Molecular mapping of the determinants involved in human Staufen–ribosome association

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The human double-stranded (ds) RNA-binding protein Staufen (hStau) is considered to have a role in RNA transport and its localization. By using sedimentation analysis on sucrose gradients, we showed that the Staufen isoform with an apparent molecular mass of 55 kDa (Stau⁵⁵) co-fractionated with ribosomes and associated with both the 40 and 60 S ribosomal subunits, suggesting that the Staufen isoform hStau⁵⁵ plays some role in translation. To map the determinant(s) involved in this association, we generated a series of deletion mutants and analysed their subcellular distribution by cell fractionation and fluorescent immunomicroscopy. Our results demonstrated that multiple determinants promote hStau⁵⁵–ribosome association via both an RNA-binding-dependent mechanism and protein–protein interaction. The RNA-binding activity of the ds

RNA-binding protein domain 3 (dsRBD3) but not that of dsRBD4 is the first determinant. Although necessary for stable association with ribosomes, dsRBD3 alone is not sufficient and needs other determinants as co-factors. Consistently, when expressed together, dsRBD4 and the tubulin-binding domain constitute the minimal Stau⁵⁵/ribosome protein–protein association domain. This region of Stau⁵⁵ is sufficient to associate with ribosomes independently, but requires the RNA-binding activity of dsRBD3 for complete association. Thus the results are consistent with a putative role for Stau⁵⁵ in the regulation of translation.

Key words: cytoskeleton, mRNA localization, mRNA transport, RNA-binding activity, RNA-binding protein.

INTRODUCTION

Mammalian Staufen, an RNA-binding protein, binds to RNAs and double-stranded (ds) RNAs with extensive secondary structures [1,2]. Staufen contains four copies of a ds RNA-binding protein domain (dsRBD) consensus sequence. Molecular dissection of the protein *in vitro* revealed that only one of them, dsRBD3, efficiently binds to ds RNA, although dsRBD4 has a weak RNA-binding activity [2]. Mammalian Staufen was also shown to bind tubulin *in vitro*, via a tubulin-binding domain similar to the MAP-1B microtubule-binding domain [2]. Electron microscopy and pharmacological studies in rat hippocampal neurons were consistent with the idea that Staufen associates with microtubules, but direct interaction *in vivo* has not been described previously [3–5]. In both neurons and fibroblasts, Staufen co-localizes mainly with markers of the rough endoplasmic reticulum (RER) [1–3]. Cell fractionation and sedimentation analyses before and after dissociation of polyribosomes by puromycin or EDTA treatment further suggested that Staufen co-fractionated with ribosomes and polysomes [1]. Interestingly, endogenous and transfected Staufen were shown to co-localize with SYTO 14-labelled granules in dendrites of hippocampal neurons in culture [3,4]. SYTO 14-labelled granules were shown to correspond to clusters of ribosomes and to co-localize with polyadenylated [poly(A)⁺] RNAs, the 60 S ribosomal subunit and the translation elongation factor 1 α [6]. Recently [7], these densely packed clusters of ribosomes were shown to be highly enriched in Staufen protein. Therefore we hypothesized that Staufen–ribosome association observed by biochemical analyses is relevant for *in vivo* Staufen function.

Staufen isoforms with apparent molecular masses of 55 kDa (Stau⁵⁵) and 63/65 kDa (Stau⁶³) have been observed by Western blotting in human, mouse and rat. cDNAs coding for Stau⁵⁵ have been isolated in these species [1–3,5]. In a previous study, we

reported a differentially spliced transcript in human cells that codes for a protein of 63 kDa and co-migrates with the 63 kDa protein revealed by the Western-blot analysis [2]. Since similar differentially spliced transcripts have not been found in the mouse and rat [5,8], it is possible that the cloned transcript is specifically expressed in humans and the 63/65 kDa isoform represents a third isoform for which a cDNA has not been isolated so far in any species.

In *Drosophila* sp., Staufen was shown to be involved in the transport and localization of specific mRNAs in oocytes and neuroblasts [9] and also essential for the derepression of oskar mRNA, once localized at the posterior pole [10]. The role of Staufen in mammals is still unclear. Human ds RNA-binding protein Staufen (hStau) was shown to be a component of RNA-containing granules that migrates in the dendrites of hippocampal neurons [4]. Also, it was shown to be encapsulated along with the HIV-1 RNA into virus particles [11], which strongly suggests an involvement in mRNA transport. In addition, its association with clusters of ribosomes in moving granules supports the hypothesis that it can be involved in translation regulation [7].

In the present study, we used multiple biochemical and cellular techniques to show that the Staufen isoform hStau⁵⁵ is associated with ribosomes. To understand the mechanism by which it associates with ribosomes, we have also studied the subcellular distribution of a series of hStau⁵⁵ mutants expressed *in trans*. These experiments allowed us to identify the molecular requirements for Stau⁵⁵–ribosome association.

