Members of the Tristetraprolin Family of Tandem CCCH Zinc Finger Proteins Exhibit CRM1-dependent Nucleocytoplasmic Shuttling*

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Members of the tristetraprolin (TTP) family of CCCH tandem zinc finger proteins can bind directly to certain types of AU-rich elements (AREs) in mRNA. Experiments in TTP-deficient mice have shown that TTP is involved in the physiological destabilization of at least two cytokine mRNAs, those encoding tumor necrosis factor α and granulocyte-macrophage colony-stimulating factor. The two other known mammalian members of the TTP family, CMG1 and TIS11D, also contain ARE-binding CCCH tandem zinc finger domains and can also destabilize ARE-containing mRNAs. To investigate the effects of primary sequence on the subcellular localization of these proteins, we constructed green fluorescent protein fusions with TTP, CMG1, and TIS11D; these were predominantly cytoplasmic when expressed in 293 or HeLa cells. Deletion and mutation analyses revealed functional nuclear export signals in the amino terminus of TTP and in the carboxyl termini of CMG1 and TIS11D. This type of leucine-rich nuclear export signal interacts with the nuclear export receptor CRM1; abrogation of CRM1 activity resulted in nuclear accumulation of TTP, CMG1, and TIS11D. These proteins are thus nucleocytoplasmic shuttling proteins and rely on CRM1 for their export from the nucleus. Although TTP, CMG1, and TIS11D lack known nuclear import sequences, mapping experiments revealed that their nuclear accumulation required an intact tandem zinc finger domain but did not require RNA binding ability. These findings suggest possible roles for nuclear import and export in the regulation of cellular TTP, CMG1, and TIS11D activity.

The tristetraprolin (TTP) family of CCCH tandem zinc finger (TZF) proteins consists of three known members in mammals, with a fourth member identified in other vertebrates such as Xenopus laevis and Danio rerio. This protein family is characterized by the presence of two zinc fingers spaced 18 amino acids apart, each containing three cysteines and one histidine with strictly defined spacing of CX(8)CX(5)CX(3)H. The TTP family is further defined by a conserved amino-terminal sequence R(K)YKTEL leading to each finger. TTP (2), also known as TIS11 (3), Nup475 (4), and GOS24 (5), was the first described and is the best studied member of the family. Experiments using TTP-deficient mice have shown that TTP normally regulates the stability at least two cytokine mRNAs, those encoding tumor necrosis factor α (TNFα) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (1, 6–10). In addition, TTP is able to regulate the stability of interleukin-3 (IL-3) mRNA in cell transfection studies, although this has not yet been shown to be a physiological role (1, 11, 12).

Decay of these cytokine mRNAs is normally regulated in part by the presence of AU-rich elements (ARE) located in their 3′-untranslated regions (13). TTP exerts a destabilizing effect on mRNA by binding directly to the ARE, leading to the accelerated destruction of ARE-containing transcripts (7–9). The mechanisms by which this accelerated degradation is accomplished remain unknown, but it is thought to be initiated by TTP-dependent deadenylation (14).

Two additional mammalian members of the TTP family have been identified to date. They are referred to here as CMG1 (15) (also known as TIS11B (16), ERF1 (17), BRF1 (18), or Berg36 (19)) and TIS11D (20) (also known as ERF2 (20) or BRF2 (21)). CMG1 and TIS11D each contain a TTP-like CCCH TZF domain, and they can, like TTP, destabilize the ARE-containing TNFα, GM-CSF, and IL-3 mRNAs in co-transfection studies (1). Although the genes encoding mouse CMG1 and TIS11D are regulated differently from the TTP gene and are expressed in different tissues, their similar mRNA binding and destabilizing properties to TTP suggest that some functional overlap may exist within this protein family.

