Identification of the sequence determinants mediating the nucleo-cytoplasmic shuttling of TIAR and TIA-1 RNA-binding proteins

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Summary

TIAR and TIA-1 are two closely related RNA-binding proteins which possess three RNA recognition motifs (RRMs) followed by an auxiliary region. These proteins are involved in several mechanisms of RNA metabolism, including alternative hnRNA splicing and regulation of mRNA translation. Here we characterize the subcellular localization of these proteins in somatic cells. We demonstrate that TIAR and TIA-1 continuously shuttle between the cytoplasm and the nucleus and belong to the class of RNA-binding proteins whose nuclear import is transcription-dependent. We identified RRM2 and the first half of the auxiliary region as important determinants for TIAR and TIA-1 nuclear accumulation. In contrast, the nuclear export of TIAR and TIA-1 is mediated by RRM3. Both RRMs contribute to TIAR and TIA-1 nuclear accumulation or export by their RNA-binding capacity. Indeed, whereas mutations of the highly conserved RNP2 or RNP1 peptides in RRM2 redistribute TIAR to the cytoplasm, similar modifications in RRM3 abolish TIAR nuclear export. Moreover, TIAR and TIA-1 nuclear accumulation is a Ran-GTP-dependent pathway, in contrast to its nuclear export which is unaffected by Ran-GTP depletion and which is independent of the major CRM1-exporting pathway. This study demonstrates the importance of TIAR and TIA-1 RNA-binding domains for their subcellular localization and provides the first evidence for distinct functions of TIAR and TIA-1 RRMs.

Key words: RNA-binding protein, Nuclear import, Nuclear export

Introduction

TIAR and TIA-1 are related U-rich RNA-binding proteins involved in multiple aspects of RNA metabolism. In the nucleus, these proteins act as RNA splicing regulators of a series of alternatively spliced pre-mRNAs (Fas, msl-2, FGFR-2, calcitonin/CGRP) (Del Gatto et al., 1997; Forch et al., 2000; Zhu et al., 2003). In the cytoplasm, TIAR and TIA-1 have been shown to regulate the translation of various mRNAs by binding to AU-rich elements (AREs) located in these mRNA 3′ untranslated regions (3′UTRs). This is the case of mRNAs encoding TNF-α, cyclooxygenase-2 (Cox-2), human matrix metallinoproteinase-13 (HMMP-13) and β2-adrenergic receptor (Dixon et al., 2003; Kandasamy et al., 2005; Piecyk et al., 2000; Wang et al., 2003). In addition to the translational silencing of selected cytoplasmic transcripts, both TIAR and TIA-1 participate in the cellular response to environmental stress as they migrate in cytoplasmic foci called stress granules (SG) (Kedersha et al., 2000). Originally found in plant cells (Nover et al., 1989), these structures have been shown to form in mammalian cells upon exposure to different stresses, such as heat shock, osmotic and oxidative stress as well as UV-irradiation. The characterization of these foci revealed that they constitute a reservoir of sequestered mRNAs maintained in an untranslated status. Indeed, mRNAs located in SGs are associated with the 48S pre-initiation complex but lack the translation initiation factor eIF2 as well as the 60S large ribosomal subunit. In fact, the formation of SGs can be induced by the phosphorylation of the α subunit of eIF-2, which blocks translation initiation by reducing the formation of the eIF2-GTP-tRNAi-Met ternary complex that loads initiator methionine onto the small ribosomal subunit (Kedersha and Anderson, 2002). TIAR and TIA-1 are particularly abundant in the brain, testis and spleen (Beck et al., 1996). Mice that lack TIA-1 or TIAR exhibit high embryonic lethality, suggesting an important role of these proteins during the embryonic development (Beck et al., 1998; Piecyk et al., 2000). Moreover, *tiar* gene inactivation leads to sterility, owing to the defective development of primordial germ cells (Beck et al., 1998).

TIAR and TIA-1 are composed of three RNA Recognition Motifs (RRMs) and a C-terminal auxiliary domain rich in glutamine. The highest degree of identity between these related proteins is observed at the level of the RRMs (more than 90%) whereas the C-terminal regions diverge more significantly (51% identity). Both TIAR and TIA-1 are expressed as two isoforms, resulting from the alternative splicing of a common precursor transcript, leading to the inclusion or exclusion of 11 or 17 amino acid peptides in RRM2 or RRM1 of TIA-1 and TIAR, respectively (Beck et al., 1996). In vitro studies revealed that the three RRMs differ in their RNA-binding capacities.

Indeed, RRM2 is the major domain involved in the binding of U-rich oligomers. RRM3 also displays RNA-binding ability but toward sequences that differ from uridylate stretches. RRM1 does not seem to contribute to TIAR/TIA-1 RNA-binding ability (Dember et al., 1996). Recently, the 100 amino acid long C-terminal sequence, which is structurally related to prion protein, was shown to mediate TIA-1 and TIAR aggregation occurring upon SG formation (Gilks et al., 2004).

In contrast to germinal cells, in which TIAR and TIA-1 are mostly cytoplasmic (Colegrove-Otero et al., 2005), most somatic cells accumulate these proteins both in the nuclear and the cytoplasm, the highest proportion being nuclear at the equilibrium (Kedersha et al., 1999). So far, the sequence determinants and the mechanisms ruling TIAR and TIA-1 subcellular distribution have not been addressed. In the present study, we established that in somatic cells, these two RNA-binding proteins shuttle between the nucleus and the cytoplasm. Indeed, like several hnRNP proteins, TIAR and TIA-1 accumulate in the cytoplasm upon transcription blockade. Moreover, the analysis of the cellular distribution of TIAR and TIA-1 deletion mutants revealed that both RRM2 and the first 50 amino acids of the C-terminal region are required for nuclear accumulation of TIAR and TIA-1. More importantly, the integrity of RRM2 RNA-binding ability seems to be crucial as point mutations of either RNP2 or RNP1 motifs of RRM2 preclude nuclear localization. In contrast, the export of TIAR and TIA-1 from the nucleus is mediated by RRM3 as its deletion prevents the cytoplasmic accumulation of TIAR and TIA-1 observed upon actinomycin-D-induced blockade of nuclear import. In addition, RRM3 is sufficient to activate the nuclear export of a protein normally confined to the nucleus. RRM3 exporting activity seems to be dependent on its RNA-binding activity as it is abolished by mutations in its RNP motifs. The RRM3-mediated nuclear export of TIAR and TIA-1 is independent of the major export CRM1-dependent pathway as it is unaffected by CRM1 inhibitor, leptomycin B. Moreover, in contrast to nuclear import, TIAR and TIA-1 nuclear export is not dependent on Ran-GTP as it is maintained upon depletion of the Ran-GTP pool.

Altogether, our results reveal that the nucleo-cytoplasmic shuttling of TIAR and TIA-1 proteins relies on sequence determinants and protein transport machinery that differ from that described so far for RNA-binding proteins, thereby further illustrating the complexity of the intracellular traffic of macromolecules.

Materials and Methods

Materials

Enzymes were purchased from Invitrogen and Roche. Oligonucleotides were purchased from Sigma or Genset. The anti-Flag M2 monoclonal antibody was purchased from Sigma-Aldrich, the anti-TIAR and anti-hnRNP A1 polyclonal antibodies were purchased from Santa Cruz Biotechnology. The secondary antibodies, the Alexa-594-coupled donkey anti-mouse IgG and donkey anti-goat IgG were products of Molecular Probes.