EXPERIMENTAL

Cell culture and microscopy

Mammalian cells grown in Dulbecco's modified Eagle's medium supplemented with 10 % foetal bovine serum, and penicillin–

Abbreviations used: ds, double-stranded; dsRBD, ds RNA-binding protein domain; DTT, dithiothreitol; F135A, Phe¹³⁵ → Ala; GFP, green fluorescent protein; HA, haemagglutinin; hStau, human ds RNA-binding protein Staufen; Ni-NTA, Ni²⁺-nitrilotriacetate; PKR, ds RNA-dependent protein kinase; RER, rough endoplasmic reticulum; Stau⁵⁵, Staufen isoform with apparent molecular mass of 55 kDa; TBD, tubulin-binding domain.

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Table 1 Oligonucleotide primers used in the construction of hStau⁵⁵ mutants

Constructs	Oligonucleotides (sense–antisense)	Restriction sites*
RBD2	5'-AATTGGTACCTGCACTGTGCGTAAACTTGA-3' 5'-TATATCTAGATTAGCGGCCGCAACCTTGGTCACAAAGTTC-3'	<i>KpnI</i> <i>XbaI</i> and <i>NotI</i>
RBD3	5'-ATATAAGCTTAA GCCACCATGG AGGTGAATGGAAGAGAATC-3'† 5'-ATATTCTAGATTAGCGGCCGCTGATTCTAGGCTTTACTCGTTC-3'	<i>HindIII</i> <i>XbaI</i> and <i>NotI</i>
RBD4	5'-ATATAAGCTTAA GCCACCATGG TCAAGCCACAGACAAGC-3'† 5'-ATATTCTAGATTAGCGGCCGCTCTCCTGTGACTTGAGTGC-3'	<i>HindIII</i> <i>XbaI</i> and <i>NotI</i>
RBD4/TBD	5'-ATATAAGCTTAA GCCACCATGG TCAAGCCACAGACAAGC-3'† 5'-TACAATCTAGATTAGCGGCCGCTCAGAGGGTCTAGTGCAGAG-3'	<i>HindIII</i> <i>XbaI</i> and <i>NotI</i>
TBD/5	5'-TACATAAGCTTAA GCCACCATGG TCAAAGTCCCGCAGCGGC-3'† 5'-TACAATCTAGATTATCAGCGGCCGCACTCCACACAGACAT-3'	<i>HindIII</i> <i>XbaI</i> and <i>NotI</i>
hStau ⁵⁵ Δ3	5'-GCAGTTGAACGAGTAAAGCCTAGAATC-3' 5'-GAGATTTTCTTCTCGGATTCTCTCC-3'	
hStau ⁵⁵ Δ4	5'-ACCAAACCCGCACTCAAGTCAGAGGAG-3' 5'-TGGGCTTGCTGTGGCTTGACTATGGG-3'	
hStau ⁵⁵ Δ5	5'-AATTGGTACCTGCACTGTGCACTGAAACTTGA-3' 5'-TATATCTAGATTAGCGGCCGCAACCTCTCGGGTATCATGGC-3'	<i>KpnI</i> <i>XbaI</i> and <i>NotI</i>
hStau ⁵⁵ /3*	5'-TTTTCCCGGGAGAGTGGCCACCCACATGAAGAAGCTGTGACCAAG-3'‡ 5'-CTACAGCCTGGGCGACCTCGG-3'	<i>SmaI</i>
hStau ⁵⁵ /4*	5'-TTTTAGGCTCCCGCGCCGAGGAGGCTGTGATGCAG-3'‡ 5'-CTACAGCCTGGGCGACCTCGG-3'	<i>StuI</i>

* Underlined nucleotides: restriction sites.

† Boldface nucleotides: Kozak's consensus sequence and ATG-initiation codon.

‡ Nucleotides in italics: mutated nucleotides as compared with those in the wild-type sequence.

streptomycin were transiently transfected with cDNAs using the calcium phosphate precipitation technique. Cells were fixed less than 16 h post-transfection to prevent overexpression of the proteins and stained with antibodies as described previously [2]. For the analysis of cytoskeleton-associated proteins, transfected cells were first extracted in 0.3% (v/v) Triton X-100 before fixation. Cells were revealed by immunofluorescence using the ×63 Plan-Apochromat objective of a Zeiss Axioskop fluorescence microscope.

Construction and molecular cloning of the fusion proteins

All mutants were constructed by PCR amplification of the cDNAs coding for hStau⁵⁵ using the Vent DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.). The resulting fragments were digested with appropriate restriction enzymes and cloned in pCDNA3/rous sarcoma virus [12]. When necessary, a haemagglutinin (HA) tag, namely HA₃, or the green fluorescent protein (GFP) was added to the C-terminus of the mutants as described previously [2]. Primers used for PCR amplification are listed in Table 1. For hStau⁵⁵Δ4 and hStau⁵⁵Δ3, the PCR was treated with *DpnI* (Roche Molecular Biochemicals, Laval, Quebec, Canada) and T4 PNK kinase (Pharmacia, Baie d'Urfé, Quebec, Canada) before being self-ligated and cloned.