Previous work from our laboratory (22) has shown that, in serum-starved fibroblasts, TTP is localized to the nucleus and that upon stimulation with mitogens it rapidly (<5 min) translocates to the cytoplasm. Moreover, in primary mouse macrophages cytosolic, but not nuclear, levels of TTP increase dramatically when cells are treated with lipopolysaccharide (7). The mechanism by which TTP translocates in response to extracellular stimuli is at present unclear. Here we present evidence that TTP, CMG1, and TIS11D can shuttle between the
nucleus and cytoplasm and that the nuclear export is mediated by a functional leucine-rich nuclear export sequence (NES) (23, 24). This sequence motif can direct export of proteins through the nuclear pore complex (NPC) into the cytoplasm (25).

This NES is at the amino terminus of TTP but at the carboxyl terminus of CMG1 and TIS11D; in all cases, the nuclear export is mediated by the export receptor CRM1. Finally we show that the nuclear import of the CCCH proteins requires the TZF domain but occurs independently of TZF RNA-binding ability.

MATERIALS AND METHODS

**Plasmid Construction**—The cDNA for mouse TIS11D (accession number M58566) was obtained by screening a mouse fibroblast NIH-3T3 cell library (CLONTECH Laboratories, Inc., Palo Alto, CA) by standard techniques with a random primed, α-32P-labeled probe consisting of nucleotides 1–1190 of the rat cM1 cDNA (15); accession number X62590; generously supplied by K.D. Brown, The Babraham Institute, Babraham, Cambridge, UK). Three positive cDNA clones were sequenced by dRhodamine dye terminator cycle sequencing (PerkinElmer Life Sciences). The longest clone, containing nucleotides 205–2659 of accession number M58566, was designated CMG1–3′cDNA.

The 5′most 200 nucleotides of mouse CMG1 were cloned by reverse transcriptase-PCR, using the Access RT-PCR System (Promega) according to the manufacturer’s instructions, with a template of 1 μg of total cellular mRNA isolated from NIH-3T3 cells. The primers used were 5′-GCGCCCTTCTCGCGACCGAGCTCCAC-3′ and 5′-ATGGATGAGATTGCGACGGGGGC-3′ (corresponding to nucleotides 1–26 and 599, respectively, of accession number M58566). A PCR product of ~600 nucleotides was gel-purified, cloned into the vector pGEM-T (Promega Corp., Madison, WI), and sequenced by dRhodamine dye terminator cycle sequencing. The resulting plasmid, containing nucleotides 1–599 of accession number M58566, was designated CMG1–5′cDNA.

The protein coding region of mouse CMG1 spans nucleotides 68–1085 of accession number M58566. A cDNA containing this entire region was constructed from the above plasmid by cloning an ~2-kb Eco47III-HindIII fragment from the CMG1–3′cDNA into the Eco47III and HindIII sites of the CMG1–5′cDNA. The resulting plasmid was sequenced and contained nucleotides 1–2220 of accession number M58566. This was designated CMG1–2210cDNA and used as a PCR template for all CMG1 constructs described here.

A mouse genomic (MG-TIS11D) clone was obtained by screening a SV129 library (Stratagene) with a random-primed, α-32P-labeled probe consisting of nucleotides 1–275 of accession number M58565, which was a PsII-PsiI fragment from EST clone AI021952. From the MG-TIS11D clone, an ~7.3-kb XbaI fragment was subcloned into the vector pBSK (CLONTECH) to create the plasmid p11D-XbaI7.5, which was sequenced as described above. From p11D-XbaI7.5, an ~400-bp EcoRI-XhoI fragment containing the 3′ end of exon 1, the single intron, and the beginning of exon 2 was excised and replaced by a 140-bp EcoRI-XhoI fragment obtained from EST clone AI327503 to reconstitute a full-length TIS11D cDNA (this corresponds to nucleotides 1–980 of accession number M58565, as well as 120 upstream nucleotides representing additional sequence thought to be present in the full-length TIS11D protein) in plasmid p11DcDNA. Base 7 of EST clone AI327503 corresponds to the initiator methionine in the putative full-length human protein (corresponding to nucleotides 1–26 and 599). Plasmid pCMV-CMG1.105–338 encodes amino acids 108–338, corresponding to nucleotides 410–899. Plasmid pCMG1-1190 encodes amino acids 178–338, corresponding to nucleotides 600–899. Plasmid pCMG1.295–338 encodes amino acids 295–338, corresponding to nucleotides 950–1086. Plasmid pCMG1.TZF encodes amino acids 108–177, corresponding to nucleotides 410–599.

