Nuclear Import and Export Functions in the Different Isoforms of the AUF1/Heterogeneous Nuclear Ribonucleoprotein Protein Family

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The heterogeneous nuclear ribonucleoprotein D family of proteins also known as AUF1 consists of four isoforms implicated in both nuclear and cytoplasmic functions. The AUF1 proteins are largely nuclear but also are found in the cytoplasm and are thought to undergo nucleocytoplasmic shuttling. The nucleocytoplasmic distribution and potential shuttling activity of the individual AUF1 isoforms have not been previously studied in detail. Therefore, we characterized the nucleocytoplasmic transport of each of the heterogeneous nuclear ribonucleoprotein D/AUF1 isoforms. All four AUF1 proteins were found to undergo rapid nucleocytoplasmic shuttling in a manner that is transcription-independent, carrier-mediated, and energy-requiring. Nucleocytoplasmic shuttling of the AUF1 proteins is shown to utilize a novel arrangement of nuclear import and export signals. Mutagenesis of the AUF1 proteins and fusion of polypeptides to a green fluorescent protein reporter demonstrated that a nuclear import signal is located in the C-terminal domain of the protein and is found only in the two smaller isoforms. Further mapping demonstrated that nuclear export is facilitated by sequences in AUF1 exon 7 found in the C-terminal domain of the two larger AUF1 isoforms. A subset of AUF1 proteins are shown to directly interact in vitro using purified recombinant proteins and in vivo in the absence of RNA. These results suggest that nuclear import of AUF1 is facilitated by sequences found only in the two smaller isoforms and that nuclear export is facilitated by sequences (exon 7 and the C-terminal domain) found only in the two larger isoforms. This novel arrangement of signals might represent a mechanism to assure co-shuttling of a subset of AUF1 proteins that interact in a heterocomplex.

Transport of proteins across the nuclear pore complex involves the recognition of specific signal sequences by transport receptors and adapter proteins (reviewed in Ref. 1). Import receptors (importins) and export receptors (exportins) shuttle back and forth across the nuclear pore complex with cargo proteins and other macromolecules. The export receptor exportin 1/CRM1 interacts directly with a leucine-rich nuclear export sequence in the cargo protein or with adapter molecules (1). The best characterized nuclear import receptors are members of the importin-β superfamily of RanGTP-binding proteins. Facilitated nuclear import involves nuclear localization sequences (NLSs) within cargo proteins containing any of several canonical basic amino acid motifs (2). Some cargo proteins utilize non-canonical NLSs for transport such as the M9 domain, which consists of a glycine-rich amino acid sequence that promotes both protein import and export, and therefore functions as a shuttling sequence (3–5). The M9 domain was originally identified in heterogeneous nuclear ribonucleoproteins (hnRNP) A1 and binds to the import receptor, transportin (5).

Apart from hnRNPs C1/C2 and U, all of the other hnRNPs appear to undergo nucleocytoplasmic shuttling (1, 2). hnRNP A1 represents one type of shuttling protein that is a nuclear protein at steady state but shuttles rapidly and accumulates in the cytoplasm with transcription inhibition (3, 6), which is associated with the presence of an M9 domain (3). The requirement for ongoing translocation in the transport of M9-domain-containing proteins is not well understood and is unlikely to simply involve co-transport by binding to mRNAs (7). hnRNP K is another type of shuttling RNA-binding protein. It contains a canonical NLS in addition to a unique shuttling domain known as KNS. Unlike hnRNP A1, the nuclear localization of hnRNP K is independent of transcriptional activity unless the NLS motif is deleted (7).

The hnRNPs D/AUF1 family, similar to many hnRNPs, is comprised of four related isoforms produced by alternate splicing of a single mRNA (Fig. 1A) (8–10). AUF1 consists of a 37-kDa core protein (p37), a 40-kDa protein (p40) with an N-terminal 19 amino acid insertion of exon 2, a 42-kDa protein (p42) with a C-terminal 49 amino acid insertion of exon 7, and a 45-kDa protein (p45) containing both exon 2 and 7 insertions (Fig. 1A) (reviewed in Ref. 10). The different AUF1 isoforms bind various types of ARE sequences, which are associated with rapid decay of short-lived cytokine and proto-oncogene mRNAs (10). The different AUF1 isoforms bind different ARE sequences, which have been attributed to the presence of exon insertions that might alter AUF1 protein conformation (9, 11). Different members of the AUF1 family also appear to have multiple functions including transcriptional activation (12, 13).