DNA constructs

DNA constructs used to express EGFP hybrid proteins were generated by introducing PCR-amplified fragments corresponding to mouse TIARb, TIA-1b full-length or partial sequences (Beck et al., 1996) (Fig. 2A and Fig. 8A) between *Eco*RI and *Bam*HI sites of the pEGFP-C2 plasmid (Clontech). TIAR-FLAG and mutant TIAR-FLAG constructs were generated by insertion of PCR-amplified fragments of TIARb coding sequence into *Eco*RI and *Bam*HI sites of pcDNA3.1(–) plasmid (Invitrogen). The FLAG-encoding sequence was included in the reverse primers used for PCR amplifications to insert the FLAG epitope at the C-terminus of the expressed proteins. The NPc-NLS-FLAG construct was made by PCR amplification of the amino acids 2-150 of *Xenopus laevis* nucleoplasmin core (NPc) using an EST (GenBank accession number BX850185) as a template. The PCR fragment containing a *Xho*I site at the 5′ end and an *Eco*RI site at the 3′ end, was cloned into the *Xho*I and *Eco*RI sites of pcDNA3.1(–). A double-stranded oligonucleotide (sense: 5′-AATTCATGAAACGCCCTGCAGAAGATATGGAAGAGGAACAA-GCATTTAAAAGATCTAGAGACTACAAAGACGATGACGACAA-GTGAG-3′; antisense: 5′-GATTCTCACTTGTCGTCATCGTCTTT-GTAGTCTCTAGATCTTTTAAATGCTTGTTCCTCTTCCATATC-TTCTGCAGGGCGTTTCATG-3′) encoding hnRNP K bipartite NLS (21-KRPAEDMEEEQAFKRSR-37) (Matunis et al., 1992) and the FLAG epitope was then inserted between the *Eco*RI and *Bam*HI sites to create the NPc-NLS-FLAG construct. TIAR and TIA-1 RRM3, RRM2 or mutated RRM3 sequences were amplified by PCR and inserted in the *Eco*RI site of NPc-NLS-FLAG to generate NPc-RRM3-NLS-FLAG, NPc-RRM3mutRNP2-NLS-FLAG, NPc-RRM3mutRNP1-NLS-FLAG, NPc-RRM2-NLS-FLAG and NPc-RRM3(TIA-1)-NLS-FLAG (Fig. 5A and 8A). All the constructs were subsequently sequenced.

Cell culture and treatments

Cos-7 cells were grown in DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL), penicillin (50 U/ml), streptomycin (50 mg/ml) and L-glutamine (2 mM). For all experiments of cell transfection, Cos-7 cells were grown on coverslips overnight and transfected using Fugene reagent (Roche). DNA (3 μg) was transfected into 2×10^5 cells with 10 μl Fugene. Cells were fixed 36 hours after transfection. Where indicated, the cells were treated with actinomycin D (5 μg/ml) (Sigma) for 3 hours in the presence or absence of cycloheximide (20 μg/ml) or leptomycin B (0.2 μM) (BioMol Research Laboratory). For ATP depletion, Cos-7 cells were washed with PBS, and incubated in glucose-free DMEM containing penicillin and streptomycin sulphate (50 U/ml), HEPES (25 mM, pH 7.4) and 10% dialyzed FCS or in the same medium containing 10 mM sodium azide and 6 mM 2-deoxyglucose for 50 minutes (Schwoebel et al., 2002).

Fluorescence microscopy

The subcellular distribution of EGFP hybrid proteins was analyzed 36 hours after transfection. Cells grown on coverslips were washed three times for 5 minutes with PBS, fixed with 2% paraformaldehyde for 10 minutes at room temperature (RT), washed three times with PBS for 5 minutes and mounted with Gel/Mount and DAPI (100 pg/ml). The cells were then examined by epifluorescence (Leica DM 4000B) or confocal fluorescence microscopy (Leica DM IRE2). The subcellular distribution of TIAR, hnRNP A1 and FLAG-tagged proteins was analyzed according to the same procedure except that after fixation in 2% paraformaldehyde and rinsing in PBS, cells were permeabilized with 0.5% Triton X-100, washed three times in PBS for 5 minutes and blocked in 10% BSA for 10 minutes at RT. The coverslips were incubated with the primary antibody diluted in 0.1% Tween 20 for 1 hour at RT (anti-TIAR dilution, 1:50; anti-FLAG dilution, 1:2000, anti-hnRNP A1 dilution, 1:50). The coverslips were then washed three times for 5 minutes in 0.1% Tween 20, blocked in 10% donkey serum for 10 minutes at RT and incubated for 30 minutes at RT with the secondary antibody: Alexa-594-conjugated donkey anti-goat to detect TIAR and hnRNP A1 (dilution 1:30,000) and Alexa-594-conjugated donkey anti-mouse to detect FLAG-tagged

proteins (dilution 1:25,000). The coverslips were washed three times for 5 minutes in 0.1% Tween 20 and mounted with 10 μl Gel/Mount and DAPI (100 pg/ml). Dilutions of all antibodies and reagents were made in PBS unless stated otherwise.

Quantification of the cytoplasmic and nuclear fluorescence was performed by integrating the fluorescent signal in identical surface units taken in each subcellular compartment (FCStandalone program, Alpha-Innotech), assuming that both nuclear and cytoplasmic compartments of Cos-7 cells are of similar surfaces. This analysis was performed on at least 20 cells and the average ratios of cytoplasmic and nuclear fluorescence was plotted as a percentage.

Cell fractionation

Cytoplasmic and nuclear extracts from Cos-7 cells were prepared as described (Gueydan et al., 2002).

Results

TIAR is a shuttling protein whose nuclear import is coupled to RNA polymerase II transcription

Previous studies have shown that hnRNPs accumulate in the nucleus by two modes, which are transcription-dependent (for example, C proteins) or transcription-independent (for example, A1 protein) (Pinol-Roma and Dreyfuss, 1991). Therefore, we investigated whether TIAR nuclear accumulation was affected by RNA polymerase II transcription blockade by actinomycin D. Treatment of cells with actinomycin D (5 μg/ml) induced the accumulation of TIAR in the cytoplasm (Fig. 1). In contrast, TIAR nuclear accumulation remained unaffected upon translational blockade by cycloheximide. This result indicates that TIAR belongs to the class of RNA-binding proteins whose nuclear import is coupled to transcription. Moreover, cycloheximide did not diminish the cytoplasmic accumulation of TIAR observed in the presence of actinomycin D. Therefore TIAR that accumulates in the cytoplasm after transcription inhibition corresponds to pre-existing protein that is exported from the nucleus. The same results were obtained when transcription was blocked by α-amanitin, which specifically inhibits RNA polymerase II transcription (data not shown), thereby confirming the coupling of TIAR nuclear import with RNA polymerase II activity.