Plasmid pCMV-CMG1 contains nucleotides 68–1085 of accession number M58566 and encodes the full-length mouse CMG1 protein, amino acids 1–338 (see accession number AAA72948). Plasmid pCMG1-CMG1.1–307 encodes amino acids 1–307, corresponding to nucleotides 68–989 of accession number M58566. Plasmid pCMG1.1–108 encodes amino acids 1–108, corresponding to nucleotides 68–337. Plasmid pCMG1.1–177 encodes amino acids 1–177, corresponding to nucleotides 68–599. Plasmid pCMG1.105–338 encodes amino acids 108–338, corresponding to nucleotides 410–899. Plasmid pCMG1.118–338 encodes amino acids 178–338, corresponding to nucleotides 600–899. Plasmid pCMG1.295–338 encodes amino acids 295–338, corresponding to nucleotides 950–1086. Plasmid pCMG1.TZF encodes amino acids 108–177, corresponding to nucleotides 410–599.

Similarly, plasmid pGFP-TTP encodes the full-length mouse TTP protein, amino acids 1–319 (see accession number P22893), corresponding to nucleotides 32–990 of accession number M57422. Plasmid pGFP-TTP.1–290 encodes amino acids 1–290, corresponding to nucleotides 32–872. Plasmid pGFP-TTP.1–94 encodes amino acids 1–94, corresponding to nucleotides 32–317. Plasmid pGFP-TTP.1–158 encodes amino acids 1–158, corresponding to nucleotides 32–506. Plasmid pGFP-TTP.15–158 encodes amino acids 15–158, corresponding to nucleotides 75–506. Plasmid pGFP-TTP.15–319 encodes amino acids 15–319, corresponding to nucleotides 75–990. Plasmid pGFP-TTP.15–319 encodes amino acids 15–319, corresponding to nucleotides 75–990. Plasmid pGFP-TTP.15–319 encodes amino acids 95–158, corresponding to nucleotides 320–990. Plasmid pGFP-TTP.TZF encodes amino acids 95–158, corresponding to nucleotides 320–990.

Epitope-tagged plasmids were similarly constructed by inserting a PCR fragment containing the CMG1 or TTP protein coding sequence into vector pCMVFLAG2 (Sigma) or pCMV-HA (CLONTECH). In all cases, the epitope tag was at the amino terminus of the fusion protein.

**Cell Culture and Transfections**—Cells were incubated in secondary antibody (Alexa Fluor 488; Molecular Probes, Inc., Eugene, OR). After drying overnight, the slides were examined and images obtained using a Zeiss LSM510 confocal microscope (Zeiss, Thornwood, NY). When indicated, leptomycin B (Sigma; 10 ng/ml) was added in fresh medium 6 h before cell fixing. To observe epitope-tagged proteins, cells grown on glass slides were fixed as described above, rinsed twice in PBS, and then incubated in an ice-cold 2:1 solution of ethanol/glacial acetic acid at −20 °C for 5 min. After rinsing four times in PBS, cells were permeabilized with ProLong Anti-fade (Molecular Probes, Inc., Eugene, OR). After drying overnight, the slides were scanned and images obtained using a Zeiss LSM510 confocal microscope (Zeiss, Thornwood, NY). When indicated, leptomycin B (Sigma; 10 ng/ml) was added in fresh medium 6 h before cell fixing.

**Immunofluorescence and Immunofluorescence—To observe cells expressing GFP fusion proteins, cells grown on glass slides were rinsed twice at room temperature in PBS, fixed for 10 min in 4% (v/v) paraformaldehyde, and then rinsed again twice with PBS before mounting with ProLong Antifade (Molecular Probes, Inc., Eugene, OR). After drying overnight, the slides were scanned and images obtained using a Zeiss LSM510 confocal microscope (Zeiss, Thornwood, NY). When indicated, leptomycin B (Sigma; 10 ng/ml) was added in fresh medium 6 h before cell fixing.

