Staufen2 isoforms localize to the somatodendritic domain of neurons and interact with different organelles

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Summary

Mammalian Staufen2 (Stau2) is involved in mRNA transport in neurons. Here, we report that Stau2 is a double-stranded RNA-binding protein that is mainly expressed in the brain. We show that Stau2 is found in the somatodendritic compartment of neurons. In dendrites, Stau2 is aligned on individual tracts and colocalizes with microtubules. Stau2 is expressed as at least three splice isoforms, which can be observed in several subcellular complexes. Although a 62 kDa isoform (Stau2^{62}) fractionates in ribosome-free fractions of light density, Stau2^{59} and Stau2^{52} are found in high-density complexes. These complexes are resistant to EDTA and to non-ionic detergent. For the first time, we also provide evidence for an interaction of some Stau2 isoforms with ribosomes, thus pointing to an interesting new role for Stau2 in translation. EDTA treatment, which dissociates ribosome subunits, does not release Stau2 from the subunits, suggesting that Stau2-ribosome associations are not mediated mainly by mRNA intermediates. Although Stau2 has many features in common with its paralogue Stau1, it does not colocalize with Stau1-containing particles, indicating that these proteins are components of different complexes in dendrites. Our findings suggest that members of the Staufen family share evolutionarily conserved properties and highlight the complexity of Staufen-mediated RNA transport in neurons.

Key words: Staufen, Ribonucleoparticle, Ribosome, RNA-binding protein, RNA transport

Introduction

Cytoplasmic mRNA transport and anchoring to defined subcellular domains allow efficient spatial and temporal restriction of genetic expression (St Johnston, 1995; Kiebler and DesGroseillers, 2000). mRNA transport is observed in a variety of cell types and organisms and plays an important role in processes such as learning and memory, synaptic transmission, axis formation during development, cell motility and asymmetric cell division. In neurons, although most of the mRNAs are restricted to cell bodies, some are found in the somatodendritic compartment. As protein synthesis occurs in dendrites, the localization of these mRNAs in dendrites and their local translation are thought to allow neurite growth and plasticity at sites distant from the cell body and the differential plasticity of each individual dendrite in response to neighboring cells (Steward and Schuman, 2001). This also provides a basis for coordinating synaptic events with nuclear gene expression.

RNA transport is thought to be initiated by the recognition of cis-acting RNA motifs by RNA-binding protein(s) and their assembly into ribonucleoprotein (RNP) complexes. RNPs are then recruited and transported on the cytoskeleton and anchored to their final destination. To restrict RNA translation to the appropriate time and place, the mechanisms of mRNA transport must be tightly coupled to those of translation: translation of transported RNAs has to be repressed during transport, and repression has to be removed after arrival at the proper location and/or following signaling events. Evidence also supports a model in which the delivery of new mRNAs to the dendrite occurs in motile structures called RNA granules. Neuronal RNA granules were described as large clusters of ribosomes, RNAs, some translation factors and proteins and can be observed in living neurons using the RNA-binding dye SYTO14 (Knowles et al., 1996). They were suggested to be translationally incompetent and to represent reservoirs of silent RNA, which upon cell stimulation release RNAs for local translation on polyribosomes (Krichevsky and Kosik, 2001).

We and others have cloned and characterized Stau1 in mammals (Marión et al., 1999; Wickham et al., 1999; Monshausen et al., 2001), a protein similar to the *Drosophila* Staufen protein (St Johnston et al., 1991). Staufen is a dsRNA-binding protein involved in mRNA localization events in *Drosophila* oogenesis and neurogenesis (St Johnston, 1995). In mammals, Stau1 is ubiquitously expressed and localizes to the rough endoplasmic reticulum (RER) in vivo and cosediments with polyribosomes. In mammalian neurons, Stau1 was detected as particles in the soma and dendrites but not in axons (Kiebler et al., 1999; Monshausen et al., 2001). A Stau1/GFP

fusion protein colocalized with SYTO14-labeled granules and moved in dendrites at approximately the same speed as RNA-containing granules (Köhrmann et al., 1999), showing that Stau1 is a component of ribonucleoprotein complexes. Immunoelectron microscopic analyses confirmed that Stau1 is present in RNA granules in neurons (Krichevsky and Kosik, 2001). These observations, along with the evolutionary conservation of the protein structure, strongly support the involvement of Stau1 in RNA transport in mammalian cells.