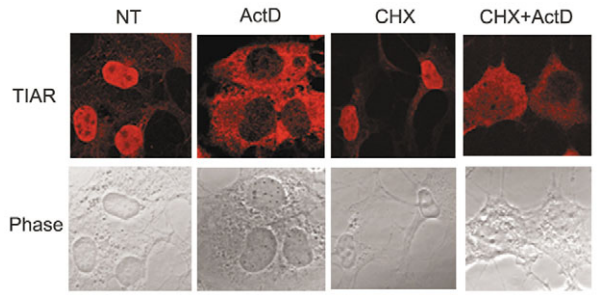


Fig. 1. TIAR accumulates in the cytoplasm of cells treated with actinomycin D. Cos cells grown on glass coverslips were incubated for 3 hours in normal medium (NT) or in medium containing cycloheximide (CHX; 20 μg/ml) or actinomycin D (ActD; 5 μg/ml) or both agents. Cells were then fixed, immunostained with anti-TIAR antibody and analyzed by confocal fluorescence microscopy as described in the Materials and Methods. Phase-contrast transmission images are also shown (Phase). Translational blockade by cycloheximide was verified by [^{35}S]methionine incorporation (data not shown).

Mapping of the sequence determinants specifying TIAR nuclear accumulation

Detailed analysis of the TIAR amino acid sequence did not reveal any sequence determinants related to nuclear localization signals identified so far. In order to identify such elements, we generated several DNA constructs to express TIAR or deletion mutants of TIAR in fusion with EGFP (Fig. 2A). These constructs were transiently transfected in Cos cells to observe the subcellular localization of the fusion proteins by immunofluorescence microscopy. This analysis first revealed that the GFP-TIAR fusion protein has a cellular distribution similar to TIAR endogenous protein (compare Fig. 1 and Fig. 2B, panel b), thereby validating the use of the EGFP tag to evaluate the subcellular distribution of TIAR mutants. Furthermore, we observed that the deletion of the first 50 amino acids of the C-terminal auxiliary domain or of the RRM2 markedly reduced the nuclear accumulation of EGFP-TIAR protein (Fig. 2B, panels f and d, respectively and Fig. 2C). In contrast, the removal of RRM1, RRM3 or the last 50 amino acids of TIAR did not disturb subcellular localization of EGFP-TIAR (Fig. 2B, panels c,e,g). These results suggested that both RRM2 and the first half of the C-terminal auxiliary domain contributed to the nuclear accumulation of TIAR. Indeed, the association of both domains to EGFP is necessary and sufficient to induce EGFP nuclear accumulation (Fig. 2B, panel h and Fig. 2C) whereas the association of either of them to EGFP is unable to do so (data not shown).

We then evaluated whether the involvement of RRM2 in TIAR nuclear accumulation relied on its RNA-binding ability by analyzing the subcellular localization of GFP-TIAR mutants in which conserved amino acids of RRM2 RNP motifs were modified (RNP2mut: F100D, G102P, L104D; RNP1mut: G139P, Y140D, F142D, V143E) (Kim and Baker, 1993). We observed that the disruption of either of RRM2 RNP motifs clearly impaired GFP-TIAR nuclear accumulation as efficiently as the deletion of the whole RRM2 domain (Fig. 2B, compare panels d and i and data not shown), thereby suggesting that RRM2 RNA-binding ability is important for TIAR accumulation in the nucleus. It should be mentioned that the same results were obtained in HeLa cells or when TIAR or TIAR mutants were fused to the FLAG epitope, excluding artefactual localization due to the GFP tag (data not shown).

We then determined whether the nuclear import activity of the first half of the TIAR auxiliary domain could be mapped to a shorter sequence. Therefore, we generated additional GFP-TIAR mutant constructs in which only portions of the first 50 amino acids of the auxiliary domain have been removed (Fig. 3A). These constructs were transfected in Cos cells and the localization of the GFP-TIAR mutants was analyzed by fluorescent microscopy. This experiment revealed that the deletion of any part of this 50-amino acid region led to an increased cytoplasmic accumulation of GFP fusion proteins compared with GFP-TIAR wild-type protein (Fig. 3B), thereby indicating that the first half of the auxiliary domain as a whole contributes to the nuclear accumulation of TIAR.

