monomeric ACF320. (A) Left, Coomassie staining of SDS–PAGE resolved affinity purified ACF64 with and without limited TPKC-trypsin digestion. Arrowheads denote prominent bands reactive in western blot analysis. (B) Western blot analysis of trypsin digested ACF64 probed with either N- or C-terminal ACF peptide reactive antibodies. (C) Coomassie staining of SDS–PAGE resolved ACF320 purified via a N-terminal 4 His tag following expression in *E. coli*. (D) Sephacryl S-100 column (GE Healthcare) fractionation of pooled fractions 3–6 from 'B'. Solid line represents OD280 and dashed lines OD260, measured in mAU units in the *y*-axis. Molecular mass distribution is on the *x*-axis as determined through calibration with protein standards. Fractions ranging from 40 to 25 in 'C' were pooled and concentrated to 2.8 mg/mL and submitted for AU. (E) Four concentrations of ACF320 were analyzed ranging from 0.12 to 1.28 mg/mL and the sedimentation coefficient distribution, $g(s^*)$, values were determined using DcDt+, version 2.1.0 [20,21] and represented graphically. (F) The continuous sedimentation coefficient distribution $c(s)$ of samples in 'D', values determined using Sedfit version 11.71 and represented graphically.

concentrations of A320 input (0.12, 0.33, 0.72 and 1.28 mg/mL) demonstrated one peak with an estimated value of 2.8S (Fig. 1E). The appearance of only one major peak with increasing protein concentration was consistent with most of ACF320 existing as monomer that did not interact with itself. The presence of a 'tail' in the $g(s^*)$ curves toward higher *S* values, however, was characteristic of a small population of larger complexes.

The AU data were fit to a two species non-interacting model in which the second species was restricted to twice the molecular weight of the main peak. The major peak represented ~95% of the sample with a predicted molecular weight of 37.3–35.9 kDa. The secondary peak accounted for most of the remaining 5% of the population. A graphical representation of the $c(s)$, calculated by removing the effects of diffusion from the $g(s^*)$ calculation, enabled a clearer visualization of the minor species (~5%) consistent with dimeric form of ACF320 (Fig. 3F). We concluded that ACF320 was predominantly a monomer in the absence of RNA.

3.3. ACF320 self-associates in vivo via an RNA-dependent interaction

Whether ACF320 was a monomer in living cells was evaluated using quenched FRET (FqRET) [16]. EGFP (fluorescence donor) was expressed as an N-terminal fusion to V5-ACF320 while REACh2 (fluorescence quencher) was cloned as either an N- or C-terminus fusion with HA-ACF320. HEK 293T cells were co-transfected with donor and quencher chimeras at a fixed amount of N-terminal EGFP-V5-ACF320 plasmid and increasing amounts of either REACh2-HA-ACF320 or HA-ACF320-REACh2 plasmids. HEK 293T cells were used for these experiments as they do not express endogenous ACF and are easily transfected. Cells co-transfected with EGFP tagged ACF320 and C-terminally REACh2 tagged ACF320 yielded maximally 5% quenching (Fig. 2A) which was not statistically significant ($p = 0.60$, $n = 111$). Western blot analysis of whole cell extracts confirmed expression of V5-tagged EGFP construct and the HA-tagged REACh2 constructs (Fig. 2B and D). In contrast, co-transfection of HEK 293T cells with EGFP-ACF320 and REACh2-ACF320 plasmid resulted in maximally 22% reduction of the fluorescence (mean gray scale value of 1396 ± 102 vs. 1090 ± 84) (Fig. 2C) and was statistically significant ($p = 0.018$, $n = 113$). Given that the maximum reduction of fluorescence observed for covalently linked FqRET partners was 50% [16], the data supported the possibility that ACF320 formed dimers wherein the N-termini of the subunits were maintained in a closer proximity than the N- and C-termini, suggesting a parallel, head-to-head orientation of ACF320 monomers. That self-association of ACF320 was not mediated by protein/protein interactions but rather involved the formation of a protein–RNA–protein bridge was suggested by the finding that HA-tagged ACF320 co-immunoprecipitated with V5 epitope tagged ACF320 only when extracts from transfected cells (Fig. 2E) were not digested with RNase A (Fig. 2F).