Recently, a second Staufen homologue (*Stau2*) located on human chromosome 8 has been reported on the basis of genomic and EST analyses (Buchner et al., 1999). In cultured neurons, the amount of Stau2 in dendrites seems to correlate with that of RNA, suggesting that, like Stau1, Stau2 plays an important role in the delivery of RNA to dendrites (Tang et al., 2001). Here, we report the identification of two additional Stau2 isoforms generated by differential splicing and the cellular and molecular characterization of these proteins. Since Stau2 is mainly expressed in the brain, our studies concentrate on neurons. We show that Stau2 has many conserved features in common with Stau1. However, they also differ in other aspects that are likely to fulfil different though complementary roles in neurons.

Materials and Methods

Molecular cloning of Stau2

Human brain cDNA (Clontech, Palo Alto, CA) and fetal mouse cDNA (a generous gift from A. Royal, Montréal, Canada) libraries (10^5 pfu each) were screened with a [^{32}P]-labeled partial mouse Stau2 cDNA fragment as described previously (Wickham et al., 1999). 5′RACE was performed on RNA isolated from human (Clontech, Palo Alto, CA, USA) and mouse brains using the Mo-MuLV reverse transcriptase and the RT-primer 5′-GACAGGCATATTTCTCTTCAGCG-3′ as described before (Wickham et al., 1999). A poly(dA) tail was added at the 3′end of the resulting cDNA using terminal transferase. This fragment was PCR amplified for 10 cycles (94°C for 2 minutes, 52°C for 2 minutes and 72°C for 2 minutes) using the Vent DNA polymerase (New England BioLabs, Mississauga, ON, Canada), the sense oligonucleotide 5′-TGAGGTGGTTGCCACAGGAGG(T)$_{20}$-3′ and the RT primer. Nested PCR was performed for 30 cycles (94°C for 2 minutes, 52°C for 2 minutes and 72°C for 2 minutes) with the primer pair 5′-TGAGGTGGTTGCCACAGGAGG-3′ and 5′-CAGCATTGT-GTCTGGCAGCTTG-3′. Positive clones were sequenced by the dideoxynucleotide method according to Sequenase protocols (United States Biochemical Corp., Cleveland, OH) or with an automatic sequencer (373A Stretch XL; Université Laval, Québec, Canada), and overlapping PCR fragments and cDNAs were ligated to generate full-length cDNAs.

RNA analysis

For northern blotting, mouse tissues were dissected and total RNA immediately prepared using Trizol (Life Technology, NY). Poly(A)$^+$ RNA was isolated with the Oligo(dT)-cellulose matrix. Poly(A)$^+$ RNAs (4 μg) were loaded on a denaturing formaldehyde agarose gel and transferred to a nylon Hybond N+ membrane. Hybridization was carried out with the ExpressHyb solution (Clontech, Palo Alto, CA, USA) as proposed by the supplier. Three [^{32}P]DNA probes were used: a fragment coding for the C-terminus of Stau2 (*Bam*HI site to the Stop codon); a fragment coding for the C-terminus of Stau1 (*Eco*RI to the Stop codon); and a fragment coding for the entire open reading frame of mouse actin.

RT-PCR assays were performed with total RNA isolated from different tissues using the RNA PCR kit (Applied Biosystems, Foster City, CA). To determine the relative abundance of the Stau2^{62} and Stau2^{59} transcripts in a tissue, we amplified isolated RNA using RT-PCR in a single reaction tube with the sense 5′-CGCAGTT-TTGTGGAGCTGTGAGGG-3′ and antisense 5′-CCATGTCTGCT-CGCCAAGACTCAG-3′ oligonucleotides. These primers flank the alternatively spliced exon and therefore amplified both transcripts. Different amounts of starting RNA (250 to 1000 ng) and PCR cycles (94°C for 30 seconds, 60°C for 30 seconds) were compared to ensure that the amplification was below the level of PCR saturation and that the ratio of the two bands did not vary with the number of cycles used.