Sequence analysis of this 50 amino acid region did not reveal

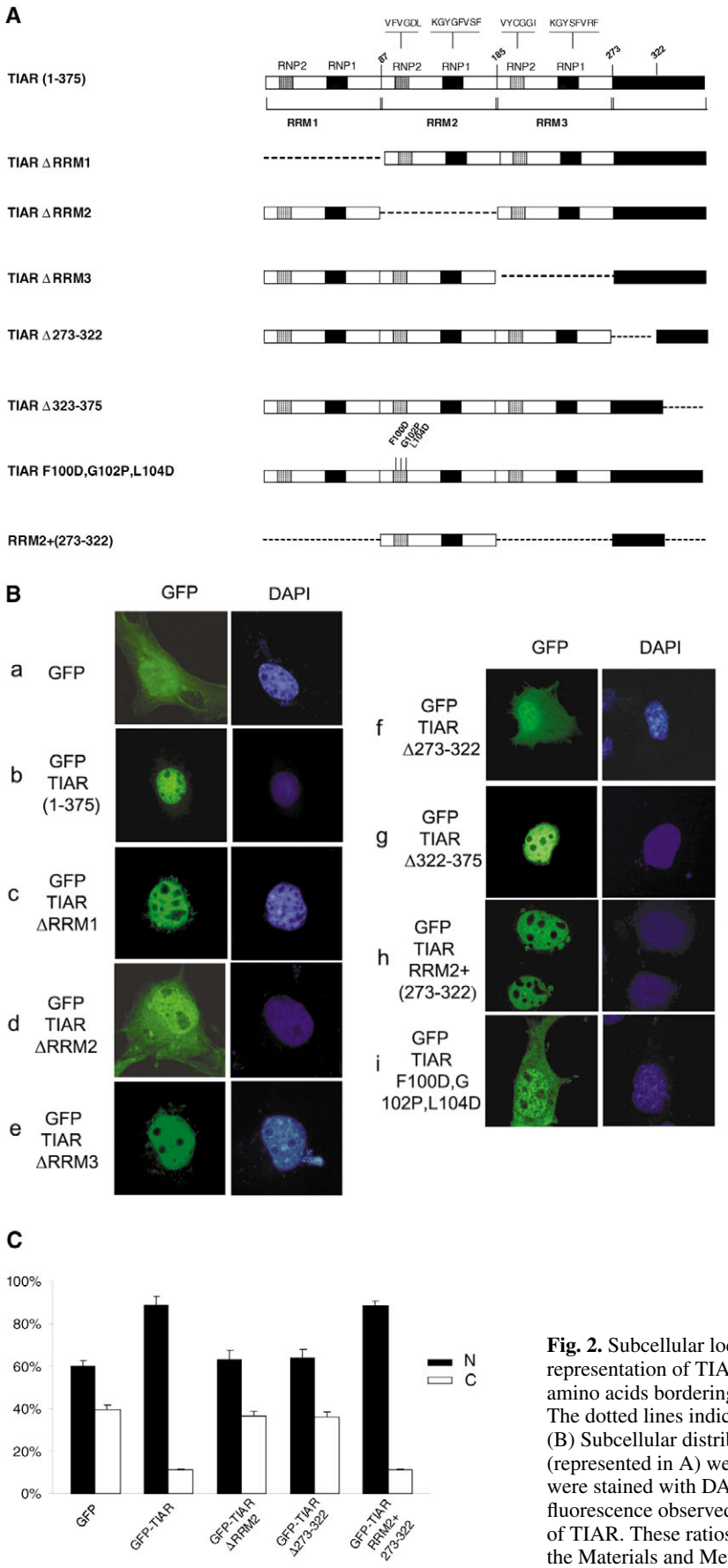


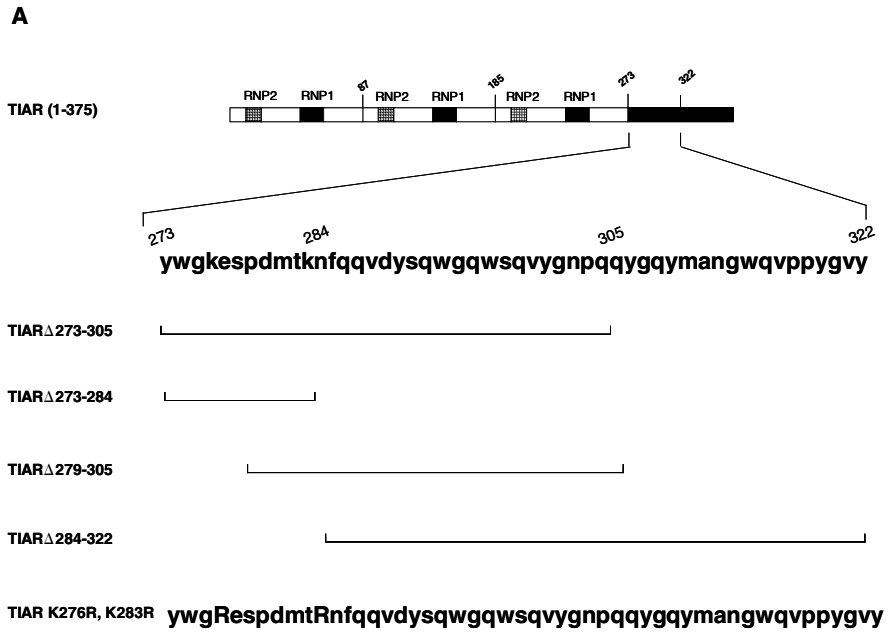
Fig. 2. Subcellular localization of EGFP-TIAR hybrid proteins. (A) Schematic representation of TIAR protein and TIAR mutants fused to EGFP protein. The amino acids bordering the different domains composing TIAR are indicated. The dotted lines indicate the deleted region in the different mutants. (B) Subcellular distribution of GFP and GFP-TIAR hybrid proteins (represented in A) were analyzed by epifluorescence microscopy. Cell nuclei were stained with DAPI. (C) Ratios of cytoplasmic (C) and nuclear (N) fluorescence observed for GFP alone or fused with TIAR or relevant domains of TIAR. These ratios are the means±s.d. and were calculated as described in the Materials and Methods.

any similarity with known nuclear localization motifs. However, it contains two lysine residues that might be important for TIAR nuclear accumulation. Indeed, lysine residues are major determinants of classical NLS (Fried and Kutay, 2003) and are target residues for sumoylation, a post-translational modification known to mediate protein nuclear import (Dohmen, 2004). We thus evaluated the importance of K276 and K283 residues for TIAR nuclear accumulation by analyzing the subcellular localization of the GFP-TIAR mutant in which these lysine residues were converted into arginine (TIAR K276R, K283R). As shown in Fig. 3B, these mutations did not modify EGFP-TIAR localization, thereby excluding the possibility that this region contains a cryptic classical NLS or requires sumoylation for its import activity.

TIAR nuclear export is mediated by the RNA binding activity of RRM3

TIAR is actively exported from the nucleus as revealed upon transcription blockade by actinomycin D (Fig. 1). We thus analyzed the localization of TIAR mutants upon actinomycin D treatment to map the sequence mediating TIAR nuclear export. FLAG-tagged TIAR or TIAR mutant DNA constructs were transfected into Cos cells which were subsequently treated or not with actinomycin D before analysis by immunostaining with anti-FLAG antibody and fluorescent microscopy. The deletion of RRM3 completely abolished TIAR nuclear export observed upon exposure to actinomycin D (Fig. 4A), thereby revealing the importance of this RRM in the nuclear export of TIAR. The deletion of any other parts of the protein did not preclude relocalization in the cytoplasm upon transcription blockade although the phenomenon was clearly attenuated for mutants lacking RRM2 or the first half of the C-terminal domain because of an increased cytoplasmic accumulation of the protein before actinomycin D treatment (data not shown).

In order to determine whether RRM3 is sufficient to mediate nuclear export, we tested whether it activated the nuclear export of a protein that is normally confined to the nucleus.



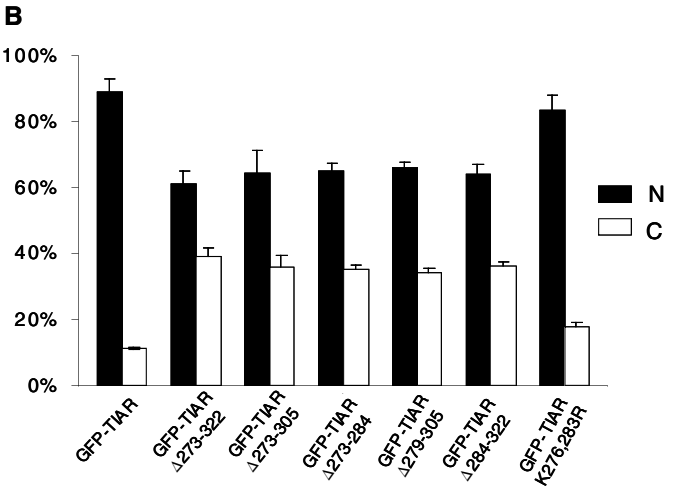


Fig. 3. Subcellular localization of EGFP-TIAR hybrid proteins lacking parts of the first half of the auxiliary domain. (A) Schematic representation of TIAR protein and TIAR mutants fused to EGFP. The amino acid sequence of the first half of the auxiliary domain as well as the deletions introduced in the different mutants is specified. (B) The subcellular distribution of GFP and GFP-TIAR hybrid proteins was analyzed by epifluorescence microscopy and quantified as for Fig. 2C.

This protein corresponds to the nucleoplasmin core domain (NPc). Several studies have shown that upon injection into the cytoplasm, NPc does not enter the nucleus and, importantly, that upon injection in the nucleus NPc does not cross the nuclear envelope into the cytoplasm (Dingwall et al., 1982; Dingwall et al., 1988; Laskey et al., 1993). We generated DNA constructs to express FLAG-tagged NPc in fusion with the classical NLS of hnRNP K (Michael et al., 1995) with or without TIAR RRM3 (Fig. 4B). These constructs were introduced into Cos and HeLa cells and the localization of FLAG-tagged proteins was analyzed as previously. We observed that the NPc-NLS-FLAG protein mostly accumulated in the nucleus as previously reported (Michael et al., 1995). However, the insertion of RRM3 in the protein clearly relocalized a significant proportion of the protein in the cytoplasm, thereby demonstrating the nuclear export activity of RRM3 (Fig. 4C and data not shown).

