A Novel Nuclear Localization Signal in the Auxiliary Domain of Apobec-1 Complementation Factor Regulates Nucleocytoplasmic Import and Shuttling*

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C to U editing of the nuclear apolipoprotein B (apoB) transcript is mediated by a core enzyme containing a catalytic deaminase, apobec-1, and an RNA binding subunit, apobec-1 complementation factor (ACF). ACF expression is predominantly nuclear, including mutant proteins with deletions of a putative nuclear localization signal. We have now identified a novel 41-residue motif (ANS) in the auxiliary domain of ACF that functions as an authentic nuclear localization signal. ANS-green fluorescence protein and ANS-green fluorescent protein chimeras were both expressed exclusively in the nucleus, whereas wild-type chimeras or an ACF deletion mutant lacking the ANS were cytoplasmic. Nuclear accumulation of ACF is transcription-dependent, temperature-sensitive, and reversible, features reminiscent of a shuttling protein. ACF relocates to the cytoplasm after actinomycin D treatment, an effect blocked by the CRM1 inhibition of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. * This work was supported by National Institutes of Health Grants HL-38180 and DK-56260 and Digestive Disease Research Core Center Grant DK-52574 (particularly the Morphology Core). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. ‡ To whom correspondence should be addressed. Fax: 314-362-2033; E-mail: nod@im.wustl.edu. § The abbreviations used are: apoB, apolipoprotein B; ACF, apobec-1 complementation factor; NLS, nuclear localization sequence; GFP, green fluorescence protein; Trn2, transportin 2; GST, glutathione S-transferase; AMD, actinomycin D; LMB, leptomycin B; DAPI, 4,6-diamidino-2-phenylindole; hnRNP, heterogeneous nuclear ribonucleoprotein; HNS, HuR nuclear localization signal; NES, nuclear export sequence; ANS, ACF nuclear localization sequence; SV-40, simian virus 40.

required in order for this minimal complex to mediate C to U RNA editing of a synthetic apoB transcript in vitro (4, 5). Physiological activity of these two core components and in vivo apoB mRNA editing efficiency may be modified through higher order interactions with other proteins that represent elements of a larger, holoenzyme complex (6–8).

The tissue-specific expression pattern and subcellular distribution of both ACF and apobec-1 has been explored in some detail (2, 3, 9, 10). Independent studies using epitope-tagged protein expression in vitro have established that ACF localizes almost exclusively to the nucleus (4, 7). These findings were corroborated in recent studies, demonstrating a marked increase in endogenous, nuclear ACF immunostaining and protein content accompanying metabolic induction of apoB mRNA editing activity after insulin or thyroid hormone administration in vivo in rodent hepatocytes (11, 12). On the other hand, apobec-1 is found in a predominantly cytoplasmic or perinuclear distribution in transfected cells (4, 7, 8, 13). In examining the cytoplasmic distribution of apobec-1, recent studies concluded that neither the putative bipartite nuclear localization sequence (NLS) nor the predicted nuclear export signal in the carboxyl terminus of apobec-1 was functional in directing import or export of a reporter (14). Thus, the mechanism by which apobec-1 is transported to the nucleus and the signals by which this process is directed remain unknown. Because C to U editing of the nuclear apoB transcript requires a physical interaction between ACF and apobec-1 (4), these two proteins must presumably exist in the same subcellular compartment (i.e. the nucleus) to effect deamination of the targeted base in apoB mRNA (13). This assumption is supported by the demonstration that co-expression of apobec-1 with ACF results in the redistribution of both ACF and apobec-1 to the nucleus (4, 7). By contrast, co-expression of an ACF mutant that lacks the apobec-1 interaction domain along with wild-type apobec-1 reveals that apo- bec-1 remains cytoplasmic, whereas the ACF mutant localizes to the nucleus (4). These observations raise the possibility that, in addition to its requisite function in the enzymatic catalysis of C to U deamination, ACF may also regulate apoB mRNA editing by controlling nuclear accumulation of apobec-1 through protein-protein interactions and directed intracellular trafficking.