3.4. ACF320 binds preferentially to apoB RNA

The apoB RNA-binding activities of full length ACF64 and ACF320 were determined using UV cross-linking of RNA–protein complexes assembled *in vitro* [18,27]. RNA cross-linking signals increased when ACF64 was titrated (0.3–40 pmol) against a fixed amount (150 fmol) of radiolabeled *apoB* RNA (Fig. 3A). A similar titration of ACF320 demonstrated binding to *apoB* RNA primarily at 40 pmol of input ACF320. The yield of cross-linked complex was comparable to that seen with 1.5 pmol ACF64 (based on PhosphorImager quantification). Non-specific RNA competition certified a selectivity of ACF320 for the *apoB* RNA editing site. Non-specific RNA, at >100× molar excess of *apoB* RNA, demonstrated that despite its reduced RNA-binding activity, ACF320 at 40 pmol input protein preferentially UV cross-linking to *apoB* RNA, comparable to that seen with ACF64 at 1.5 pmol input of protein (Fig. 3B).

3.5. ACF320 binds to APOBEC-1 and complements apoB editing

V5-ACF320 or V5-ACF64 were immunoprecipitated from extracts of HEK 293T cell that had been co-transfected with HA

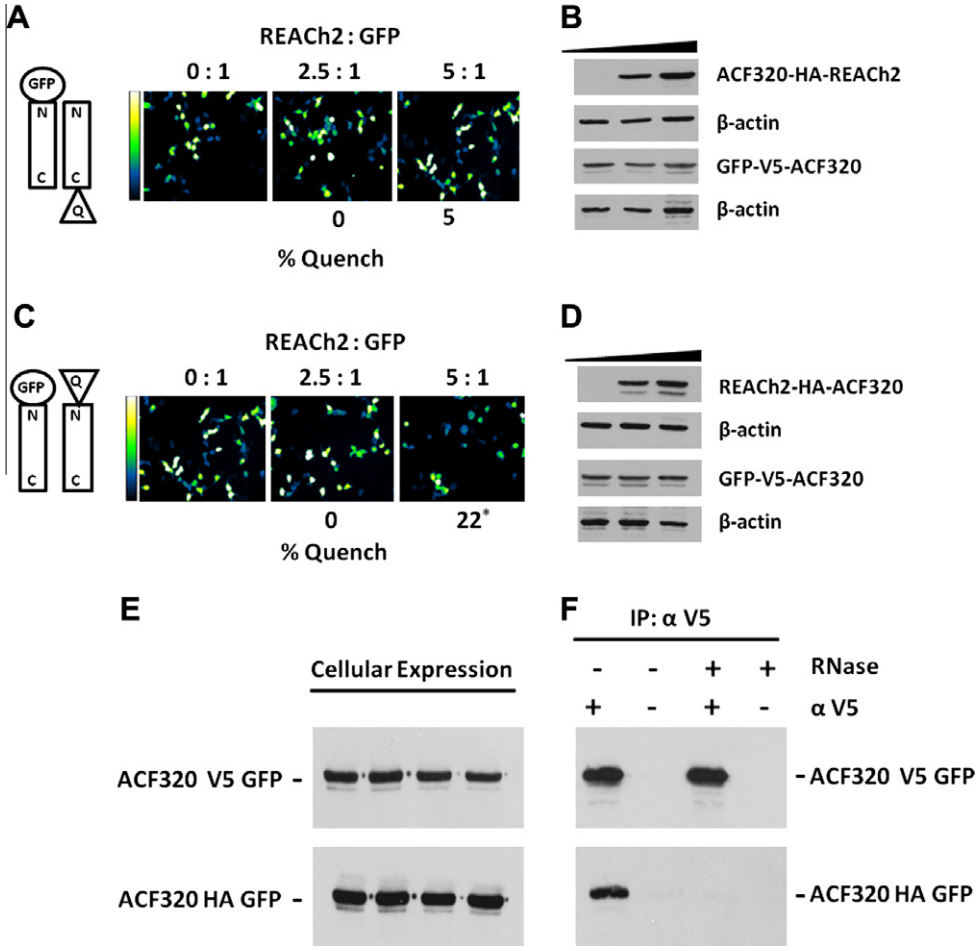

Fig. 2. ACF320 self-association *in vivo is due to RNA-bridging*. (A) FqRET pairs EGFP (donor) and REACh2 (acceptor) fused to ACF320 were co-transfected in HEK 293T fibroblasts. EGFP was always N-terminal while REACh2 was either C- (A) or N-terminal (C) shown graphically on the far left of each panel. Fluorescence microscopy of cells co-transfected with the indicated ratio of plasmids (indicated below the micrographs). The intensity of fluorescence in pseudocolor increases from blue to white as denoted by the scale left of the images. Percentage of quenching relative to the signal from cells transfected with EGFP-ACF320 alone, (' * ') denotes a statistically significant quenching ($p = 0.018$). Western blot analysis (B, D) of equal microgram amounts lysate from transfected cells were reacted with anti-V5, anti-HA or anti-actin to validate expression of chimeric proteins and similar loading of total cellular protein for each sample. (E) Equal microgram aliquots of whole cell extracts prepared from 293T cells that had been co-transfected with V5-ACF320-EGFP and HA-ACF320-EGFP were resolved by SDS–PAGE and western blotted with V5 or HA reactivity antibodies. (F) Co-immunoprecipitation using a monoclonal antibody recognizing V5 was performed with or without RNase A digestion of the cell extract. Western blots were probed sequentially for HA and the V5 (top right panel) after stripping the blot. The result is representative of two independent experiments.