We then investigated whether RRM3 mediated TIAR nuclear export via its RNA-binding capacity by mutating RNP2 and RNP1 motifs independently. As illustrated in Fig. 4C, the disruption of either RNP motif abolished the nuclear export activity of RRM3. Moreover, the nuclear export of TIAR relies specifically on RRM3 as RRM2 or other portions of the TIAR protein do not activate the nuclear export of NPc-NLS-FLAG (Fig. 4C and data not shown). Altogether, these results indicate that TIAR is exported from the nucleus in association with RNA bound to RRM3.

Verification of the import and export determinants of TIAR by nucleo-cytoplasmic fractionation

To confirm the data obtained by microscopy, Cos-7 cells were transfected with FLAG-tagged TIAR or relevant mutants and the distribution of the proteins between the nucleus and the cytoplasm was examined after cell fractionation by western blot. These experiments confirmed that TIAR and the mutant lacking RRM3 mostly accumulated in the nucleus. In contrast, the deletion of RRM2 induced a significant relocalization of the protein in the cytoplasm (Fig. 5). Upon transcription blockade by actinomycin D, TIAR and the mutant lacking RRM2 moved to the cytoplasm, whereas the mutant lacking RRM3 remained mostly nuclear. The exporting activity of RRM3 was also confirmed as its fusion to NPc-NLS-FLAG protein induced a major shift of the protein from the nuclear to the cytoplasmic fraction. Moreover, as previously demonstrated, the mutation of RNP2 markedly inhibited RRM3 exporting activity. To control the fractionation procedure, cytoplasmic and nuclear extracts were probed for FUSE-binding proteins whose localization is predominantly nuclear (He et al., 2000).

Altogether, these results confirmed the importance of RRM2 for TIAR nuclear accumulation and the exporting activity of RRM3.

TIAR nuclear export is independent of the CRM1 nuclear export pathway

One major nuclear export pathway relies on the export receptor

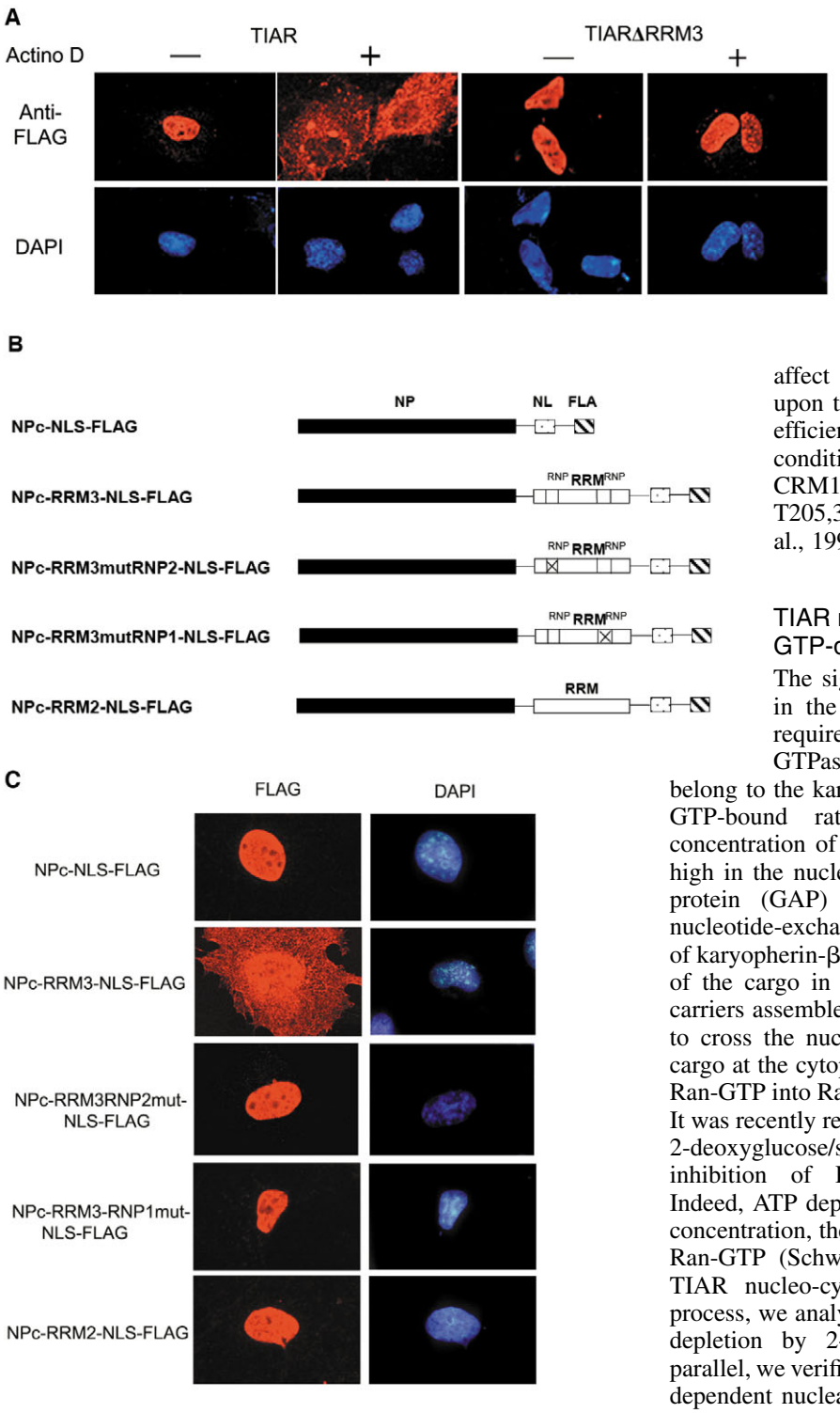


Fig. 4. RRM3 mediates TIAR nuclear export. (A) Subcellular localization of TIAR-FLAG and TIARΔRRM3-FLAG mutant in Cos cells before and after actinomycin D (5 μg/ml) treatment for 3 hours. Cells were fixed, immunostained with anti-FLAG antibody, and analyzed by epifluorescence microscopy. (B) Schematic representation of NPc-NLS- FLAG constructs with or without different parts of the TIAR protein. Mutations in the NPc-RRM3mutRNP2-NLS-FLAG and NPc-RRM3mutRNP1-NLS-FLAG were: Y208D, C209R, G210P, G211P, I212D and G241P, Y242D, F244D, V245E, F247D, respectively. (C) Subcellular localization of proteins illustrated in B.

CRM1. CRM1 is the most versatile of all export factors, being involved in the export of many different classes of proteins such as cell cycle regulators, transcription factors, RNA binding proteins and numerous others (Fried and Kutay, 2003). Therefore, we investigated whether TIAR nuclear export was CRM1-dependent by analyzing the effect of the CRM1 inhibitor, leptomycin B on TIAR cytoplasmic accumulation and upon transcription blockade by actinomycin D. Leptomycin B does not affect TIAR cytoplasmic accumulation observed upon transcription blockade (Fig. 6). To verify the efficiency of leptomycin B in our experimental conditions, we analyzed its capacity to block the CRM1-dependent nuclear export of the GFP-MK2-T205,317E constitutively exported mutant (Engel et al., 1998) (Fig. 6B).