Phenylalanine residues at position 135 in dsRBD3 and at position 238 in dsRBD4 were mutated into alanine residues [Phe¹³⁵ → Ala (F135A), Phe²³⁸ → Ala (F238A)] using two sense primers with mutations and an antisense primer located downstream from the endogenous *EcoRI*-restriction site (Table 1). The resulting fragments were digested with *SmaI*–*EcoRI* and *StuI*–*EcoRI* respectively, substituted for the corresponding wild-type fragment in hStau⁵⁵ cDNA and then sequenced.

All the fusion and mutated proteins were first expressed in COS7 cells and analysed by SDS/PAGE to test their level of expression and stability. We fixed or harvested the cells 16 h post-transfection to prevent overexpression of the proteins in

subsequent studies. At this time point, the amount of transfected proteins is usually less than that of the endogenous Stau⁵⁵ protein (right-hand side panels in the Figures). The half-life of wild-type and of several mutants has been described previously [11].

Protein purification, Western blotting and RNA-binding assay

To determine the RNA-binding capacity of hStau⁵⁵/3* and hStau⁵⁵/3*/4*, we substituted the HA₃ tag with a His₆ tag at the C-terminal end of the mutants by using the annealed oligonucleotides 5'-GGCCACCATCACCATCACCATT-3' and 5'-GGCCTAATGGTGATGGTGATGGT-3'. After transfection into HEK-293 cells, the cells were harvested and lysed. hStau⁵⁵–His₆ fusion proteins were isolated on Ni²⁺-nitrilotriacetate (Ni-NTA) columns as described previously [2]. Expression of the mutants was determined by Western blotting. Their RNA-binding capacity was determined by Northwestern blotting as described previously [2].

Cell fractionation on sucrose gradients

Cell fractionation was done by sedimentation analysis on sucrose gradients [13]. COS7 cells were rinsed with cold (4 °C) PBS (pH 7.5), and harvested. Approximately 4 × 10⁷ cells were resuspended in 3 ml of lysis buffer [10 mM Hepes (pH 7.5), 12.5% sucrose, 1 mM EDTA, 1 mM PMSF, 1 µg/ml pepstatin, 1 µg/ml aprotinin] and were subjected to at least 10 strokes in a Dounce homogenizer. Two centrifugations at 700 g and 1000 g were performed and the resulting supernatant was layered on to a step gradient containing nine fractions of 22–60% (w/v) sucrose in 10 mM Hepes (pH 7.5) and 1 mM MgCl₂. The gradients were centrifuged at 37000 rev./min (SW41 rotor; Beckman Instruments, Spinco Division, Palo Alto, CA, U.S.A.) for 2.5 h at 4 °C. Fifteen fractions (0.77 ml) were taken sequentially from the top of the tube and aliquots of each fraction were analysed by SDS/PAGE. Proteins were transferred on to a nitrocellulose mem-

brane and probed with anti-HA, anti-hStau [2], anti-calnexin (Stressgene, BC, Canada), anti-ribosomal L7a or anti- α -tubulin antibodies (ICN Biomedicals, Irvine, CA, U.S.A.). Quantification of the protein amounts that co-fractionated with ribosomes, RER or soluble proteins was done with an HP ScanJet (6100C) using the NIH image processing and analysis program.

For ribosome analysis, COS7 cells were washed in cold (4 °C) PBS (pH 7.5), and in an isotonic buffer [110 mM potassium acetate/2 mM MgCl_2 /1 mM dithiothreitol (DTT)/10 mM Hepes (pH 7.5)]. Cells were recovered with a rubber policeman in a hypotonic buffer [10 mM potassium acetate/25 mM EDTA/1 mM DTT/5 mM Hepes (pH 7.5)], supplemented with 1 unit/ml RNase inhibitors (Applied Biosystems, Foster City, CA, U.S.A.) and EDTA-free 'complete' protease inhibitor cocktail (Roche, Indianapolis, IN, U.S.A.). Cells were broken by two sets of 20 strokes in a 23-gauge syringe followed by centrifugation at 1500 *g* for 10 min. Supernatants were pooled, adjusted to 100 mM KCl and 0.5 % Nonidet P40. Extracts were left on ice for 30 min and centrifuged on a continuous 10–40 % sucrose gradient containing 100 mM KCl, 10 mM potassium acetate, 25 mM EDTA, 1 mM DTT and 5 mM Hepes (pH 7.5) for 4 h at 38000 rev./min in a SW41 rotor. Fractions (0.8 ml) were recovered and the RNA sedimentation profile was monitored spectrophotometrically at 254 nm. Total RNA was prepared from the fractions by phenol–chloroform extraction and ethanol precipitation and RNA was separated on denaturing formaldehyde–agarose gel. Proteins were recovered by acetone precipitation and analysed by Western blotting. Images were obtained on a Bio-Rad PhosphorImager.