3 The accession numbers cited throughout this work are from GenBank™.
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**RESULTS**

Transfected TTP, CMG1, and TIS11D Fusion Proteins with GFP Are Predominantly Cytoplasmic at Steady State in 293 Cells—To examine the subcellular localization of wild-type TTP, CMG1, and TIS11D, 293 cells were transfected with plasmids encoding GFP fusion proteins, fixed, and examined by confocal microscopy. GFP alone was expressed in both the nuclear and cytoplasmic compartments (Fig. 1A), whereas GFP-TTP, GFP-CMG1 and GFP-TIS11D were predominantly cytoplasmic (Fig. 1, B–D). To show that fusion to GFP had no effect on protein localization, 293 cells were also transfected with epitope-tagged versions of TTP and CMG1 and then examined by immunocytochemistry. TTP-FLAG and CMG1-FLAG were predominantly cytoplasmic and were indistinguishable from GFP-TTP and GFP-CMG1 in their subcellular localization (Fig. 1, E and F). In all cases, the transfections were performed at least three times, and ~200 fluorescent cells were examined for each expression construct. The individual cells shown in the figures are representative of the overall cell population. Most of the same fusion proteins were also expressed in HeLa cells, in each case with identical subcellular localization in the two cell types (data not shown).

**TPP, CMG1, and TIS11D Subcellular Localization Are Lepptomycin B-sensitive**—The antifungal agent leptomycin B (LMB) is a selective inhibitor of the nuclear export receptor CRM1 (29), which transports proteins across the nuclear pore complex into the cytoplasm (30). To determine whether the subcellular localization of the CCCH proteins was sensitive to LMB, 293 cells transfected with GFP fusion proteins were treated with carrier (100% methanol; final concentration 0.001%, v/v) or a final concentration of 10 ng/ml LMB for 6 h, fixed, and examined by confocal microscopy. In the absence of LMB, GFP was distributed throughout the cells (Fig. 2A), whereas GFP-TTP (Fig. 2C), GFP-CMG1 (Fig. 2E), and GFP-TIS11D (Fig. 2G) were predominantly cytoplasmic. In the presence of LMB, GFP remained distributed throughout the cells (Fig. 2B), whereas GFP-TTP (Fig. 2D), GFP-CMG1 (Fig. 2F), and GFP-TIS11D (Fig. 2H) were predominantly nuclear, with nucleolar exclusion. Essentially identical results were obtained in similar experiments performed in HeLa cells (data not shown). Thus, when protein export from the nucleus was blocked by LMB, localization of TTP, CMG1, and TIS11D shifted from the cytoplasm to the nucleus. These data indicate that, when transfected into these mammalian cells, TTP, CMG1, and TIS11D shuttle between the nucleus and the cytoplasm.

Expression of the Dominant Negative Nuclearoporin Mutant ΔCAN Can Cause Nuclear Accumulation of TTP, CMG1, and TIS11D—In mammalian cells, the CAN/Nup214 protein is a component of the nuclear pore complex, and its binding to CRM1 is required for CRM1-mediated export (31). CRM1 activity in cells can be efficiently blocked by overexpression of the dominant negative mutant ΔCAN, a truncated form of the nuclearoporin CAN/Nup214 containing only the CRM1-binding site (27). To determine the effect of ΔCAN on TTP, CMG1, and TIS11D nuclear export, plasmids expressing the GFP fusion proteins were co-transfected with ΔCAN or a control plasmid into 293 cells. In the absence of ΔCAN, GFP was distributed throughout the cells (Fig. 3A), whereas GFP-TTP (Fig. 3C), GFP-CMG1 (Fig. 3E), and GFP-TIS11D (Fig. 3G) were predominantly cytoplasmic. In the presence of ΔCAN, GFP remained distributed throughout the cells (Fig. 3B), whereas GFP-TTP (Fig. 3D), GFP-CMG1 (Fig. 3F), and GFP-TIS11D (Fig. 3H) were predominantly nuclear, with nucleolar exclusion. These data, together with the results shown in Fig. 2, demonstrate that TTP, CMG1, and TIS11D can shuttle in and out of the nucleus. Furthermore, nuclear accumulation of the CCCH proteins in the presence either of LMB or ΔCAN indicates that nuclear export of TTP, CMG1, and TIS11D depends on the CRM1 export receptor.