TIAR nuclear import but not export is a Ran-GTP-dependent mechanism

The signal-mediated accumulation of import cargo in the nucleus or export cargo in the cytoplasm requires energy, most frequently linked to the small GTPase Ran. Indeed, the majority of nuclear carriers belong to the karyopherin-β superfamily and bind Ran in the GTP-bound rather than the GDP-bound form. The concentration of Ran-GTP is kept low in the cytoplasm and high in the nucleus by localizing the Ran-GTPase-activating protein (GAP) to the cytoplasm and the Ran-guanine nucleotide-exchange factor (GEF) to the nucleus. The binding of karyopherin-β carriers to Ran-GTP leads to the dissociation of the cargo in the nucleus. Inversely, most nuclear export carriers assemble into cargos upon association with Ran-GTP to cross the nuclear envelope. Dissociation of the exported cargo at the cytoplasmic side is triggered by the hydrolysis of Ran-GTP into Ran-GDP by RanGAP (Fried and Kutay, 2003). It was recently reported that cellular ATP depletion induced by 2-deoxyglucose/sodium azide treatment results in the inhibition of Ran-dependent nucleo-cytoplasmic traffic. Indeed, ATP depletion induces a major drop in the free-GTP concentration, thereby decreasing the nuclear concentration of Ran-GTP (Schwoebel et al., 2002). To determine whether TIAR nucleo-cytoplasmic shuttling was a Ran-dependent process, we analyzed TIAR subcellular localization after ATP depletion by 2-deoxyglucose/sodium azide treatment. In parallel, we verified that ATP depletion inhibited the Ran-GTP-dependent nuclear import of the shuttling hnRNP A1 (Siomi et al., 1997). A 50 minute exposure to 2-deoxyglucose/sodium azide led as expected, to a detectable cytoplasmic accumulation of hnRNP A1. Under the same conditions, TIAR became mostly cytoplasmic (Fig. 7). The cytoplasmic accumulation of both hnRNP A1 and TIAR induced by ATP depletion most probably resulted from the blockade of their nuclear import without preventing their nuclear export. Indeed, cycloheximide did not diminish the cytoplasmic accumulation of hnRNP A1 and TIAR observed upon ATP depletion, thereby confirming that hnRNP A1 and TIAR accumulation in the cytoplasm after

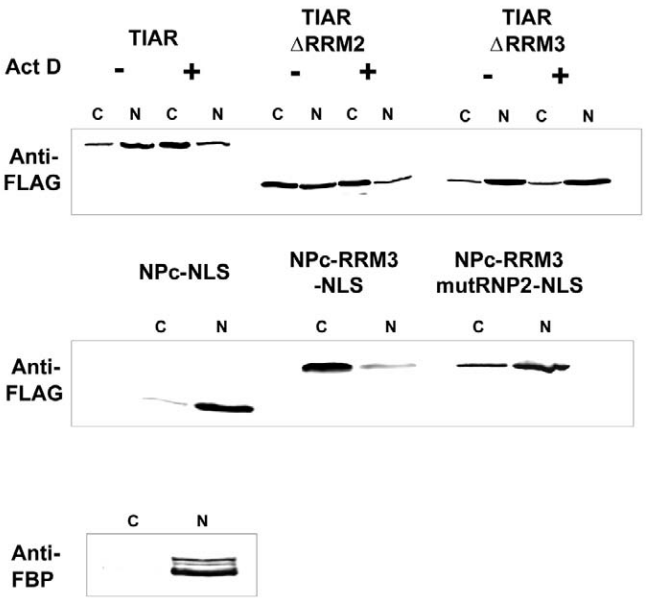


Fig. 5. Characterization of the nucleo-cytoplasmic distribution of TIAR and TIAR mutants by cell fractionation. Cos-7 cells were transfected with the indicated TIAR and TIAR mutants fused with the FLAG epitope. Where indicated, cells were then treated with actinomycin D (5 μg/ml, 3 hours) before cell fractionation. Cytoplasmic (C) and nuclear (N) extracts were analyzed by western blot using anti-FLAG and anti-FBP to control the fractionation procedure.

ATP depletion corresponds to pre-existing proteins that are still exported from the nucleus but which are unable to be re-imported (data not shown). In conclusion, these experiments suggest that TIAR nuclear import but not export is a Ran-dependent mechanism.

TIAR and TIA-1 nucleo-cytoplasmic shuttling rely on the same sequence determinants

TIAR and TIA-1 proteins share a strong degree of identity, mostly within the RRMs (more than 90% identity) with the C-terminal auxiliary domain diverging more significantly (50% similarity). We thus analyzed whether TIA-1 subcellular localization was ruled by the same sequence determinants as TIAR. Therefore, as for TIAR, we generated different DNA constructs to express TIA-1 or deletion mutants of TIA-1 in fusion with EGFP (Fig. 8A). These constructs were transiently transfected in Cos cells to observe the subcellular localization of the fusion proteins by immunofluorescence microscopy. EGFP-TIA-1 mostly accumulated in the nucleus (Fig. 8B). However, as observed with TIAR, the deletion of either RRM2 or the first half of the C-terminal auxiliary domain leads to a major accumulation of EGFP-TIA-1 in the cytoplasm. In contrast, the association of both of these domains of TIA-1 to the EGFP is sufficient to restore a subcellular distribution identical to the full-length TIA-1 protein. These results revealed that the nuclear accumulation of TIA-1 relies on the same sequence determinants as TIAR. We then tested the importance of RRM3 for TIA-1 nuclear export by generating