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The abbreviations used are: CRM1, chromosomal region maintenance protein 1; p37, 37-kDa core protein; p40, 40-kDa core protein; p42, 42-kDa core protein; p45, 45-kDa core protein; ARE, AU-rich element; AdoX, adenosine-2',3'-diformate; CTD, C-terminal domain; BSA, bovine serum albumin; hnRNP, heterogeneous nuclear ribonucleoprotein; LMB, leptomycin B; NLS, nuclear localization signal; PEG, polyethylene glycol; GFP, green fluorescent protein; EGFP, enhanced GFP, CMV, cytomegalovirus; CHO, Chinese hamster ovary; GST, glutathione S-transferase; RBD, RNA-binding domain.

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and binding to nucleolin (14), telomeric single-stranded DNA (15), and components of the translation initiation apparatus (16). Nevertheless, all four AUF1 isoforms associate with hnRNP A1 (17), which is involved in mRNA transport (18) and splicing (19).

Although AUF1 proteins are found in both the nucleus and cytoplasm, steady state analysis demonstrates a much higher nuclear accumulation of all four proteins (16, 20). Heat-shock or down-regulation of the ubiquitin-proteasome network mediates even greater nuclear accumulation of AUF1 and the increased and subcultured p37 and p40 proteins (16, 21). Therefore, we sought to characterize the mechanisms for AUF1 nucleocytoplasmic shuttling and that the shuttling pathway used by the AUF1 proteins is transcription-independent and carrier-mediated.

We found that the uninterrupted C-terminal domain (CTD) found in the p37 and p40 AUF1 isoforms possesses a strong nuclear import activity, whereas the CTD interrupted by exon 7 promotes cytoplasmic localization, which is inserted in the CTD of the p42 and p45 isoforms. We show that a subset of AUF1 proteins can directly interact in vitro and in vivo in the absence of RNA, which suggests that nuclear import is probably facilitated by the p37 and p40 isoforms, whereas nuclear export is facilitated by the p42 and p45 isoforms as a part of a larger AUF1 complex of proteins.

EXPERIMENTAL PROCEDURES

Cell Culture, Heterokaryon Fusions, Drug Treatments, and Labeling—Cells were maintained in Dulbecco’s modification of Eagle’s medium supplemented with 10% bovine calf serum and 50 μg/ml gentamicin (as described below). Monoclonal anti-hnRNP D (Invitrogen) coupled to fluorescein isothiocyanate or rhodamine (Jackson Immunoresearch Laboratories, West Grove, PA) was generously provided by Gideon Dreyfuss (University of Pennsylvania). Antibodies were passed over immobilized GST (Amersham Biosciences) and then affinity-purified using the p37 resin. Serums were passed over immobilized GST (Amersham Biosciences) and then affinity-purified using the p37 resin.

Steady State Distribution of AUF1 Protein Isoforms—All of the recombinant proteins were expressed in BL21(DE3) Escherichia coli cells. Bacterial lysate purification and purification with glutathione-Sepharose 4B was performed as recommended by the manufacturer (Amersham Biosciences). His-tagged AUF1 proteins were purified using TALON resin (Clontech). Protein concentrations and purity were determined by Coomassie Blue staining and comparison to a titration of BSA.

Antibody Production and Purification—Rabbits were immunized with GST-p37 following a standard protocol (Cocalico Biologicals, Inc.). Thrombin cleavage of the GST moiety produced nearly full-length p37 protein, which was then coupled to Affi-Gel-10 (Bio-Rad). Crude antisera were passed over immobilized GST (Amersham Biosciences) and then affinity-purified using the p37 resin.

In Vitro AUF1 Protein Interaction Analysis—Recombinant proteins were incubated in 100 μl of binding buffer (10 mM Hepes-KOH, pH 7.5, 100 mM potassium acetate, pH 7.5, 5 mM magnesium acetate, 0.5% Nonidet P-40, 0.1 mg/ml BSA) for 1 h at 4 °C. 15 μl of glutathione-Sepharose 4B slurry was then added followed by incubation for 1 h at 4 °C. Bound proteins were washed three times with binding buffer, eluted by boiling in SDS-loading dye, and then resolved by 10% SDS-PAGE. Immunoblotting was performed using polyclonal anti-GST antibody from Santa Cruz Biotechnology and monoclonal anti-T7 antibody from Novagen.