**Putative Nuclear Export Sequences Are Present in TTP, CMG1, and TIS11D**—The nuclear export receptor CRM1 is known to interact directly with the leucine-rich NES originally characterized in the viral RNA export protein Rev (23). At their carboxy termini, TTP, CMG1, and TIS11D each contain a cluster of four characteristically spaced hydrophobic residues, similar to this type of NES. In addition, TTP contains a second putative NES at its amino terminus. An alignment of the putative carboxy-terminal NES in TTP, CMG1, and TIS11D, compared with known NES in other shuttling proteins, is shown in Fig. 4A. In the CCCH proteins, the critical NES residues are strongly conserved among family members from different animal species, including mammals, amphibians, and fish (Fig. 4B). Strikingly, the putative CMG1 and TIS11D NES showed exact amino acid identity across all vertebrate species examined. Therefore, the deletion and mutation analyses discussed below used only CMG1 as a representative of both CMG1 and TIS11D.

CMG1 and TTP Contain Discrete Regions Mediating Nuclear Import and Export—We wished to determine which, if any, of the NES in TTP, CMG1, and TIS11D were able to direct nuclear export of the proteins. In addition we wished to identify the protein region(s) required for nuclear import of the CCCH proteins. To this end, a panel of GFP-TTP and GFP-CMG1 deletion mutants was created. Each mutant was transfected into 293 cells and HeLa cells, treated with either carrier or LMB as described under Materials and Methods, and examined by confocal microscopy.

The CMG1 deletion mutants tested are illustrated schematically in Fig. 5A, and the results from confocal microscopy are summarized on the right side of the figure. The amino acid numbers indicate the residue number in accession number P23950. Confocal images (see Fig. 2, E and F) revealed that wild-type CMG1 appeared predominantly cytoplasmic and accumulated in the nucleus following LMB treatment. Taken together, the results summarized in Fig. 5A indicate a probable NES between amino acids 308 and 338 and a probable nuclear localization in the two cell types (data not shown).
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Subcellular localization of TTP, CMG1, and TIS11D is LMB-sensitive. 293 cells (1 × 10^6 cells per well) were plated on 2-chamber multiwell slides and transfected with 10 ng/well of DNA from the following plasmids: GFP (A and B), GFP-TTP (C and D), GFP-CMG1 (E and F), or GFP-11D (G and H). The total amount of DNA was brought up to 300 ng with pcDNA3.1. Cells were grown for an additional 24 h and then treated either with carrier (−; top panels) or 10 ng/ml LMB (+; bottom panels). After 6 h of treatment, cells were fixed and images were obtained as described in the legend to Fig. 1.

Localization signal (NLS) between amino acids 108–177 in CMG1. These domains correspond to amino acids 482–491 for an NES and 122–190 for an NLS in mouse TIS11D (accession number P23949). The NES-containing region of CMG1 is conserved exactly in the carboxyl terminus of TIS11D in all vertebrate species examined (Fig. 4), strongly suggesting that TIS11D also contains a functional NES in this region.

Similar experiments were performed using the TTP mutants as summarized in Fig. 5B; amino acid numbers refer to the residues in accession number P22893. Confocal images (see Fig. 2, C and D) revealed that wild-type TTP appeared to be predominantly cytoplasmic and accumulated in the nucleus following LMB treatment. Surprisingly, the data summarized in Fig. 5B revealed the probable presence of an NES between amino acids 1 and 15, as well as a probable NLS between amino acids 95 and 158 in TTP. The carboxyl-terminal NES-like sequence in TTP thus appears to be inactive.