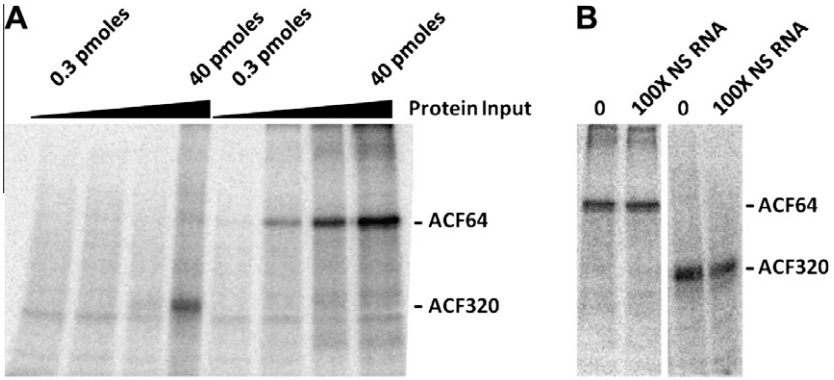

Fig. 3. ACF320 specifically binds *ApoB* RNA. (A) UV cross-linking of ACF to RNA was conducted using increasing molar amounts (0.3, 1.5, 7.5 and 40 pmol) of purified ACF64 or ACF320 by irradiating reactions with 254 nM light, for 5 min, on ice and then digested with RNase A. Samples were resolved by SDS–PAGE and visualized by autoradiography. Results are representative of 3 trials. (B) 40 pmol of ACF64 or ACF320 were cross-linked as in 'A' in the presence or absence of 100-fold molar excess of non-specific (NS) as unlabeled competitor RNA. Results are representative of two trials.

epitope tagged APOBEC-1 (Fig. 4A). APOBEC-1 co-immunoprecipitated with both ACF64 and ACF320 though comparatively, the yield of APOBEC-1 co-immunoprecipitated with ACF320 was much less than that recovered with ACF64 (Fig. 4B).