In Vivo AUF1 Protein Interaction Analysis—CHO cells were transfected with vectors expressing GFP-p37 AUF1 and FLAG-tagged p37, p40, p42, or p45 AUF1 proteins as described above. Cells were incubated with buffer (100 mM NaCl, 20 mM Hepes, pH 7.5, 2.5 mM magnesium chloride, 1 mM dithiothreitol, 10 mM NaF, 1 mM sodium orthovanadate, 2.5 mM β-glycerophosphate, 0.5% Nonidet P-40, supplemented with Complete protease inhibitor (Roche Applied Science) and then harvested using Nonidet P-40 lysis buffer (150 mM NaCl, 20 mM Hepes, pH 7.5, 0.5% Nonidet P-40, supplemented with Complete protease inhibitor (Roche Applied Science)). Cells were disrupted by four passages through a 29-gauge needle followed by incubation on ice for 20 min. This procedure disrupts nuclei and produces whole cell lysates that contain soluble nuclear and cytoplasmic protein. Lysates were cleared by microcentrifugation at 4 °C for 15 min. Protein concentrations were determined using the Bradford assay (Bio-Rad).

Plasmids—FLAG-AUF1 constructs were made by PCR amplification of each AUF1 isoform cDNA to generate a HindIII site upstream of the initiating AUG codon and an EcoRI site downstream of the termination codon. PCR products were purified by gel-electrophoresis (Qiagen), digested with HindIII and EcoRI, and then ligated into pFLAG-CMV-2 (Sigma) digested with the same enzymes. The resulting construct contains the 9-amino acid FLAG epitope fused in-frame to the N terminus of each AUF1 coding region. The truncation mutants, FLAG-p37(CTD) and FLAG-p40(CTD), were created by digesting FLAG-p37 or FLAG-p42 plasmids using a 5′-HindIII primer and the 3′-EcoRI primer into pEGFP-C3 (Clontech) digested with the same enzymes. Plasmids encoding glutathione S-transferase (GST) fusions of each AUF1 isoform were also constructed by PCR cloning. An EcoRI site upstream of the initiating AUG codon was introduced as well as a XhoI site downstream of the termination codon. PCR products were purified by gel-electrophoresis, digested with EcoRI and XhoI, and then cloned into pGEX-4T-3 (Amersham Biosciences) digested with the same enzymes. To generate His-tagged AUF1 proteins, cDNAs were PCR cloned into pET-23b (Novagen) using the BamHI and XhoI sites, allowing in-frame fusion with the N-terminal T7 tag and the C-terminal His tag. Specific details of any oligomer are available upon request. Each reading frame was confirmed by DNA sequencing. Plasmids encoding Myc-tagged AUF1 isoforms and FLAG-AUF1 were purified using TALON resin (Clontech). Protein concentrations and purity were determined by Coomassie Blue staining and comparison to a titration of BSA.

RESULTS

Steady State Distribution of AUF1 Protein Isoforms—Indirect immunofluorescence staining was carried out using affin-
ity-purified antiserum that recognizes all four AUF1 isoforms. Endogenous AUF1 protein produced a strong nuclear signal with nucleolar exclusion and a weak cytoplasmic signal in CHO and COS-1 cells that was evident only when images were overexposed as shown in Fig. 1B, upper panels. This pattern was independent of different fixation and permeabilization techniques and was also found in HeLa and NIH 3T3 cells (data not shown). To determine whether AUF1 isoforms exhibit differential localization, cDNAs were cloned for all four AUF1 proteins linked to an N-terminal FLAG epitope tag. COS-1 cells were transiently transfected with expression vectors for each isoform. Indirect immunofluorescence staining of the FLAG epitope revealed that all four AUF1 isoforms (at a similar staining intensity) localized largely in the nucleus (Fig. 1B, lower panels), which was independent of the amount of plasmid transfected and the abundance of protein produced (data not shown). Identical results were obtained in CHO, HeLa, and NIH 3T3 cells (data not shown). Therefore, all four AUF1 isoforms localized largely but not exclusively to the nucleus at steady state. Immunoblot analysis of FLAG-AUF1 proteins, HeLa cells transfected as above were lysed and equal amounts of protein were resolved by SDS-PAGE and detected by immunoblot using anti-FLAG antibodies.