CMG1 and TTP Contain Functional Leucine-rich Nuclear Export Sequences—The putative CMG1 NES spans amino acids 327–335 of accession number P23950 (see Fig. 4), whereas the analogous region in TIS11D spans amino acids 481–494 (accession number P23949). Previous studies (32) have indicated that the conserved hydrophobic residues, often leucines, in NESs are required for nuclear export activity. Therefore, we mutated critical hydrophobic NES residues within the CMG1 NES-like region to alanine (mutants L327A and F330A). The subcellular localization of these mutants is shown in Fig. 6. Again, wild-type CMG1 was almost exclusively cytoplasmic (Fig. 6A), whereas the L327A and F330A mutants were each predominantly nuclear (Fig. 6, C and D, respectively). The truncation mutant GFP-CMG1.1–307, lacking the entire putative NES, appeared to be entirely nuclear (Fig. 6B). Taken together, these results demonstrate the presence of a functional NES in CMG1, spanning amino acids 327–335 of accession number P23950, and that this NES is required for CMG1 nuclear export. By extension, these data suggest that the identical sequence in TIS11D is also a functional NES.

The putative amino-terminal TTP NES spans amino acids 3–15 of accession number P22893 (see Fig. 4). The critical hydrophobic residues in the amino-terminal TTP NES were mutated to alanine (L3A and I6A). The subcellular localization of these mutants is shown in Fig. 7. Compared with the cytoplasmic wild-type TTP (Fig. 7A), TTP mutants L3A and I6A were each predominantly nuclear (Fig. 7, C and D, respectively). The amino-terminal truncation mutant GFP-TTP.15–319, lacking the entire NES, appeared to be entirely nuclear (Fig. 7B). These results demonstrate the presence of a functional NES in TTP, spanning amino acids 3–13, and that this NES is required for TTP nuclear export.

Nuclear Localization of CMG1 and TTP Does Not Require RNA Binding—As demonstrated above, mutant TTP and CMG1 proteins in which the NES is disrupted (either by deletion or amino acid substitution) accumulate in the nucleus, and this requires the NLS-containing region within residues 108–177 in CMG1 (accession number P23950) and within residues 95–158 in TTP (accession number P22893). In each protein, the apparent NLS region coincides with the tandem zinc finger (TZF) domain that mediates RNA binding. We tested whether the ability to bind RNA is required for TTP and CMG1 nuclear import using point mutants in which one Cys in the first CCCH zinc finger of each protein was mutated to Arg or Ser. These mutants have been shown previously (1) to beunable to interact with ARE-containing mRNAs. The point mutant fusion proteins GFP-TTP.C147R and GFP-CMG1.C129S were transfected into 293 cells and treated with either carrier or 10 ng/ml LMB for 6 h, as shown in Fig. 8. Each of the mutant fusion proteins was predominantly cytosolic at steady state in the absence of LMB (Fig. 8, A and B). LMB treatment resulted in
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**DISCUSSION**

In this study, we provide evidence that the three known mammalian members of the TTP family of CCCH TZF proteins, TTP, CMG1, and TIS11D, are nucleocytoplasmic shuttling proteins. Macromolecular traffic between the nucleus and cytoplasm in eukaryotic cells occurs via the NPC, a large protein channel passing through the nuclear envelope (33). Unique receptors for import and export have been identified, as well as specific targeting sequences in the substrates being transported (34). The leucine-rich NES, first identified in the viral protein HIV-Rev (23), is composed of 3–5 hydrophobic amino acids with defined spacing (32, 35). NES motifs of this type have been shown to mediate nuclear export of a diverse group of mammalian nucleocytoplasmic shuttling proteins including mitogen-activate protein kinase/extracellular signal-regulated kinase/mitogen-activated protein kinase (36), c-AbI (37), cyclin B1 (38), IRF-3 (39), p53 (40, 41), IκBα (42), BTK (43), STAT1 (44, 45), SMAD4 (46), BRCA1 (47), APC (48, 49), and E2F4 (50). Here we demonstrate the presence of functional NES-like sequences in TTP and in the other two known mammalian members of its family of CCCH tandem zinc finger proteins, CMG1 and TIS11D.