Co-immunoprecipitation

Transfected cells were washed in isotonic buffer and the extracts were prepared as described above for the ribosomal gradient. Immunoprecipitations were performed with the anti-ribosomal P protein human antiserum as described previously [14].

RESULTS

Stau⁵⁵–HA₃ is associated with ribosomes

To determine the subcellular localization of Stau⁵⁵ in COS7 cells and also to determine whether transfected hStau⁵⁵–HA₃ has the same distribution and therefore can be used to map the determinant(s) involved in Staufen distribution, we compared their sedimentation profile in a 22–60 % sucrose gradient. Transfected cells were harvested about 16 h post-transfection to prevent overexpression of the protein. Each fraction of the gradient was analysed by Western blotting with anti-hStau, anti-HA, anti-calnexin, anti-L7a and anti- α -tubulin antibodies to detect endogenous Stau⁵⁵, transfected hStau⁵⁵–HA₃, RER, ribosomes and soluble proteins respectively. As shown in Figure 1(A), endogenous Stau⁵⁵ in untransfected cells was found predominantly in ribosome-containing fractions like L7a. The cloned hStau⁵⁵–HA₃ presented a similar profile and also co-fractionated with ribosomes (Figure 1B).

To confirm that the endogenous Stau⁵⁵ and hStau⁵⁵–HA₃ are associated with ribosomes and to separate the ribosome subunits, COS7 cell extracts were separated in a sharper sucrose gradient (10–40 %) and subjected to a longer centrifugation time in the presence of EDTA. Under these conditions, the co-migration of endogenous Stau⁵⁵ (Figure 2A) and hStau⁵⁵–HA₃ (Figure 2B) with both the 40 and 60 S ribosomal subunits demonstrated their association with ribosomes. Altogether, these results indicate that Stau⁵⁵ is associated with ribosomes, which transiently express hStau⁵⁵–HA₃, has the same subcellular distribution as the en-

Figure 1 Association of the Stau⁵⁵ isoform with ribosomes

(A) Untransfected COS7 cells were homogenized and fractionated on a sucrose gradient. Each fraction was collected and the proteins were separated by SDS/PAGE. Staufen, RER, ribosomes and soluble proteins were detected as indicated with anti-Staufen (α Stau), anti-calnexin (α CNX), anti-L7a (α L7a) and anti- α -tubulin (α Tub) antibodies respectively. Molecular mass (kDa) of the proteins is indicated on the right-hand side. The fast migrating proteins of approx. 53 kDa are generated from Stau⁵⁵ by differential translation initiation at different ATGs (M. Luo and L. DesGroseillers, unpublished work). R, ribosome. (B) COS7 cells were transfected with cDNAs coding for hStau⁵⁵–HA₃, lysed 16 h post-transfection and analysed as described in (A), except that fusion proteins were detected with the anti-HA (α HA) antibody. L7a is also detected in RER fractions on overexposed blots. Schematic representation of transfected protein is shown below the gel. White and grey boxes: major and minor RNA-binding domains respectively, whereas black boxes represent regions with RNA-binding consensus sequence but lacking RNA-binding activity *in vitro*. Hatched box: position of the region similar to the MAP-1B microtubule-binding domain. Right panel: Western-blot analysis of mock (M) and hStau⁵⁵–HA₃-transfected (T) COS7 cells. Western-blot analyses were probed with anti-hStau antibody. Transfected hStau⁵⁵–HA₃ (60 kDa) is not overexpressed as compared with endogenous (63 and 55 kDa) Stau proteins.

ogenous protein, and that it can be used to identify the molecular determinant(s) involved in Stau⁵⁵–ribosome association.

Staufen RNA-binding activity is one of the determinants for ribosome association

As hStau⁵⁵ contains a strong dsRBD3 and a weak dsRBD4, is RNA-binding activity involved in hStau⁵⁵–ribosome association? To find an answer, we introduced point mutations in dsRBD3 alone (F135A) or in both dsRBD3 (F135A) and dsRBD4 (F238A) to generate hStau⁵⁵/3*–His⁶ and hStau⁵⁵/3*/4*–His⁶ (Figure 3). We first tested whether the point mutations introduced in the dsRBDs eliminated the ds RNA-binding capacity of the protein. hStau⁵⁵–His⁶, hStau⁵⁵/3*–His⁶ and hStau⁵⁵/3*/4*–His⁶ were expressed in mammalian cells and purified on the Ni-NTA columns. Levels of expression were determined by Western blotting (Figure 4A) and RNA-binding activity by Northwestern-blot assay (Figure 4B). This experiment showed that the mutations are sufficient to eliminate the ds RNA-binding capacity of the mutants and that dsRBD3 is critical for this activity.