A putative, SV-40 type NLS motif (PKTKKKRE) is present in the amino-terminal portion of ACF, overlapping the second RNA recognition motif (RRM2). Point mutations or deletion of this motif, however, failed to alter nuclear distribution of the mutant ACF, suggesting that another active NLS domain directs nuclear import (4). In this study we have characterized a functional NLS located in the auxiliary domain of ACF. The function of this motif and the regulation of nuclear-cytoplasmic transport of ACF and the ACF-apobec-1 complex is the focus of this report.
MATERIALS AND METHODS

Plasmid Constructs and Protein Expression—The construction of the ACF deletion mutants has been previously described (4). The ΔN35-ACF deletion fragment was cloned into the eukaryotic expression vector pCMV2B using the restriction sites BamHI and XhoI. All the green fluorescence protein (GFP) fusion proteins were constructed following a two-step PCR method (15). The full-length PCR products were sequenced and subcloned into the eGFP-N1 vector (Clontech) using KpnI-BamHI sites. This cloning generates an NH2-terminal GFP fusion protein. The ANS-β-galactosidase fusion was constructed following a two-step PCR strategy using pTYB1-ACF and pSV-β-galactosidase vector (Promega) as template. The full-length PCR product was inserted into pCMV2B vector (Stratagene) using the BamHI-XhoI restriction sites. Importin-α, transportin 1, and transportin 2 (Trn2) were cloned following PCR amplification and inserted into pCMV2B or -3B vectors (Stratagene). This cloning resulted, respectively, in the expression of an NH2-terminal FLAG or Myc-tagged fusion protein. Trn2 recombinant protein was expressed as a GST fusion (a generous gift of I. Gallouzi, McGill University) and purified to homogeneity using glutathione-Sepharose 4B affinity chromatography as recommended by the manufacturer (Amersham Biosciences). After extensive washes, the recombinant protein was eluted in the presence of 50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 120 mM NaCl, 0.1% Triton X-100, and protease inhibitors and dialyzed against Dignam D buffer (7).

Protein-Protein Interaction Studies—COS-7 cells were transiently transfected with 2 μg of plasmid encoding FLAG-tagged wild-type ACF, ΔN35-ACF, or HuR (a generous gift of I. Gallouzi, McGill University). 48 h post-transfection, cell lysates were prepared in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and 1 mM EDTA, and pSV-β-galactosidase inhibitor mix. Each extract was treated with RNase A for 30 min at room temperature and loaded on GST or GST-Trn2-coupled glutathione-Sepharose beads for 12 h at 4 °C. The unbound material was washed with 20 μl HEPES, pH 8.0, 100 mM KCl. Bound proteins were eluted with 2× Laemmli loading buffer, fractionated through SDS-10% PAGE, and analyzed by Western blotting using rabbit anti-FLAG antibody (Affinity BioReagents, Inc.). In vitro translation of epitope-tagged constructs encoding importin-α, transportin 1, transportin 2, FLAG-tagged ACF, ΔN35-ACF, or FLAG-ANS was conducted as described previously using T7 coupled reticulocyte lysate (Promega). 35S-Radiolabeled Trn2 protein was mixed with unlabeled FLAG-tagged ACF, ΔN35-ACF, or ANS, and the complex was recovered by immobilization on immune-coupled protein A beads (4, 7).