Rapid and Facilitated Shuttling of All Four AUF1 Protein Isoforms—The heterokaryon fusion assay was used to determine whether all four AUF1 isoforms possess nucleocytoplasmic shuttling activity. HeLa cells were transfected with Myc-epitope-tagged hnRNP A1, hnRNP C1, or a FLAG-epitope tagged AUF1 cDNA expression vectors, plated on non-transfected murine NIH 3T3 cells, and fused using PEG. Cells were immunostained for the epitope tag 2 h post-fusion and treated with Hoechst 33258 dye to distinguish punctate staining murine nuclei (arrowheads) from human HeLa cell nuclei. Phase-contrast and immunofluorescence images represent typical results observed in three independent experiments. For immunoblot analysis of FLAG-AUF1 proteins, HeLa cells transfected as above were lysed and equal amounts of protein were resolved by SDS-PAGE and detected by immunoblot using anti-FLAG antibodies.
transfected HeLa cell nuclei. Identical results were obtained using COS-1 cells instead of HeLa cells to donate FLAG-AUF1 proteins (data not shown), indicating that AUF1-shuttling activity is a rapid and general phenomenon. FLAG-AUF1 protein, Myc-C1, and Myc-A1 proteins were identified in equal amounts of whole cell lysates and shown to be undegraded and of proper molecular weight.

The nucleocytoplasmic movement of AUF1 isoforms could occur by diffusion or by facilitated transport. Because at 4 °C both receptor-mediated nuclear import and export are inhibited whereas diffusion is unaffected (3), nuclear localization at 4 °C indicates use of a receptor-binding pathway, whereas cytoplasmic localization implies passive diffusion. As a control for receptor-mediated transport, a plasmid expressing the GFP fused to the SV40 NLS was used (24). Nuclear import of a GFP-NLS control was blocked at 4 °C, and diffusion was unopposed, allowing cytoplasmic accumulation beyond nuclear borders (data not shown), whereas endogenous AUF1 protein localization at 4 °C was unchanged from that at 37 °C, indicating that AUF1 export is energy-dependent (data not shown).

**Transcription and CRM1-independent Transport of AUF1 Protein**—Transcriptional inhibition alters the subcellular localization of many but not all RNA-binding proteins. Several hnRNP family members, HuR, and poly(A)-binding protein 1, which contain non-canonical NLSs, are dependent on transcription for nucleocytoplasmic shuttling (1). Studies also indicate an active role for transcription acting on the M9 domain itself in hnRNP A1 (25). Therefore, cells were treated with actinomycin D or vehicle control for 4 h to inhibit transcription prior to immunostaining to determine whether endogenous AUF1 protein localization is dependent on active transcription. As expected, Myc-tagged hnRNP A1 demonstrated a clearly visible and significant increase in cytoplasmic accumulation with transcriptional inhibition (Fig. 3, compare actinomycin D (Act. D) −/+ panels). There was no such change observed in endogenous AUF1 distribution with transcription inhibition, although nucleolar exclusion was lost. Identical results were obtained for FLAG-AUF1 proteins expressed from transfected plasmids, which was independent of the concentration of actinomycin D or increasing the time of treatment to 6 h (data not shown). Therefore, localization of AUF1 proteins is independent of ongoing transcription.

LMB inhibits CRM1-mediated nuclear export (1). LMB was used to determine whether AUF1 proteins are exported from the nucleus to the cytoplasm in a CRM1-dependent manner. Cells were transiently transfected with vectors expressing either FLAG-AUF1 or GFP-IxB fusion protein whose localization has been shown to be sensitive to LMB treatment (26). Cells were then treated with LMB or vehicle control for 1 h prior to immunostaining endogenous or transfected AUF1 proteins as described previously (26). Images were somewhat overexposed to make cytoplasmic AUF1 protein more readily detectable. Endogenous AUF1 and FLAG-p37 proteins displayed low level cytoplasmic staining, which was unchanged by LMB treatment (Fig. 4, AUF1 panel). GFP-IxB showed a marked increase in nuclear staining and decreased cytoplasmic staining compared with mock-treated cells. Collectively, these results indicate that AUF1 is exported from the nucleus via a CRM1-independent and transcription-independence mechanism.