Analysis of transiently transfected COS7 cells by immunofluorescence showed that hStau⁵⁵/3*–HA₃ and hStau⁵⁵/3*/4*–HA₃ were randomly distributed in the cells, which demonstrated that RNA-binding activity was essential for their stable

Figure 2 Endogenous and transfected Stau⁵⁵ co-fractionate with ribosomal subunits

Untransfected COS7 cells (**A**) or cells transfected with a cDNA coding for hStau⁵⁵-HA₃ (**B**) were incubated in the presence of 25 mM EDTA and loaded on to a linear 10–40% sucrose gradient. Each fraction was collected and screened for ribosomal RNA by ethidium bromide staining of formaldehyde-agarose gel and for Stau⁵⁵ by Western blotting using anti-Stau (**A**) or anti-HA (**B**) antibodies.

association with cell structures. However, when the cells were treated with Triton X-100 before fixation to remove the soluble proteins, significant amounts were retained in Triton-resistant structures (results not shown). Consistently, when analysed by sucrose density centrifugation, more than 35% of both hStau⁵⁵/3*/4*-HA₃ (Figure 4C) and hStau⁵⁵/3*-HA₃ (Figure 4D) were found along with the soluble proteins, as compared with 3% for hStau⁵⁵-HA₃. Approximately 50% of both proteins still co-fractionated with the ribosome marker. A similar point mutation that eliminated the weak RNA-binding activity of dsRBD4 alone (hStau⁵⁵/4*-HA₃) had no effect on its distribution (Figure 4E), which is similar to that of hStau⁵⁵-HA₃. These results indicate that dsRBD3 RNA-binding activity plays a role(s) in the subcellular localization of hStau⁵⁵, especially on the amount and strength of hStau⁵⁵-ribosome association. However, it did not completely eliminate the co-fractionation of the protein with ribosomes, which implies that protein-protein interaction also occurs.

dsRBD4/TBD is necessary and sufficient for protein-protein interaction with ribosomes

We mapped the minimal domain that is required for hStau⁵⁵/ribosome protein-protein interaction. When expressed alone, dsRBD2-GFP, dsRBD3-GFP (results not shown), dsRBD4-GFP (Figure 5, upper panels) and TBD-GFP [2] were

Figure 3 Schematic representation of hStau mutants

Left panel: schematic representation of Stau⁵⁵ mutants. The colour code is the same as in the legend of Figure 1(A): mutations F135A or F238A. Right panel: summary of results obtained by immunofluorescence and cell fractionation (gradients). IF: +, complete association with subcellular organelles/structures in the absence or presence of Triton X-100; —, random cytosolic/nuclear distribution before Triton treatment and complete protein removal after Triton treatment; +/-, random cytosolic/nuclear distribution before Triton treatment but significant association with subcellular organelles/structures after Triton treatment. Gradients: +, co-fractionation of Stau⁵⁵ with ribosomes; +/-, co-fractionation with ribosomes and with soluble proteins; —, lack of co-fractionation with ribosomes.

both randomly distributed in the cell and extracted by Triton X-100. Consistently, none of the dsRBD2-HA₃, dsRBD3-HA₃, dsRBD4-HA₃ or TBD-HA₃ co-fractionated with ribosomes in the sucrose gradients (results not shown). However, when dsRBD4 and TBD were fused together, large amounts of RBD4/TBD-GFP were retained with the Triton X-100-insoluble materials (Figure 5, lower panels). RBD4/TBD-HA₃ also co-fractionated with ribosomes in the sucrose gradient (Figure 6A). Approximately 35% of RBD4/TBD-HA₃ was found in the fractions with soluble proteins, as described above for hStau⁵⁵/3*/4*-HA₃. When the F238A point mutation was introduced in dsRBD4 to generate RBD4*/TBD-HA₃, both immunofluorescence analyses in the presence or absence of Triton X-100 (results not shown) and cell fractionation (Figure 6B) indicated that the mutation that eliminated the RNA-binding activity of dsRBD4 had no effect on the RBD4*/TBD-HA₃ distribution, when compared with that of RBD4/TBD-HA₃. In contrast, TBD/5-HA₃ was shown to be randomly distributed and completely removed by Triton treatment in transfected cells (results not shown), and was found in the fractions containing soluble proteins in the sucrose gradient (results not shown). These results demonstrate that the region encompassing dsRBD4/TBD is necessary and sufficient for the localization of Stau⁵⁵ to ribosomes through protein-protein interaction and constitutes the minimal RNA-independent ribosome-binding domain. How-

Figure 4 hStau⁵⁵ RNA-binding activity is involved in ribosome association

cDNAs coding for either hStau⁵⁵-His⁶ (lane 1), hStau⁵⁵/3*-His⁶ (lane 2) or hStau⁵⁵/3*/4*-His⁶ (lane 3) were transfected in HEK293 cells and the His-tagged proteins were purified on an Ni-NTA column. (A) Western-blot analysis with anti-hStau antibodies. (B) RNA-binding assay. The fusion proteins were electrophoresed on a 10% polyacrylamide gel, transferred on to the nitrocellulose membrane and incubated with ³²P-labelled bicoid 3' untranslated region RNA as described previously [2]. After extensive washing, RNA binding was detected by autoradiography. COS7 were transfected with hStau⁵⁵/3*/4*-HA₃ (C), hStau⁵⁵/3*-HA₃ (D) or hStau⁵⁵/4*-HA₃ (E) and fractionated on the sucrose gradient. Each fraction was collected and the proteins were separated by SDS/PAGE and analysed as described in Figure 1. Right panel: Western-blot analysis of transfected cells. Western blots were probed with anti-hStau antibody. Transfected proteins (60 kDa) are not overexpressed as compared with endogenous (63 and 55 kDa) Stau proteins.

ever, this region is not capable of providing full association as observed with hStau⁵⁵-HA₃.