Transfection, Immunofluorescence, and Confocal Microscopy—COS-7 cells, HeLa cells, and NIH3T3 (ATCC) were maintained in Dulbecco's modified Eagle's medium as described previously (7). Where indicated, cells were incubated with actinomycin D (AMD) (Sigma) at 10 μg/ml for 3 h or as indicated in the relevant figure legend. In some experiments, cells were transfected with leptomycin B (LMB) at 10 ng/ml (a generous gift from Dr. Minoru Yoshida, RIKEN Institute, Japan) (16, 17) for 6 h before fixation and staining. Cycloheximide (Sigma) was used where indicated for 3 h or as indicated in the relevant figure legend. In some experiments, cells were treated with leptomycin B (LMB) at 10 ng/ml (a generous gift from Dr. Minoru Yoshida, RIKEN Institute, Japan) (16, 17) for 6 h before fixation and staining. Cycloheximide (Sigma) was used where indicated for 3 h or as indicated in the relevant figure legend. In some experiments, cells were transfected with 2 μg of appropriate eukaryotic expression vector using 6 μl of FuGENE 6 (Roche Molecular Biochemicals). 48 h after transfection, cells were fixed with 10% formalin solution (Sigma), permeabilized with 0.5% Triton X-100, and probed with rabbit anti-FLAG IgG and/or mouse monoclonal anti-Myc IgG followed by Cy3 secondary antibody or fluorescent isothiocyanate-conjugated secondary IgG (Jackson ImmunoResearch). For standard immunofluorescence microscopy, nuclei were identified using 4',6-diamidino-2-phenylindole (DAPI, Vector). Slides were examined using a Zeiss Axiaiphot 2 MOT microscope equipped with a 40× plane neofluar objective and a 3-CCR camera (DAGE-MTI, Inc.). A Zeiss Astrocarr variable intensity lamp was used with filter sets configured for Cy3 and 4,6-diamidino-2-phenylindole (DAPI, Vector). For confocal microscopy, nuclei were detected using TO-PRO3 iodide (Molecular Probes, Oregon). Preparations were visualized using a 63× Zeiss plane apochromatic 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, 4–8 images at planes separated by 0.5 μm were collected. Images were processed using Adobe Photoshop 4.0 software.

Heterokaryon Assay—The interspecies human-mouse heterokaryons were generated as described previously (18) with minor modifications. Briefly, HeLa cells grown on 60-mm dishes were transfected with 3 μg of plasmid expressing amino-terminal FLAG-tagged ACF using 25 μl Polyfect (Qiagen). 24 h after transfection, HeLa cells were seeded onto coverslips in 35-mm dishes at 3 × 104 cells/coverslip. After overnight incubation, NIH3T3 cells preincubated for 30 min in the presence of 100 μg/ml cycloheximide were seeded onto the same coverslips at 3 × 104 cells/coverslip. The co-cultures were grown for 3 h with 100 μg/ml cycloheximide and then were fused in the presence of 50% polyethylene glycol 3350 (Sigma) for 3 min. Cells were washed with phosphate-buffered saline, cultured in the same medium containing 100 μg/ml cycloheximide for 3 h, and fixed for immunostaining as described above. To visualize the HeLa-NIH3T3 heterokaryon, Hoechst dye 33258 (Sigma) was used at 1 μg/ml during the incubation with the secondary antibody to permit discrimination between human and murine nuclei (18).

RESULTS

ACF Has a Non-conserved Nuclear Import Sequence in the COOH-terminal Auxiliary Domain—Previous studies demonstrated that deletion of the putative SV-40 type NLS in the amino-terminal domain of ACF (2, 3) (Fig. 1A) failed to alter its nuclear distribution (4). Thus, to identify the active NLS within ACF we constructed a series of deletion mutants spanning the entire protein (Fig. 1B). Each mutant was transiently expressed in COS-7 cells, and its intracellular distribution was examined by immunofluorescence microscopy. Most of these deletion mutants revealed a nuclear staining pattern indistinguishable from wild-type ACF (Fig. 1B). However, there were two exceptions, mutants Δ-(331–385) and Δ-(380–402), which appeared cytoplasmic (Fig. 1B). These results suggested that the region spanning residues 331–402 may contain an NLS. Further deletion mapping (data not shown) indicated that the region spanning residues 360–401 contains the minimal nuclear localization sequence. A mutant ACF construct with an internal deletion spanning residues 360–401 showed a cytoplasmic, perinuclear localization pattern when expressed in COS-7 cells (data not shown). Sequence alignment of the region spanning residues 360–401 revealed no clusters of basic residues suggestive of a canonical SV-40 type or bipartite NLS. Furthermore, this region revealed no homology with either the NLS consensus of heterogeneous nuclear ribonucleoproteins (hnRNP) A1 or hnRNP K (M9 and KNS, respectively) or with the HNS (HuR nuclear localization signal) in HuR (Fig. 1C). We will hereafter refer to this apparently novel motif (residues 360–401 of ACF) as ANS for ACF nuclear localization sequence.