The nuclear export receptor Xpo1/CRM1 (51) mediates nuclear export of leucine-rich NES-containing proteins by binding directly to the NES (30, 52, 53). In the nucleus, the CRM1-NES interaction is stabilized by cooperative binding of the small GTPase Ran in its GTP-bound form (RanGTP) (54); it is this trimeric CRM1-RanGTP-NES complex that is able to travel through the NPC. Export of CRM1 complexes through the NPC is not completely understood but is known to require a direct association between CRM1 and multiple NPC proteins, including the nucleoporin CAN/Nup214 (31). Once through the NPC, the high cytoplasmic concentration of RanGAP causes hydrolysis of RanGTP to RanGDP and subsequent release of NES-containing proteins from CRM1 (55). CRM1-dependent nuclear export is selectively blocked by the cytotoxin LMB, which binds to CRM1 and interferes with its interactions with the NES (29, 31, 52, 56, 57). We found that a dramatic shift in the localization of TTP, CMG1, and TIS11D from the cytoplasm to the nucleus could be achieved by blocking the NES-CRM1 interaction using LMB or by blocking the CRM1-CAN interaction using a dominant negative ΔCAN mutant. The same localization shift occurred when either the carboxyl-terminal NES in CMG1 or the amino-terminal NES in TTP was deleted, but not when the carboxyl-terminal NES-like sequence of TTP was deleted. It is possible that deletion of the entire NES in this way could cause abnormal protein folding leading to the abnormal localization of the mutant fusion proteins. We believe that this is unlikely, since mutating individual key hydrophobic residues in either active NES led to a similar nuclear localization, whereas similar mutations in the carboxyl-terminal NES-like sequence had no effect on subcellular localization.2 These data provide a mechanism for the previously noted export of TTP from the nucleus (22), as well as the first description of nucleocytoplasmic shuttling by CMG1 and TIS11D.

We have also provided evidence that, in addition to being required for mRNA binding, the TZF domain of both TTP and CMG1 (and presumably of TIS11D) is required for nuclear import of the proteins. This ability of the TZF to mediate nuclear import is apparently unrelated to its ability to interact with ARE-containing mRNA, in that mutations that are known to disrupt ARE binding by the CCCH zinc fingers (and that presumably disrupt the integrity of the zinc fingers) do not affect the ability of the TZF domain to direct nuclear localization. Further studies are underway to determine the mechanism by which the TZF region mediates nuclear import, as well as the critical amino acid sequences required for this process.

The data presented here suggest that CRM1-mediated nuclear export of the CCCH proteins could serve as a means to

\[\text{A} \quad \text{TTP (N)} \quad \text{CMG1} \quad \text{TIS11D} \quad \text{HIV-Rev} \quad \text{IRS-3} \quad \text{MEK1} \quad \text{E1F-4} \]

\[\text{B} \quad \text{kTTP (N)} \quad \text{mTTP (N)} \quad \text{xTTP (N)} \quad \text{hCMG1} \quad \text{mCMG1} \quad \text{xCMG1} \quad \text{zCMG1} \quad \text{hTIS11D} \quad \text{mTIS11D} \quad \text{xTIS11D} \quad \text{zTIS11D} \]

**FIG. 4. Identification of potential NES in TTP, CMG1, and TIS11D.** These alignments show the spatial conservation of the four key hydrophobic residues characteristic of the leucine-rich type of NES (shaded). In each case, the numbers before and after the sequence indicate the amino acid number of the proteins whose accession numbers are listed below. A, putative NES-like amino acid sequences in mouse TTP, both amino-terminal (N) and carboxyl-terminal (C), mouse CMG1 and mouse TIS11D are aligned with previously characterized NES-like sequences in human TTP and in the other two known mammalian members of its family of CCCH tandem zinc finger proteins, CMG1 and TIS11D.
modulate their ability to promote cytoplasmic and possibly nuclear mRNA decay. To date, TTP is the only member of the family of CCCH zinc finger proteins that has been shown to have a physiological effect on mRNA stability in intact animals and in primary cells derived from them. However, numerous other ARE-binding proteins have been identified, including heterogeneous nuclear ribonucleoprotein D/AUF-1 (58), which can destabilize ARE-containing transcripts in hematopoietic cells (59); TIA-R (60) and TIA-1, translational silencers of the TNFα gene (61); and members of the embryonic lethal abnormal vision family (62). HuR, the best studied embryonic lethal abnormal vision protein, can bind to ARE-containing mRNA (63) and can increase the stability of ARE-containing mRNA when overexpressed in mammalian cells (64–67). HuR can also shuttle into and out of the nucleus using a novel shuttling signal that is neither a leucine-rich NES nor a typical NLS (68).