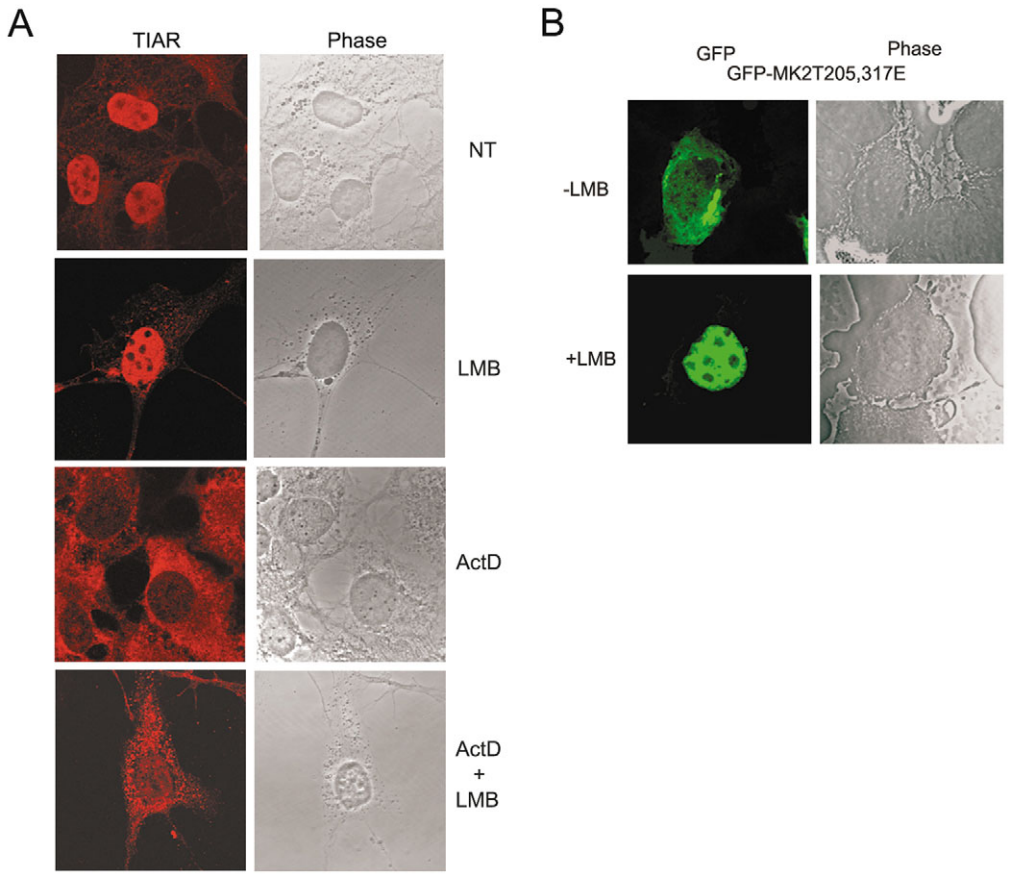


Fig. 6. TIAR nuclear export is independent of CRM1 export pathway. (A) Cos cells grown on glass coverslips were incubated for 3 hours in normal medium (NT) or in medium containing actinomycin D (ActD; 5 μg/ml) or leptmomycin B (LMB; 0.2 μM) or both agents. Cells were then fixed, immunostained with anti-TIAR antibody and analyzed by confocal fluorescence microscopy as described in the Materials and Methods. Phase-contrast images are also shown (Phase). (B) Cos cells grown on coverslips were transfected with the GFP-MK2-T205,317E construct. Thirty-six hours after transfection, cells were incubated or not with leptomycin B and GFP fluorescence was analyzed as in A.

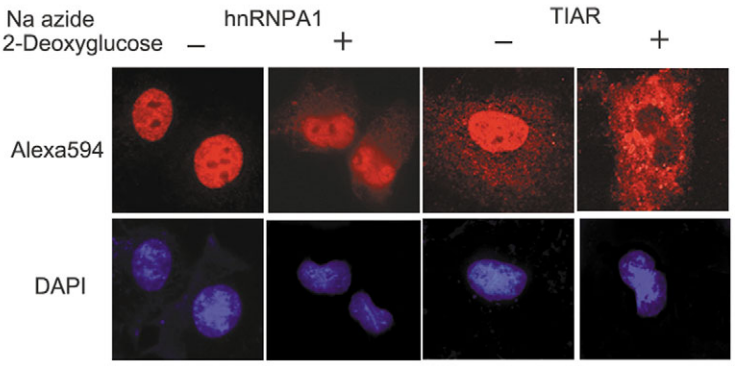
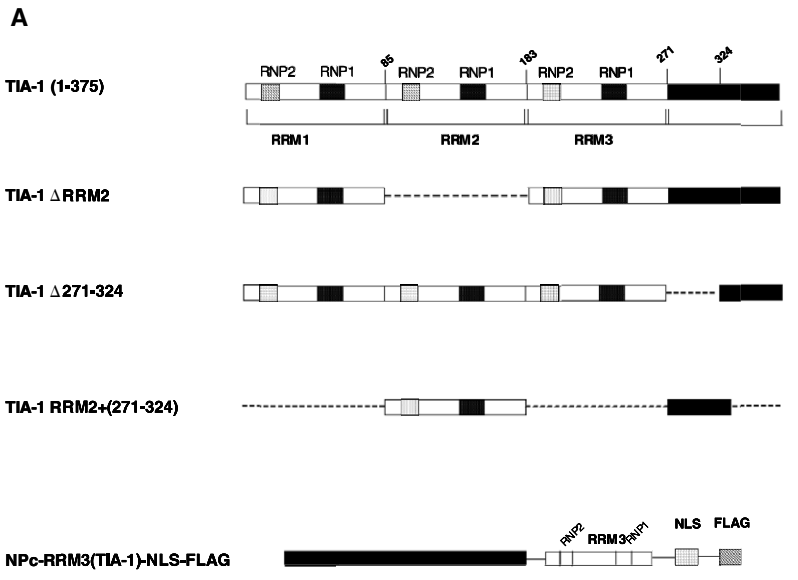


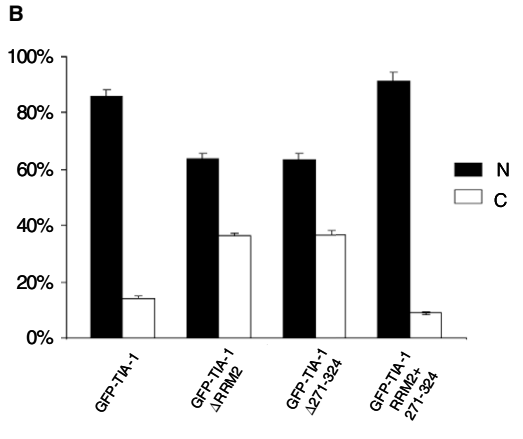
Fig. 7. ATP depletion leads to the cytoplasmic accumulation of hnRNP A1 and TIAR. Cos cells grown on coverslips were ATP-depleted and immunostained. The subcellular localization of hnRNP A1 and TIAR was analyzed as described in Fig. 1.

a DNA construct to express FLAG-tagged NPc-NLS fused with TIA-1 RRM3 (Fig. 8A). This construct was transfected into Cos cells and the subcellular distribution of NPc-RRM3(TIA-1)-NLS-FLAG was analyzed by immunostaining with anti-FLAG antibody and fluorescent microscopy. RRM3 activated the nuclear export of NPc-NLS-FLAG protein (Fig. 8C). This result combined with the observation that a TIA-1 mutant lacking RRM3 does not accumulate in the cytoplasm upon blockade of transcription (data not shown), indicates that as for TIAR, RRM3 mediates the nuclear export of TIA-1.

Discussion

In this work, we characterized the subcellular localization of TIAR and TIA-1 as well as we defined the sequence determinants involved in their distribution in the nuclear and





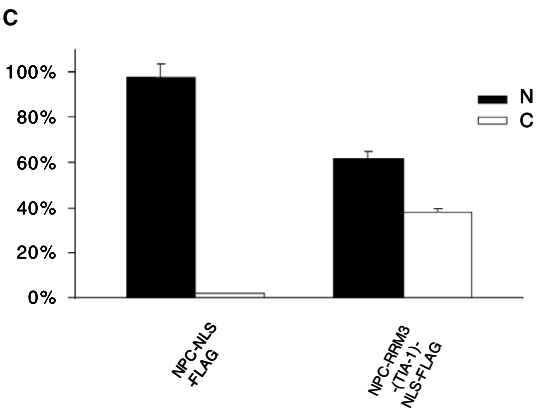


Fig. 8. TIAR and TIA-1 subcellular localization relies on the same sequence determinants. (A) Schematic representation of the TIA-1 constructs. The amino acids bordering the different TIA-1 domains are indicated. The dotted lines indicate the deleted region in the different mutants. (B,C) Subcellular distribution of GFP-TIA-1, GFP-TIA-1 mutants and NPc-RRM3(TIA-1)-NLS-FLAG described in A. The experiment was performed as described in Fig. 2B and Fig. 4C and ratios of cytoplasmic and nuclear fluorescence were calculated as previously described.

cytoplasmic compartments. First, we demonstrated that TIAR and TIA-1 shuttle between the nucleus and the cytoplasm and that their nuclear accumulation is a transcription-dependent process as for other RNA-binding proteins such as hnRNP A1 (Pinol-Roma and Dreyfuss, 1991) and HuR (Peng et al., 1998). We also showed that TIAR/TIA-1 nuclear import depends on the Ran-GTPase energy system. Therefore, the transcription dependence of TIAR and TIA-1 nuclear import most probably ensures that energy is not wasted to re-import these proteins into the nucleus if the nuclear pool of their RNA substrates is low.