The Nuclear Localization Signal of ACF Is Functional—To confirm that the ANS domain is functional, we examined its capacity to restrict nuclear expression of GFP. Expression of wild-type GFP revealed diffuse fluorescence throughout the cell, including the cytoplasm and nucleus (Fig. 2a). When expressed as a (331–402)-GFP fusion protein, fluorescence was restricted to the nucleus (Fig. 2b). This observation suggests that the ANS domain is biologically active in nuclear targeting. We further investigated if the minimal ANS (i.e. residues 360–401) could function to target a large, heterologous protein such as β-galactosidase to the nucleus. Accordingly, an epitope-tagged ANS-β-galactosidase chimera was expressed, and its subcellular localization was determined by examining both protein activity and immunohistochemical distribution. The wild-type construct demonstrated the expected perinuclear distribution, whereas the ANS-β-galactosidase chimera was efficiently targeted to the nucleus (Fig. 3, A and B). These findings collectively suggest that the ANS functionally directs protein import into the nucleus, both in cis as well as in trans.

ACF Can Redistribute between the Nucleus and Cytoplasm—Sequence alignment and phylogenetic analysis suggest that ACP is distantly related to hnRNPs, some of which continuously shuttle between the nucleus and cytoplasm (19). These studies used the RNA polymerase II inhibitor AMD to demonstrate transcription dependence of hnRNP shuttling (20). To examine the transcription dependence of ACF distribution,
COS-7 cells were treated with AMD for 3 h, which revealed a shift in distribution of ACF from the nucleus to the cytoplasm (Fig. 4, panel A). There was no effect on the nuclear localization of ACF when cells were treated with cycloheximide (20 μg/ml) (Fig. 4B). These findings suggest that the cytoplasmic accumulation of ACF after AMD treatment was likely the result of transcription inhibition rather than defects in protein synthesis.
reversible upon restoring transcription, as revealed in studies where AMD-treated cells were incubated in an AMD-free medium at 37 °C for 3 h (Fig. 4C). In summary, these data indicate that nuclear accumulation of ACF is a transcription and energy-dependent process.

**ACF Shuttles between the Nucleus and Cytoplasm**—To formally demonstrate nuclear-cytoplasmic shuttling of ACF, we analyzed its migration between nuclei in a human-mouse heterokaryon assay (18, 20). HeLa cells transfected with FLAG-ACF were fused with NIH3T3 cells to produce heterokaryons. Three hours after fusion, ACF was localized by immunofluorescence microscopy within murine nuclei of a representative heterokaryon (Fig. 5, a–c), establishing shuttling as a mechanism to account for ACF migration from the human to the mouse nucleus. Human nuclei were distinguished from the mouse nuclei using Hoechst dye (18). These data demonstrate that ACF, like HuR (Fig. 5, d–f), shuttles between the nuclear and cytoplasmic compartments.

**Nuclear Transport of ACF Involves Interaction with a Member of the Transportin Family**—Distinct shuttling/NLS/NES motifs are recognized by specific protein carriers. Proteins containing a classical NLS motif are bound by the importin α/β complex and directed to the nuclear pore complex (23). Motifs like M9 or KNS in hnRNPs proteins or the HNS motif in HuR interact with transportin family members transportin 1 and transportin 2, respectively (24–26). Our findings to this point suggest that ACF uses a novel NLS for nuclear import. Accordingly, we asked whether ACF could interact with the known carrier proteins, importin-α, transportin-1, and transportin-2. We first performed immunoprecipitation assays in which recombinant ACF was mixed with a panel of [35S]Met-labeled carrier proteins, importin-α, and transportin-1. In summary, these data indicate that nuclear accumulation of ACF is a transcription and energy-dependent process.