**AUF1 Nuclear Uptake Is Mediated by the Uninterrupted C-terminal Domain**—The M9 domain of hnRNP A1 is sufficient to mediate transportin binding and nuclear import (28). It is loosely characterized as a region that is rich in glycine, arginine, and asparagine (25), although many transportin-binding proteins contain little if any homology to the M9 consensus sequence (1, 28). Transportin reportedly interacts *in vitro* with the CTD of AUF1 proteins, although only the p42 and p45 isoforms were tested (28). The role of the AUF1 CTD in transport is unknown. All four AUF1 isoforms share a common CTD but it is interrupted in the p42 and p45 isoforms by insertion of exon 7 (see Fig. 1A). Therefore, we created truncation mutants of FLAG-p37 and FLAG-p40 to determine whether the uninterrupted 35-amino acid CTD mediates AUF1 nuclear uptake (Fig. 5A). *In vitro* binding studies showed that deletion of the CTD does not affect the ARE binding ability of AUF1 proteins (29). Deletion of the CTD in FLAG-p37ΔCTD and FLAG-p40ΔCTD shifted AUF1 distribution to a predominantly cytoplasmic localization compared with predominantly nuclear distribution for wild type p37 and p40 (Fig. 5B). Thus, the CTD of the two smaller AUF1 isoforms promotes nuclear import or, less likely, retention. It is very unlikely that deletion of the CTD altered AUF1 protein localization because of misfolding of the protein, because similar mutational analysis showed that AUF1 was still capable of binding RNA and therefore retained activity (29). Inspection of the CTD of AUF1 indicates a weak similarity to the M9 domain: a 12-amino acid glycine-arginine-rich sequence (AUF1 M9-like motif, WGRSGGGFAGGR; consensus M9 domain, Y(F/W)V/XJXSSXXG(P/K)/O(M/L)V/(K/R)) and several adjacent RGG repeats that might serve as RNA-binding sites. Therefore, a small deletion was introduced in FLAG-p37 to specifically excise the potential transportin-binding site (Fig. 5A, Flag-p37Δ12). Compared with wild type FLAG-p37 protein, FLAG-p37Δ12 displayed increased cytoplasmic accumulation but was clearly still retained in the nucleus as well (Fig. 5B). Immunoblot analysis of equal amounts of whole cell lysates confirmed that each AUF1 mutant was expressed at levels similar to wild type (Fig. 5C). Thus, these results indicate that a potential transportin-binding site in the CTD is probably involved in nuclear uptake of AUF1 proteins but that additional CTD elements also participate in AUF1 nuclear import or retention.

The entire CTD that was deleted in the FLAG-p37ΔCTD mutant contains three RGG motifs, which can act as accessory
unchanged compared with untreated cells (Fig. 6A). Fixation of cells and immunostaining following identical experimental conditions demonstrated that AUF1 localization was dramatically reduced overall cellular protein methylation (Fig. 6B). Treatment with AdOX resulted in a reduction of overall cellular protein methylation. The position of co-electrophoresed protein molecular mass markers (not shown) is included. B, CHO cells were treated with or without 100 μM AdOX for 24 h and then fixed, immunostained for endogenous AUF1 proteins, and imaged.