The factors/mechanisms governing which ARE-binding protein is able to occupy an ARE at a given time are far from clear. Recently, it has been demonstrated that in fibroblasts, overexpression of HuR cannot overcome the destabilizing effect of overexpressed TTP on ARE-containing mRNA; however, in combination with activation of the p38 signaling pathway, HuR

![Fig. 5. Deletion analysis of CMG1 and TTP to identify functional nuclear import and export sequences. A, the figure represents a diagram of wild-type CMG1 and CMG1 deletion mutants, showing the relative positions of the TZF domain (gray shading) and the potential carboxyl-terminal NES (solid shading). The corresponding amino acid numbers in the expressed proteins are shown on the left and compared with the numbering of accession number P23950. The columns on the right indicate the subcellular localization of each expressed protein, in the absence (–) or presence (+) of LMB. N, nuclear; C, cytoplasmic; N+C, both. B, the figure represents a diagram of wild-type TTP and TTP deletion mutants, showing the relative positions of the TZF domain (gray shading) and the potential carboxyl-terminal (hatched shading) and amino-terminal NES (solid shading). The corresponding amino acid numbers in the expressed proteins are shown on the left, and compared with the numbering of GenBank™ accession number P22893. The columns on the right indicate the subcellular localization of each expressed protein, as described for A.](image)

![Fig. 6. Confocal images of 293 cells expressing GFP-CMG1 NES point mutants. 293 cells (1 × 10⁵ cells per well) were plated on 2-chamber, multwell slides and transfected with 10 ng/well GFP fusion plasmid DNA, as indicated. The total amount of DNA was brought up to 300 ng with pcDNA3.1. Cells were grown for an additional 30 h and fixed, and images were obtained as described in the legend to Fig. 1. A, GFP-CMG1; B, GFP-CMG1.1–307; C, GFP-CMG1.L327A; D, GFP-CMG1.F330A.](image)

![Fig. 7. Confocal images of 293 cells expressing GFP-TTP NES point mutants. 293 cells (1 × 10⁵ cells per well) were plated on 2-chamber, multwell slides and transfected with 10 ng/well of GFP fusion plasmids. The total amount of DNA was brought up to 300 ng with pcDNA3.1. Cells were grown for an additional 30 h and fixed, and then images were obtained as described in the legend to Fig. 1. A, GFP-TTP; B, GFP-TTP.15–319; C, GFP-TTP.L3A; D, GFP-TTP.I6A.](image)
is able to block TTP-mediated mRNA decay (12). Previous data from our laboratory (22) showed that TTP can be induced to move out of the nucleus rapidly (<5 min) upon treatment of fibroblasts with mitogen. These observations, taken together with the data presented in this study, suggest that CRM1-mediated export of TTP may be regulated by one or more intracellular signaling cascades.

A number of viral proteins that shuttle between the nucleus and cytoplasm also bind to and export RNA (69–72). Since TTP can both bind to mRNA and shuttle between the nucleus and cytoplasm, we speculated that it too may act as an RNA export factor. However, using a recently described assay for nucleocytoplasmic RNA export ability (73), we found no evidence that TTP could export unspliced mRNA from the nucleus. Whether or not TTP and other CCCH proteins have functions in the nucleus, their regulated nuclear import and export, mediated by leucine-rich NES and still yet-to-be-determined NLSs, are likely to be key regulatory mechanisms for controlling their activities.

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