**ACF migrates between nuclei in an interspecies heterokaryon assay.** HeLa cells were transiently transfected with FLAG-ACF, fused with NIH3T3 cells to form heterokaryons in the presence of 50% polyethylene glycol, and incubated for an additional 3 h in the presence of cycloheximide. The co-culture was then fixed and immunostained with anti-FLAG IgG. Mouse nuclei (indicated with arrows) were distinguished from human nuclei by Hoechst dye, which reveals mouse nuclei through their punctate pale blue staining compared with the homogenous staining revealed in human nuclei. FLAG-HuR shuttling activity was examined as a positive control.

**Fig. 4. Characterization of ACF nuclear localization activity.** A, nuclear localization activity of ACF depends on RNA polymerase II transcription. FLAG-tagged ACF was transiently expressed into COS-7 cells. Forty-eight hours post-transfection cells were incubated with either AMD at 10 μg/ml (a–d), LMB at 10 ng/ml (e–f), or both actinomycin D and leptomycin B (g–h). The cellular distribution of ACF was then determined by immunofluorescence staining using anti-FLAG IgG. Nuclei were identified by DAPI staining. A control performed without AMD or LMB is shown (a and b). B, cycloheximide (CHX) alone has no effect on the nuclear localization of ACF. As described above, COS-7 cells transiently expressing FLAG-ACF were incubated in the absence (a) or presence (b) of cycloheximide at 20 μg/ml for 3 h. Cells were fixed and immunostained with anti-FLAG IgG. DAPI staining identifies nuclei (c and d). C, cytoplasmic accumulation of ACF upon AMD treatment is temperature-dependent and reversible. B, COS-7 cells transiently expressing FLAG-ACF were incubated in the presence of AMD at 10 μg/ml for 3 h at 37 °C and then fixed and analyzed with anti-FLAG IgG. c, cells were treated following the conditions described for b except that incubation with AMD was performed at 4 °C. d, cells were incubated with AMD for 3 h at 37 °C. After removal of the growth medium, cells were washed and placed in fresh medium without AMD for another 2 h before fixation and immunostaining. Nuclei were identified by DAPI staining (e–h). A control performed at 37 °C in the absence of AMD is shown (a). This is representative of three independent assays.

**Fig. 5. ACF migrates between nuclei in an interspecies heterokaryon assay.** HeLa cells were transiently transfected with FLAG-ACF, fused with NIH3T3 cells to form heterokaryons in the presence of 50% polyethylene glycol, and incubated for an additional 3 h in the presence of cycloheximide. The co-culture was then fixed and immunostained with anti-FLAG IgG. Mouse nuclei (indicated with arrows) were distinguished from human nuclei by Hoechst dye, which reveals mouse nuclei through their punctate pale blue staining compared with the homogenous staining revealed in human nuclei. FLAG-HuR shuttling activity was examined as a positive control.
was determined by Western blotting using anti-FLAG IgG (beads is shown in lane 5). The expression of each epitope-tagged protein was determined by Western blotting using anti-FLAG IgG (left panel). Lane 1, negative control, COS-7 cells transfected with an empty vector. B, FLAG-tagged ACF and Myc-tagged Trn2 were co-expressed in COS-7 cells and analyzed for their cellular distribution by immunostaining using anti-FLAG and anti-Myc IgG. Nuclei were identified using TO-PRO3 staining. The confocal images were merged, revealing colocalization of both proteins at the nuclear envelope.

7) and as expected, accumulated in the cytoplasm of transfected cells (see Fig. 3B). By way of a positive control, the binding of HuR to Trn2 was confirmed in this assay (Fig. 6A, lane 8).