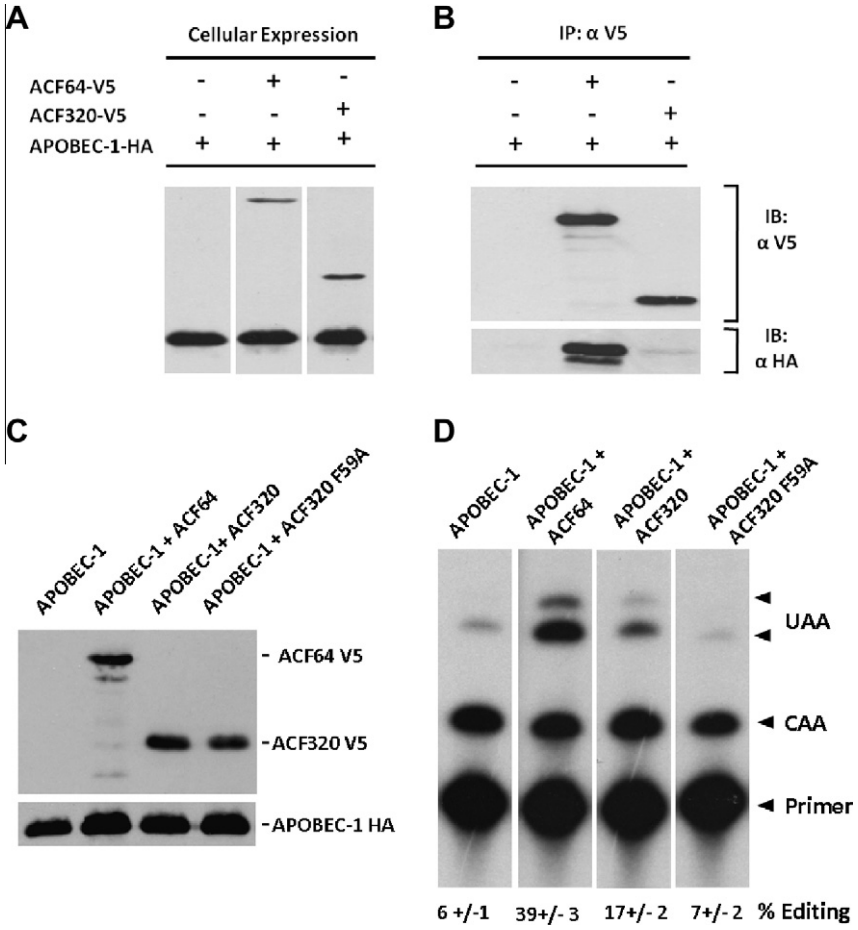

Fig. 4. ACF320 complements *ApoB* editing activity. (A) Equivalent microgram input from HEK 293T whole cell extract were western blotted simultaneously with V5 reactive antibody to detect ACF isoforms and with anti-HA antibodies to detect APOBEC-1 in transfected cells. (B) Co-immunoprecipitation of HA tagged APOBEC-1 with V5 epitope pull-down of ACF64 or ACF320 from extracts in 'C' were western blotted with the respective antibodies. The image is representative of duplicate trials. Extracts from HEK 293T cells co-transfected with APOBEC-1, *apoB* RNA editing reporter and *acf64* or *acf*320 were split into two aliquots. (C) One aliquot was western blotted for the ACF variants with the V5 epitope and APOBEC-1 with the HA epitope. (D) RNA was extracted from the second aliquot and poison-primer extension quantification of RNA editing. UAA represents edited transcript while CAA represents unedited message. Percent editing was calculated by ((UAA)/(CAA + UAA)) × 100, results and SD were calculated based on an $n = 3$.

HEK 293T cells co-transfected with the *apoB* reporter, HA-tagged APOBEC-1 and V5-tagged ACF320 or ACF64 also were evaluated for *apoB* mRNA editing. All transfectants displayed similar expression levels of ACF and APOBEC-1 as observed by western blotting (Fig. 4C). Only background levels of *apoB* RNA editing were observed in the absence of APOBEC-1 and in the absence of exogenous ACF64, APOBEC-1 only supported low levels of editing activity (6% ±1) (Fig. 4D). The highest level of editing complementation (39% ±3) was observed when APOBEC-1 and full length ACF64 were co-expressed. ACF320 complementation of APOBEC-1 editing activity (17% ±2) was approximately half as efficient as that observed with ACF64. The F59A mutant of ACF320 did not complement APOBEC-1, thereby confirming that ACF320 and its *apoB* RNA binding activity supported editing.

4. Discussion

We used biochemical and computational methods to predict a well-folded polypeptide of ACF comprised of the three RRMs. The resulting protein (ACF320) was cloned and expressed as a monodisperse soluble protein. ACF320 bound to *apoB* RNA preferentially, and its interaction with APOBEC-1 was sufficient to complement C to U RNA editing. We show that ACF320 can be isolated as a monomeric subunit with limited protein–protein self-association. However, dimers and higher-order oligomers form in cells through RNA-bridging. Live cell FqRET analysis suggested that RNA-bridged ACF320 subunits might be organized such that their N-termini are proximal.

ACF320 was approximately 27-fold less efficient in binding *apoB* RNA compared with full length ACF64. These data were of interest because ACF320 was 57 amino acids smaller than the minimal truncation mutant previously shown to have both RNA-binding and APOBEC-1 complementation activities and yet it retained approximately >100-fold higher RNA-binding activity than previously tested constructs [9,10]. Prior studies predicted the C-terminal boundary of the three RRM domain in ACF to end at residue 391 based on an alignment only with GRY-RBP [9,10]. Our studies suggested that C-terminal truncations beyond a.a. 391 would expose a disordered region (a.a. 331–377) that may have a negative impact on RNA binding. In this regard, ACF320 was designed to eliminate this disordered region but include an alpha helical region C-terminal to the end of RRM3 (a.a. 301–320).

Yeast two hybrid analyses suggested that ACF64 and its truncated variants [1,2,14,28–30] had the capacity for self-association [14,31]. The RNA dependence of ACF320 self-association makes it tempting to speculate that its mode of binding had structural and functional similarities to the RNA-induced, self-association seen with U1A. Monomers of U1A formed dimers in a parallel orientation

when bound to PIE RNA [32]. The helices C-terminal to U1A RRMs functioned to stabilization U1A binding to RNA. We propose that the mooring sequence RNA-bridged, head-to-head self-association of ACF may be critical for editosome function in that it orients dimers of APOBEC-1 [33–35] to a specific site for C to U editing.

5. Funding

This work was supported in part by a Public Health Services Grant DK043739 and AI058789 awarded to HCS.

Acknowledgment

The authors are grateful to Jenny Smith for assistance in figure preparation.

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