**FIG. 6.** AUF1 localization is independent of arginine methylation. A, CHO cells were untreated or treated with 100 μM AdOX for 24 h and then metabolically labeled during the last 3 h of treatment with 10 μCi/ml [3H]methionine in the presence of 100 μg/ml cycloheximide and 40 μg/ml chloramphenicol. 200 μg of whole cell lysate were resolved by SDS-PAGE and fluorographed. The position of co-electrophoresed protein molecular mass markers (not shown) is included. B, CHO cells were treated with or without 100 μM AdOX for 24 h and then fixed, immunostained for endogenous AUF1 proteins, and imaged. demonstrate that the uninterrupted CTD of the p37 and p40 AUF1 isoforms directs nuclear accumulation or retention. However, the p42 and p45 AUF1 isoforms contain a 49-amino acid exon 7 insert, which interrupts the CTD. Therefore, GFP fusion proteins were created to determine whether interruption of the CTD by exon 7 affects its nuclear localization function. Full-length p37 AUF1 fused to the C terminus of GFP served as a control and was compared in transfected cells to GFP fused to the uninterrupted p37 CTD or the CTD containing the exon 7 insert (Fig. 7A). Immunoblot analysis of whole cell extracts indicated that all of the GFP proteins were expressed and accumulated to levels within 3-fold of each other (Fig. 7B). GFP-p37 localized largely to the nucleus (Fig. 7C) similar to native AUF1, whereas GFP-p37 alone was distributed in both the nucleus and cytoplasm. GFP-CTD, which contains the interrupted p37 C terminus, displayed a predominantly nuclear-staining pattern with slight cytoplasmic decoration as expected from the results of Fig. 5. In contrast, GFP-CTD/exon 7, which contains the p42/p45 C terminus, demonstrated strong cytoplasmic localization but retained some nuclear distribution. This pattern indicates that within the context of the CTD, exon 7 promotes either nuclear export or cytoplasmic retention. Because additional mutagenesis of the p37 AUF1 protein, including deletion of most of the N terminus, failed to reveal additional import or export signals (data not shown), these data in combination with those of Fig. 5 indicate that the interrupted CTD with exon 7 sequences promotes cytoplasmic accumulation, either by impairing the nuclear import activity of the uninterrupted CTD or by actively facilitating nuclear export.

**AUF1 Isoforms Interact both in Vitro and in Vivo—**All four AUF1 isoforms display similar steady state cellular distributions and nucleocytoplasmic shuttling behavior. The interrupted CTD in p37 and p40 isoforms promotes strong nuclear import or retention, and the CTD/exon 7 configuration promotes p42 and p45 nuclear export or interferes with nuclear import. Thus, the nuclear import and export functions in the two smaller and two larger AUF1 proteins are segregated. However, if either of the two smaller AUF1 isoforms interacts with either of the two larger AUF1 isoforms, the p42 and p45 proteins could promote nuclear export of the p37 and p40 isoforms, which could in turn provide a nuclear import signal in trans. This possibility was investigated in vitro and in vivo. An in vitro binding assay was performed using purified recombi-
nant AUF1 proteins expressed in E. coli as GST fusions or tagged with a hexahistidine and T7 epitope. A previous study (46) shows that appending a foreign epitope to the N terminus of AUF1 does not inhibit AUF1 interactions. Following independent incubation of each GST-AUF1 isoform with each of the four His/T7-tagged AUF1 isoforms, glutathione-Sepharose was used to recover the GST-AUF1 isoform and any interacting AUF1 proteins, which were resolved by SDS-PAGE and detected by immunoblotting for T7 or GST (Fig. 8A). Equal amounts of His/T7-AUF1 and GST-AUF1 isoforms were used as shown, although some degradation of the GST fusion isoforms was evident. Proteins were identified by immunoblotting with anti-T7 or anti-GST antibodies. In control studies, GST alone failed to bind any AUF1 isoforms. Each GST-AUF1 fusion protein interacted (to different extents) with other members of the AUF1 family in vitro (Fig. 8A, a’T7 panels). The strongest interaction was for GST-p37 with the His/T7-p37 protein (i.e. p37 with itself), although each isoform interacted with other isoforms. The trans-isooform interaction was strongest between p37 and p42 or p45 (GST-p42 or GST-p45 with His/T7-p37). p40 AUF1 was the least interactive isoform, the significance of which is not known.

Studies were next carried out to determine whether the smaller (p37and p40) and larger (p42 and p45) AUF1 isoforms directly interact in vivo. GFP-p37 was co-expressed with FLAG-p37, FLAG-p40, FLAG-p42, or FLAG-p45 proteins. There were lower levels of FLAG-p37 and FLAG-p40 proteins than FLAG-p42 or FLAG-p45 (Fig. 8B) as expected because of the greater turnover of the two smaller isoforms (16). FLAG-AUF1 proteins were immunoprecipitated from equal amounts of whole cell extracts with or without RNaseA treatment to destroy RNA bridging (16). Proteins were resolved by SDSPAGE and probed with antibodies to determine the level of immunoprecipitated AUF1 isoforms. The position of the immunoglobulin heavy chain is indicated (μg). Asterisks indicate positions of FLAG-AUF1 isoforms.