To further confirm the interaction between Trn2 and ACF, we transiently expressed epitope-tagged proteins in COS-7 cells and confirmed their nuclear colocalization (Fig. 6B). The merged images show an overlapping signal at the border of the nuclear envelope, suggesting the presence of ACF associated with Trn2, possibly at the nuclear pore complex. Clearly, however, further studies will be required to examine this possibility in detail. Nevertheless, these data allowed us to examine the hypothesis that transport of ACF requires a physical interaction between the ANS motif and the Trn2 carrier. Accordingly, FLAG-tagged fusion proteins were prepared using wild-type and Δ-ACF mutants and a FLAG-ANS chimera (Fig. 7, A and B). Transportin 2 was expressed in vitro as a 35S-radiolabeled protein and mixed with the indicated unlabeled protein in an immunoprecipitation pull-down experiment to examine the physical interaction between the ANS and Trn2 (Fig. 7C). The data reveal a strong interaction with both wild-type ACF and with the FLAG-ANS recombinants but not with the Δ-ANS mutant of ACF (Fig. 7C). We interpret these findings as evidence of a physical interaction between Trn2 and the ANS domain of ACF.

**FIG. 6.** ACF nuclear localization requires physical interaction between Trn2 and ANS motif. A, COS-7 cells were transiently transfected with plasmids encoding wild-type FLAG-ACF or FLAG-ΔANS ACF. Forty-eight hours post-transfection cell extracts were prepared, treated with RNase A, and loaded on either GST or GST-Trn2-glutathione-Sepharose columns (“Materials and Methods”). The bound proteins were eluted, fractionated through SDS-10% PAGE, and analyzed with anti-FLAG IgG. The interaction of FLAG-HuR with Trn2 was examined as a positive control (lane 8). Control for the absence of nonspecific binding of FLAG-ACF to the GST-glutathione-Sepharose beads is shown in lane 5. The expression of each epitope-tagged protein was determined by Western blotting using anti-FLAG IgG (left panel). Lane 1, negative control, COS-7 cells transfected with an empty vector. B, FLAG-tagged ACF and Myc-tagged Trn2 were co-expressed in COS-7 cells and analyzed for their cellular distribution by immunostaining using anti-FLAG and anti-Myc IgG. Nuclei were identified using TO-PRO3 staining. The confocal images were merged, revealing colocalization of both proteins at the nuclear envelope.

**FIG. 7.** ACF interacts in vitro with Trn2 via the ANS motif. A, schematic representation of each protein used as bait in the immunoprecipitation assays. The deleted portion of ACF is indicated as dashed lines. The resulting protein is identified as FLAG-ΔANS. Numbers below the scheme indicate the amino acids present in the protein. B, FLAG-ANS protein contains the ANS motif encompassing the residues 360–401. B, FLAG-ACF, FLAG-ΔANS, and Trn2 were synthesized in vitro using a coupled TnT (IVTT) lysate in the presence of 35S-methionine. Lanes 1–3 are representative of the products separated on a 10% PAGE-SDS and revealed by autoradiography. Molecular weight markers are indicated on the left. C, co-immunoprecipitation (IP) of Trn2 and ACF. In vitro 35S-radiolabeled Trn2 was mixed with unlabeled FLAG-ACF (lane 1), FLAG-ΔANS (lane 2), or FLAG-ANS (lane 3), respectively. After incubation for 30 min at 30 °C, anti-ACF antibody was added to all reactions, and incubations were performed at 4 °C for 2 h. Protein complexes were collected on protein A-Sepharose beads and resolved by SDS-PAGE. The presence of Trn2 was revealed by autoradiography. A control reaction was performed without unlabeled bait (lane 4). The arrow indicates the position of 35S-radiolabeled Trn2.

**DISCUSSION**

In this study we have characterized a novel 41-residue motif in ACF that functions as an authentic NLS in directing ACF as well as heterologous proteins to the nucleus. In addition, we demonstrate that the transport of ACF into and out of the nucleus is an energy-dependent process that requires active transcription. We demonstrate that ACF shuttles between the nucleus and cytoplasm and that this shuttling likely involves at least two candidate transport proteins, transportin 2 and CRM1.