RNA-binding-dependent and -independent Stau⁵⁵-ribosome association

To confirm these results and study the relationship between the RNA-binding-dependent and -independent mechanisms, we deleted dsRBD4 from the full-length protein as a possible way to eliminate Stau⁵⁵/ribosome protein-protein interaction. An additional mutant was constructed in which the deletion was paired with a point mutation in dsRBD3 that abolished its RNA-

Figure 5 RBD4/TBD is necessary and sufficient for ribosome association

COS7 cells were transfected with cDNAs coding for RBD4-GFP (upper panels) or RBD4/TBD-GFP (lower panels). Untreated (left panels) or Triton X-100-treated (right panels) cells were fixed and revealed by autofluorescence. Scale bar: 20 μm.

Figure 6 RBD4/TBD is the minimal RNA-independent ribosome-association domain

COS7 cells were transfected with cDNAs coding for (A) RBD4/TBD-HA₃ or (B) RBD4*/TBD-HA₃ and fractionated on the sucrose gradient. Each fraction was collected and the proteins were separated by SDS/PAGE and analysed as described in Figure 1. Right panels: Western-blot analysis of transfected (T) cells. Western blots were probed with anti-hStau antibody. Transfected proteins (28 kDa) are not overexpressed as compared with endogenous (63 and 55 kDa) Stau proteins.

binding activity. As controls, we also deleted dsRBD3 and dsRBD5 to generate hStau⁵⁵Δ3-HA₃ and hStau⁵⁵Δ5-HA₃ (Figure 3). Each mutant was transfected in COS7 cells and their

Figure 7 Role of dsRBD3 and dsRBD5 in ribosome association

COS7 cells were transfected with cDNAs coding for (A) hStau⁵⁵Δ3-HA₃ or (B) hStau⁵⁵Δ5-HA₃ and fractionated on the sucrose gradient. Each fraction was collected and the proteins were separated by SDS/PAGE and analysed as described in Figure 1. Right panel: Western-blot analysis of transfected (T) cells. Western blots were probed with anti-hStau antibody. Transfected hStau⁵⁵Δ5-HA₃ (50 kDa) was not overexpressed as compared with endogenous (63 and 55 kDa) Stau proteins.

Figure 8 Critical role of dsRBD3 and dsRBD4 in ribosome association

COS7 cells were transfected with cDNAs coding for (A) hStau⁵⁵Δ4-HA₃ or (B) hStau⁵⁵/3*/Δ4-HA₃ and fractionated on the sucrose gradient. Each fraction was collected and the proteins were separated by SDS/PAGE and analysed as described in Figure 1. Right panels: Western-blot analysis of transfected (T) cells. Western blots were probed with anti-hStau antibody. Transfected proteins (50 kDa) were not overexpressed as compared with endogenous (63 and 55 kDa) Stau proteins.

subcellular localization was determined by immunofluorescence microscopy and velocity sedimentation. As expected, expression of a mutant with deletion of dsRBD3, resulted in the same

Figure 9 hStau⁵⁵-HA₃ and RBD4/TBD-HA₃ co-immunoprecipitate with ribosomes

(A) Cellular extracts from untransfected (lanes 1, 5) and the cells transfected with cDNAs coding for RBD4/TBD-HA₃ (lanes 2, 6), RBD4/TBD-HA₃ (lanes 3, 7, 9) or hStau⁵⁵-HA₃ (lanes 4, 8, 10) were immunoprecipitated with a human anti-ribosome autoimmune serum (P) (lanes 5–8) or a normal human serum (lanes 9 and 10). Total extracts (lanes 1–4) and immunoprecipitates (lanes 5–10) were loaded on to a SDS/PAGE (10% gel) and analysed by Western blotting using anti-HA and anti-L7a ribosomal protein (as control) antibodies. *, non-specific bands detected by the anti-HA antibody. (B) Cellular extracts from cells transfected with a cDNA coding for hStau⁵⁵-HA₃ were immunoprecipitated with a rabbit anti-hStau (Stau) antibody or with the pre-immune serum (Pi). Immunoprecipitates were analysed by Western blotting using anti-P ribosomal protein antibody.

phenotype as hStau⁵⁵/3*-HA₃ (see Figures 7A and 4D); significant amount of the protein was found with soluble proteins but approx. 50% of hStau⁵⁵Δ3-HA₃ was able to get associated with ribosomes. Deletion of dsRBD5 (hStau⁵⁵Δ5-HA₃) had no effect on hStau⁵⁵ distribution, which still co-fractionated with ribosomes (Figure 7B). Therefore dsRBD5 is not involved in the Stau⁵⁵/ribosome protein–protein interaction.