**FIG. 7.** Exon 7 encodes a nuclear export sequence. A, schematic representation of fusion proteins between GFP and p37 AUF1, the AUF1 CTD, and the AUF1 CTD-containing exon 7 sequences. B, COS-1 cells were transfected with GFP fusion vectors, and equal amounts of whole cell lysates were subjected to immunoblot analysis using a GFP-specific antibody. C, COS-1 cells were transfected with GFP fusion vectors, fixed 24 h later, and imaged.

**FIG. 8.** AUF1 protein interaction. A, 1 μg of each recombinant purified GST-AUF1 fusion protein (GST-p37, GST-p40, GST-p42, or GST-p45) or GST alone was incubated with 0.3 μg of each isoform of a His/T7-AUF1 fusion protein for 1 h at 4 °C. GST-AUF1 proteins were recovered with glutathione-Sepharose, resolved by 10% SDS-PAGE, and immunoblotted with a T7-specific antibody to detect each interacting His-AUF1 protein isoform (upper panels). Blots were stripped and reprobed with a GST-specific antibody (lower panels) to visualize recovered GST-AUF1 protein. Inset, equal amounts of input His/T7-AUF1 proteins were used in the interaction assays. B, CHO cells were co-transfected with vectors expressing GFP-p37 AUF1 and either FLAG vector alone (−) or FLAG-p37, FLAG-p40, FLAG-p42, or FLAG-p45. Equal amounts of whole cell lysates were resolved by SDS-PAGE and immunoblotted with anti-FLAG antibodies to determine the total level of each FLAG-AUF1 isoform or with anti-GFP antibodies to determine GFP-p37 AUF1 protein levels. C, FLAG-AUF1 was immunoprecipitated from samples described above with or without 100 μg/ml RNaseA and resolved by SDS-PAGE. Immunoblots were probed with antibodies to GFP for interacting GFP-p37 AUF1 or FLAG epitope to determine the level of immunoprecipitated AUF1 isoforms. The position of the immunoglobulin heavy chain is indicated (μg). Asterisks indicate positions of FLAG-AUF1 isoforms.
with FLAG-AUF1, indicating that binding is not through the GFP moiety (data not shown). Although a comprehensive characterization of the AUF1 isoform-binding preferences and hierarchy of interactions are currently in progress, these data demonstrate interaction both in vitro and in vivo between the two larger exon 7-containing AUF1 isoforms (p42 and p45) and the two smaller AUF1 isoforms that lack exon 7 (p37 and p40). Thus, a subset of the different AUF1 proteins form higher molecular weight heterocomplexes that contain signals for both nuclear import and nuclear export.

**DISCUSSION**

The aim of our study was to determine whether the four AUF1 proteins differ in cellular localization or shuttling activity. It is not surprising that the steady state localization of AUF1 is predominantly nuclear, because AUF1 was also identified as a member of the hnRNPs (17) using an antibody that recognizes all four isoforms (33, 34). Although AUF1 shuttling activity was first described several years ago (34), it was not possible at that time to determine the shuttling activity of individual AUF1 family members, particularly the least abundant p37, which is most strongly linked to ARE-mRNA decay. We have now investigated the nucleocytoplasmic movement of all of the AUF1 isoforms.