Neither TIAR nor TIA-1 shares any homology with nuclear localization signals identified in other shuttling RNA-binding proteins. Our deletion analysis revealed RRM2 and the first 50 amino acid sequence of the auxiliary domain as important determinants for TIAR and TIA-1 nuclear accumulation. More importantly, we showed that mutations of RNP motifs in RRM2 led to a significant cytoplasmic retention of TIAR and TIA-1. RNP motifs constitute the most conserved sequence elements of RRMs. Crystallographic analysis of U1A RRM revealed the importance of the RNP motifs for its RNA-binding capacity (Hoffman et al., 1991; Nagai et al., 1990; Oubridge et al., 1994) and this structure seems to be conserved in other members of the RRM gene family (Kenan et al., 1991). Moreover, UV crosslinking (Merrill et al., 1988) and filter binding assays (Schwemmle et al., 1989) have shown that RNP motifs are directly involved in single-stranded nucleic acid binding. Therefore, our experiments indicate that RRM2 most probably contributes to TIAR/TIA-1 nuclear accumulation by its capacity to bind RNA.

The first 50 amino acids of the TIAR auxiliary domain constitute another major determinant for TIAR nuclear accumulation. This sequence does not appear to be related to any known nuclear determinant. Moreover, we excluded the existence of a cryptic classical NLS in this region as well as a sumoylation-dependent nuclear import activity of this sequence. Interestingly, the same region of TIA-1 protein that displays 51% identity with TIAR, contributes similarly to TIA-1 nuclear accumulation. Both RRM2 and the first half of the C-terminal auxiliary domain are required to induce TIAR and TIA-1 nuclear accumulation as the deletion of either of these elements disturbed this process. Moreover, we showed that the fusion of these elements to GFP is as efficient as the full-length TIAR protein to induce GFP nuclear accumulation. Therefore, the mechanism of TIAR and TIA-1 nuclear import significantly differs from that documented for other nuclear proteins. Indeed, most nuclear or shuttling proteins are imported into the nucleus via nuclear localization signals or nucleo-cytoplasmic shuttling sequences which independently of the rest of the protein recruit the nuclear import machinery (Fried and Kutay, 2003). This is clearly not the case for TIAR and TIA-1 for which RNA-binding activity seems to largely contribute to their nuclear accumulation. Moreover, neither RRM2, nor the first half of the auxiliary domain is by itself able to induce the nuclear accumulation of the GFP reporter protein (data not shown). To this point, we can hypothesize that the first half of the auxiliary domain acts as a Ran GTPase-dependent NLS whose activity is weak when compared to classical NLS or known shuttling signals. The low efficiency of this sequence to mediate nuclear import would encounter for its incapacity to induce the nuclear accumulation of GFP, which can exit passively from the nucleus (Shulga et al., 2000). RRM2 would contribute to the transient retention of the protein by mediating the binding of the protein to its nuclear RNA substrates. In support of our hypothesis, it is worth noting that at the equilibrium, TIAR and TIA-1 accumulate in the cytoplasm in higher proportions than other shuttling RNA-binding proteins (e.g. hnRNPA1, hnRNP K).

To date, there are more than 20 identified karyopherins mediating nuclear import and export in mammalian cells, whose activity is controlled by the Ran GTPase system. Although the transport substrates as well as the signal sequences involved in their recruitment are known for many of them, much less is known of the others (Jakel et al., 2002; Koch et al., 2000; Kutay et al., 2000). It is thus possible that TIAR/TIA-1 nuclear import is mediated by one of these less well-known karyopherins.

TIAR/TIA-1 nuclear export is mediated by RRM3. Indeed, we showed that RRM3 is necessary and sufficient to trigger the nuclear export of TIAR/TIA-1 and of NPc, a protein normally confined to the nucleus. Like RRM2 for nuclear accumulation, RRM3 most probably activates nuclear export by its RNA-binding activity. As TIAR/TIA-1 nuclear export is a Ran- and CRM1-independent mechanism, we suggest that TIAR and TIA-1 exit from the nucleus in association with their RNA substrates bound to RRM3. The nuclear export of the majority of cellular mRNAs occurs by Ran- and CRM1-independent pathways involving the Aly/TAP nucleoporin-binding protein complex (Fried and Kutay, 2003). However, HuR, another ARE-binding protein, was shown to facilitate the nuclear export of ARE-containing mRNAs (Gallouzi and Steitz, 2001). Indeed, HuR serves as an adapter for *fos* mRNA export through two pathways, one relying on the HuR-shuttling domain, HNS, the other involving two protein ligands of HuR which contain leucine-rich nuclear export signals (NES) recognized by the export receptor CRM1 (Brennan et al., 2000; Rebane et al., 2004). The question whether TIAR and TIA-1 facilitate the nuclear export of their RNA substrates remains to be addressed. However, if this is the case, it would occur by a different mechanism based on the absence of similarity between TIAR/TIA-1- and HuR-exporting signals. Independently of a potential role of TIAR and TIA-1 in the nuclear export of ARE-containing mRNAs, the observation that TIAR and TIA-1 exit from the nucleus in association with their RNA substrates supports the idea that RNA-binding proteins regulating mRNA metabolism in the cytoplasm bind their substrates very rapidly after their synthesis.

Interestingly, TIAR and TIA-1 nuclear accumulation and export rely on distinct RNA recognition motifs: RRM2 and RRM3, respectively. In the nucleus, TIAR and TIA-1 participate in selective intron removal by recruiting the spliceosome onto intronic U-rich sequences (Del Gatto et al., 1997; Forch et al., 2000; Zhu et al., 2003). In the cytoplasm, these proteins bind to AREs present in the 3′ UTR of several mRNAs (Dixon et al., 2003; Kandasamy et al., 2005; Lewis et al., 1998; Piecyk et al., 2000; Wang et al., 2003). Our results suggest that whereas RRM2 would be the major binding entity involved in TIAR/TIA-1 splicing activity, RRM3 would allow the binding of TIAR and TIA-1 to their cytoplasmic ARE-containing RNA substrates. In support of this hypothesis, previous in vitro binding studies revealed that whereas TIAR and TIA-1 RRM2 selectively retained U-rich sequences,

RRM3 was unable to bind to the same sequences but interacted with cellular RNAs (Dember et al., 1996).

In conclusion, this study demonstrates that TIAR and TIA-1 distinguish themselves from other RNA-binding proteins as their nucleo-cytoplasmic shuttling determinants differ from that described so far for other shuttling RNA-binding proteins (hnRNP A1, A2, D, E, K, HuR). Indeed, they constitute the first example of RNA-binding proteins whose subcellular localization largely depends on RNA-binding domains. Moreover, we provide the first evidence for distinct functions of TIAR and TIA-1 RRM 2 and 3.

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