ApoB mRNA editing is almost certainly a nuclear event. Evidence from earlier in vivo studies using rat liver (27) as well as more recent approaches using reconstituted cell-based assays with splicing-competent or -defective reporter constructs have demonstrated that C to U editing occurs on a spliced but
proteins across the nuclear envelope occurs through nuclear pores and involves several soluble carriers, particularly transportin and other factors recently identified as key regulators of mRNA export (23). CRM1 is a member of the importin-β family and a major RNA export receptor in eukaryotes (34). CRM1 is responsible for the export of shuttling proteins containing a leucine-rich NES, with the most fully characterized being within human immunodeficiency virus 1, Rev (35). No such leucine-rich region or NES-like motif could be identified in the primary sequence of ACF, and thus, the domain responsible for CRM1 interaction remains to be identified. We considered the possibility that apobec-1 contains the interacting leucine-rich NES, although previous work by Yang and Smith (14) demonstrates that apobec-1 failed to accumulate in the nucleus after LMB treatment. Although this possibility will require formal evaluation, our preliminary observations indicate that the nuclear accumulation of ACF observed upon AMD and LMB co-treatment is diminished when apobec-1 is co-expressed; both proteins demonstrated a predominantly cytoplasmic localization pattern.2 We interpret these preliminary observations to suggest that the interaction of apobec-1 with ACF renders the CRM1-interacting domain inaccessible. Under these conditions the cytoplasmic distribution of apobec-1 and ACF implies the existence of an alternative, CRM1-independent pathway for nuclear export, a possibility that will require more complete evaluation in future studies.

A further question raised by our findings concerns the possibility that ACF and apobec-1 are coordinately directed for nuclear import. Bidirectional transport functions, for example, have been ascribed to shuttling motifs within hnRNP A1 and hnRNP K (19, 25). The current findings suggest that the ANS motif is responsible for the nuclear targeting of ACF but leave open the possibility that this motif directs import of the ACF-apobec-1 core enzyme complex. Clearly, the relationship between the shuttling of free ACF versus ACF functionally contained within an apobec-1 core enzyme complex will require further study. However, our findings permit us to conclude that the shuttling of free ACF (Fig. 5) occurs through an LMB-sensitive, CRM1-dependent pathway (Fig. 4).

The current findings also suggest that the ANS domain is responsible for an interaction with transportin 2 and further demonstrate that ACF colocalizes with Trn2 in the nucleus (Fig. 6). These findings raise the possibility that ACF may also exit the nucleus via a CRM1-independent mechanism. In this regard it bears emphasis that the interaction between the ANS of ACF and Trn2 in no way precludes the possibility that Trn2 is involved in the nuclear export of ACF. Precedent exists for diverse pathways of nuclear export of RNA-binding proteins, exemplified by findings with HuR (36). HuR interacts with two nuclear phosphoproteins, pp32 and APRIL, that contain leucine-rich NES motifs and which shuttle via a CRM1-dependent pathway. In addition, HuR is exported to the cytoplasm via a motif referred to as HNS, that interacts with Trn2 and which may be involved in HuR nuclear import as well as its export (26). Further work will be required to resolve the importance of Trn2 in the nucleocytoplasmic distribution of ACF and its relationship to the compartmentalization of the ACF-apobec-1 complex.

Finally, the results of these studies bear directly on the physiological significance of directed nuclear import of apobec-1. Previous studies have established that forced transgenic overexpression of apobec-1 is associated with promiscuous C to U RNA editing, suggesting that mechanisms likely exist in vivo through which the expression or localization of this protein is constrained. These issues take on greater relevance in the context of findings that both apobec-1 and ACF are AU-rich
RNA binding proteins with targets (at least for apobec-1) that extend beyond apoB (37). These and other questions concerning the nucleocytoplasmic distribution of both ACF and the ACF-apobec-1 complex will be the focus of future reports.

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