We show that all four AUF1 proteins rapidly shuttle and do so in a transcription-independent manner (Fig. 2, 3), that nuclear import is LMB-insensitive and therefore CRM1-independent (Fig. 4), and that nuclear export is not regulated by arginine methylation (Fig. 6). We found that the uninterrupted CTD of the p37 and p40 isoforms facilitated nuclear uptake, whereas the p42 and p45 CTD, which is interrupted by exon 7, promoted cytoplasmic accumulation, either by impairing CTD nuclear uptake function or by directly facilitating cytoplasmic transport (Fig. 7). Mutational analysis also showed that a putative transportin-binding site in the CTD is involved in cytoplasmic accumulation but is not sufficient (Fig. 7). The previous observation that the CTD of p42 and p45 can bind transportin in vitro strongly suggests that the AUF1 proteins are also substrates of transportin (25). While our studies were in progress, Kawamura et al. (35) examined the localization of the hnRNP D-like JKT-binding proteins. Similar to our results, they identified a localization motif in a C-terminal 25-amino acid segment of the JKT-binding protein. This domain is 72% identical to the CTD of AUF1 and mediates transportin binding. However, in contrast to AUF1, the JKT-binding proteins exhibited differential shuttling activity that is sensitive to transcriptional inhibition. Thus, structural organization of the hnRNP D-like proteins may be significantly conserved, but different mechanisms exist for transport of these various proteins. Because transportin shuttling is independent of transcription (36), it is possible that transportin also mediates nuclear export of AUF1 but probably in conjunction with other export receptors.

One study (37) reported that only the p37 and p40 isoforms shuttle, that this activity is transcription-dependent, and that the p42 and p45 AUF1 proteins are bound to the nuclear matrix, scaffold attachment factor-B, which is itself a stationary hnRNP (38). It should be noted, however, that hnRNP proteins are generally associated with the nuclear matrix, including hnRNPs that shuttle (39). Thus, the association of hnRNP D proteins with scaffold attachment factor-B is not in itself indicative of nuclear retention. Moreover, a recent study (40) has shown that the class of nuclear messenger RNPs, which do not contain stationary hnRNPs, includes p37 and p40 AUF1 proteins (40), consistent with our results. Additionally, biochemical fractionation of AUF1 protein from a number of laboratories is consistent with our results, demonstrating some cytoplasmic accumulation of all four AUF1 isoforms, although the relative ratio of cytoplasmic to nuclear AUF1 can vary (20, 41, 42). Thus, we cannot explain the difference in results between our study, other studies consistent with ours (20, 41, 42), and that of Arao et al. (37). A caveat to the biochemical analysis of cellular protein distribution is that nuclear leakage to the cytoplasm typically occurs during fractionation. This contamination is less likely when cells are fixed and analyzed by microscopy, so we therefore investigated AUF1 shuttling activity using indirect immunofluorescence.

Another potential means for regulating protein shuttling is arginine methylation (31, 32), although the role of arginine methylation in protein shuttling is not clear. Arginine methylation has been shown to regulate the cellular localization of hnRNP A2 but not hnRNP A1, which is also asymmetrically methylated (32). Importantly, the in vivo arginine methylation sites in hnRNP A1 have been identified and found to be distinct from the M9 localization domain (43). Therefore, it may not be surprising that the AUF1 proteins, which are reported to undergo arginine methylation (22), do not relocalize following methytransferase inhibition.

A unique feature of our work was the identification of AUF1 isoform interaction or heterocomplex formation as a probable means to facilitate nuclear-cytoplasmic shuttling of the family of AUF1 proteins. Oligomerization of the AUF1 isoforms not only serves to explain the identical shuttling kinetics of the four protein isoforms, but it also fits a general paradigm of shuttling hnRNPs. It has been shown using in vitro binding studies that hnRNPs E2, I, K, and L all form homomeric and heteromeric complexes (44). Moreover, hnRNP A1 interacts with the core A/B and C1 proteins and weakly with D, E, I, M, and K proteins (45). Thus, the interaction of p42/p45 AUF1 proteins with the p37 and p40 isoforms would be expected to promote their nuclear export, and p37/p40 AUF1 isoforms would be expected to promote nuclear import of p42 and p45 isoforms. The interaction of isoforms can account for an otherwise paradoxical symmetry that should segregate the p37 and p40 proteins in the nucleus and the p42 and p45 proteins in the cytoplasm. The exact composition and stoichiometry of the AUF1 heterocomplexes are currently under investigation, although the AUF1 oligomer would be of sufficient size to limit diffusion (Fig. 3). It is also not yet clear whether all AUF1 proteins interact and shuttle or whether only a subset do so, which would indicate that a proportion of the protein is stationary and segregated in different nuclear compartments. Studies are currently in progress to also determine how AUF1 protein shuttling activity is regulated, because this might serve as a key mechanism for separating nuclear from cytoplasmic functions.

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