Deletion of dsRBD4 from hStau⁵⁵ (hStau⁵⁵Δ4-HA₃) only had a minor effect on the strong ribosome association of the protein (Figure 8A). However, when deletion of dsRBD4 was paired to a point mutation in dsRBD3 that eliminated Stau⁵⁵ RNA-binding activity, hStau⁵⁵/3*/Δ4-HA₃ showed random cytoplasmic/nuclear distribution by immunofluorescence (results not shown) and co-fractionated with soluble proteins in the gradient (Figure 8B). Therefore hStau⁵⁵Δ4-HA₃ associates with ribosomes only via an RNA-binding-dependent mechanism in contrast with hStau⁵⁵-HA₃, which relies on both RNA-binding activity and protein–protein interaction. Results obtained with these two mutants confirm that RNA-binding activity is involved in the Stau⁵⁵-HA₃/ribosome association and that dsRBD4, but not its weak RNA-binding activity (cf. hStau⁵⁵/3*/4*-HA₃ with hStau⁵⁵/3*/Δ4-HA₃), plays a significant role in the hStau⁵⁵/ribosome protein–protein interaction.

hStau⁵⁵-HA₃ and dsRBD4/TBD co-immunoprecipitate with ribosomes

To acquire further evidence that hStau⁵⁵ and RBD4/TBD are bound to ribosomes, we immunoprecipitated ribosomes and tested for their presence in the immunoprecipitates. Therefore

hStau⁵⁵–HA₃, RBD4/TBD–HA₃ and TBD/5–HA₃, used as controls, were expressed in the COS7 cells. Transfected cell extracts were immunoprecipitated with anti-human ribosome antibody. Immunoprecipitates were analysed by Western blotting with anti-L7a (used as control) and anti-HA (Figure 9A) antibody to monitor ribosome precipitation and test putative co-immunoprecipitation of HA-tagged proteins respectively. As expected from Figures 2 and 6, both hStau⁵⁵–HA₃ (Figure 9A, lane 8) and RBD4/TBD–HA₃ (Figure 9A, lane 7) were detected in the immunoprecipitates, whereas TBD/5–HA₃ was not (Figure 9A, lane 6). The reverse experiment confirmed the Stau⁵⁵–ribosome association (Figure 9B). When cell extracts were immunoprecipitated with anti-Staufen antibody, the ribosomal P protein was detected in the resulting immunoprecipitate by Western blotting. Used as controls, normal human serum (Figure 9A) and rabbit pre-immune serum (Figure 9B) were unable to co-immunoprecipitate Stau⁵⁵–HA₃, RBD4/TBD–HA₃ and ribosomal proteins respectively. Altogether, these results further confirm Staufen's presence in a complex with ribosomes and that RBD4/TBD is capable of performing the RNA-independent interaction.

DISCUSSION

Stau⁵⁵ is associated with ribosomes

It was previously suggested that Staufen is involved in mRNA transport in neurons [4,5,15]. This suggestion was based on the fact that Staufen–GFP-labelled granules move from the cell body to the dendrites in a microtubule-dependent manner. The present study demonstrates that Stau⁵⁵ may play an additional and complementary role(s) in the cell. Our results on endogenous and transfected Staufen demonstrated that Stau⁵⁵ is mainly associated with the ribosomes. Its distribution in the same fractions as ribosomes in the sucrose gradients and its relocalization with ribosomal subunits in the presence of EDTA are strong pieces of evidence for a physical interaction between Stau⁵⁵ and the ribosomes. In addition, Stau⁵⁵ was also associated with ribosomes after RNase treatment, in contrast with the polyadenylated [poly(A)⁺]-binding protein, which was dissociated from the ribosome-enriched fractions and found in the supernatant (T. F. Duchaine and L. DesGroseillers, unpublished work). This result demonstrates that Stau⁵⁵ is not only indirectly associated with the ribosomes via binding of mRNAs. We do not exclude the possibility that Stau⁵⁵–ribosome complexes are localized close to or linked to membranes *in vivo*, since Staufen mainly co-localizes with markers of RER when observed by fluorescence microscopy [1–3].

Therefore we propose that Stau⁵⁵ is involved in a ribosome-related function. Since Stau⁵⁵ is ubiquitously expressed in mammals, it may be involved in a general pathway of translation and regulation. This is unlikely, because the number of Stau⁵⁵ molecules within a cell is far less than that of the ribosomes. Stau⁵⁵ only defines a very small population of ribosomes and therefore should not have an impact on translation of most of the mRNA. Alternatively, Stau⁵⁵ may define a functional subpopulation of ribosomes, e.g. those recruited for RNA localization, which is a phenomenon described in every cell type but more easily established in neurons. In neurons, Staufen was described in RNA granules, consisting of densely packed clusters of ribosomes [7]. These authors hypothesized that Stau⁵⁵ may play an important role in a dynamic and multi-step pathway involved in the transport of translationally silent RNA granules and transfer of specific mRNAs to active polysomes after cell activation. Genetic studies involving oskar mRNA and Staufen in *Drosophila* sp. [10] are consistent with the possibility that

Staufen modulates translation. It will be interesting to determine whether Stau⁵⁵ is involved in the derepression of mRNA translation as that of its *Drosophila* sp. homologue [16] or whether it plays other role(s).

Several members of the ds RNA-binding protein family, such as the ds RNA-dependent protein kinase (PKR), the *Xenopus laevis* RNA-binding protein A, the TAR–RNA-binding protein and NF90, were also shown to bind the ribosomes [17–20]. The molecular mechanism for ribosome association may be similar between members of the family since association of PKR with ribosomes involves both the presence of functional dsRBDs and an additional RNA-independent ribosome-association site [19,20]. Therefore Stau⁵⁵ may compete for ribosome-binding sites or modulate the function(s) of other ribosome-bound members of the dsRBP family. Heterodimer formation between members of the family has been described previously [21,22].

Ribosome association through dsRBD3 and protein–protein interaction

Our analyses of mutants demonstrated that dsRBD3 is the major RNA-binding domain and one of the molecular determinants for Stau⁵⁵ association with ribosomes. A point mutation in the conserved phenylalanine residue was shown to eliminate the RNA-binding activity of both *Drosophila* sp. Staufen and mammalian PKR dsRBDs [23–27] and also to disrupt the dsRBD3 RNA-binding activity of Stau⁵⁵. This mutation also impaired Stau⁵⁵ association with ribosomes and had the same phenotype as the one with the complete deletion of dsRBD3, suggesting that the RNA-binding activity of dsRBD3 is crucial for ribosome association. In contrast, the weak RNA-binding activity of dsRBD4 observed *in vitro* is not necessary for these functions. Indeed, dsRBD4 seems to contribute very little, if any, to the RNA-binding activity of the full-length protein *in vivo*. Interestingly, the K64E (Lys⁶⁴ → Glu) point mutation in PKR dsRBD1 also impaired both RNA-binding activity and PKR–ribosome association [19]. This lysine residue was shown to be present at the RNA–protein interface, where it interacted with the ds RNA [28,29], and its mutation did not modify the structure of the domain, adopting the same conformation as that of the wild-type protein [28]. Altogether, these results suggest that RNA-binding activity plays some role in the ribosome association by members of the dsRBP family. Alternatively, we do not exclude the possibility that protein–protein interaction mediated by dsRBD3 is involved in ribosome association. A point mutation in dsRBD1 of PKR that eliminates the RNA-binding activity of the protein was also shown to abolish PKR interaction with the 60 S ribosomal protein L18, which suggests that L18 and ds RNA compete for the dsRBD1-binding site [30].

If Stau⁵⁵ RNA-binding activity is indeed required as a molecular determinant for the ribosome association, what is the RNA target? One possibility is that Stau⁵⁵ directly binds to ribosomal RNA. This would be consistent with the fact that Stau⁵⁵ binds to both ribosomal subunits as shown in the present study and that the NMR solution structure of *Drosophila* sp. dsRBD3 shares many features common to the N-terminal domain of the bacterial ribosomal RNA-binding protein S5 [23]. If bound to ribosomal RNA, Stau⁵⁵ may not be directly linked with the mRNAs. Alternatively, Stau⁵⁵ may bind to mRNAs and this binding may trigger a conformational switch in Stau⁵⁵ that unmasks the protein–protein determinant.

Our results also demonstrated that dsRBD4/TBD is responsible for the protein–protein interaction of Stau⁵⁵. Protein–protein interactions mediated by dsRBDs have been described for *Drosophila* sp. Staufen and other members of the ds RNA-

binding protein family [22,31–33]. Similarly, the region of *Drosophila* sp. Staufen that corresponds to the mammalian TBD was shown to bind inscuteable [31], a cytoskeletal adaptor. TBD in hStau⁵⁵ binds to tubulin *in vitro* [2], and both the distribution [3] and the movement [4] of Stau⁵⁵-containing ribosome clusters within dendrites of neurons in culture are sensitive to drugs that disrupt microtubules. Since ribosomes are also associated with the cytoskeleton [34], it is possible that hStau⁵⁵ binding to ribosomes involves previous or simultaneous association with the microtubule network via TBD. Altogether, the results are consistent with the hypothesis that the ds RNA-binding domain dsRBD3 interacts with ribosomal RNA, whereas dsRBD4/TBD interacts with a separate site on the ribosome through protein–protein interaction. Further studies should determine the relative affinity of each determinant for ribosome association and identify the ribosomal or ribosome-associated RNA and protein partners required for interaction.

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