Northwestern assay

To construct the maltose-binding protein (MBP)-Stau2^{59} fusion protein (MBP-FL), the full-length λGT10 cDNA insert was first cloned into Bluescript SK (Stratagene, La Jolla, CA) and then digested with *Sal*I and *Sac*I for cloning in the corresponding sites of the pMalC vector (New England Biolabs, Mississauga, ON, Canada). To construct GST-1 to GST-5, DNA fragments coding for different Stau2 domains were PCR amplified with the Vent DNA polymerase (New England Biolabs, Mississauga, ON, Canada), cloned into Bluescript SK at the *Eco*RV site and subcloned into pGEX-T (Amersham Pharmacia, Baie d'Urfé, QC, Canada) using the *Sal*I and *Not*I restriction sites. To construct the GST-1 fusion protein, we used the sense 5′-TTCTCTCCAAGATAAAATGGCAAACCC-3′ and antisense 5′-AGACTTTTCTGGAATTGGCTCAATCTG-3′ primers. Similarly, GST-2, GST-3, GST-4 and GST-5 fusion proteins were constructed with 5′-GAGGGATACGGAAGTTTGATC-3′ and 5′-AGACTTTTCTGGAATTGGCTCAATCTG-3′; 5′-ACAGATTGAG-CCAATTCCAGAAAAGTCT-3′ and 5′-GTTTTGGCTTCTCTAC-CACAGG-3′; 5′-TCCTGTGGTAGAGAAGCCAAAAC-3′ and 5′-GAGCGGATCCTGAAGACTGGTG-3′, and with 5′-CACCAGTC-TTCAGGATCCGCTC-3′ and 5′-ATTCGTTTCCTAGAACACAG-ACACC-3′ oligonucleotides, respectively. Northwestern assays were performed as previously described (Wickham et al., 1999) using [^{32}P]bicoid 3′UTR RNA as probe.

Production of Stau2-specific antibodies

Monoclonals

A Stau2-GST fusion protein encoding the C-terminal portion of Stau2 (Thr^{379} to Stop) was expressed in BL21 pLysS DE3 (Invitrogen, Burlington, ON, Canada) and affinity purified on a Glutathione Sepharose matrix (Amersham Pharmacia, Baie d'Urfé, QC, Canada). The C-terminal Stau2 peptide was eluted from the column by cleavage with thrombin. Mice were immunized by multiple injections of 10 μg of antigen per injection, and spleens from Stau2-positive mice were isolated, and monoclonal antibodies (1C6) were prepared as described previously (Crine et al., 1985).

Polyclonals

Alternatively, the same *E. coli* overexpressed and purified Stau2-GST antigen (non thrombin-cleaved) was used for injecting mice intraperitoneally four times with 50 μg of antigen per injection. Samples of blood were then taken through the tail vein and antisera prepared as described previously (Harlow and Lane, 1988): diluted 1:1 in glycerol and stored at –20°C. Polyclonal antibodies were also prepared by injecting rabbits with 40 μg of the thrombin-cleaved antigen per injection, as described previously (Wickham et al., 1999). All these immune sera were tested for their specificity. First, no Stau2-specific signal was obtained with the pre-immune serum isolated from the corresponding mouse or rabbit. Second, pre-incubation of the antibodies with the Stau2-GST fusion protein antigen coupled to sepharose abolished the Stau2-specific signal. Finally, the mouse

antibodies recognize Stau2 but not Stau1 when overexpressed in BHK21 cells (data not shown).

Western blot assay

Western blotting was carried out as previously described (Wickham et al., 1999). Human autoimmune P serum (a generous gift of M. Reichlin, Oklahoma Medical Research Foundation, Oklahoma, USA) and rabbit anti-L7 antiserum (a generous gift of A. Ziemiecki, University of Berne, Berne, Switzerland) were used to detect ribosomes. Monoclonal anti-calnexin (Stressgen, Victoria, BC, Canada), anti-α-tubulin (ICN, Irvine, CA) and AE-4 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used to detect endoplasmic reticulum, α-tubulin and histone H1, respectively.

Primary cultures of neurons

For the biochemistry experiments, cortical neurons were isolated and cultured as described before (Shaw et al., 1985; Bassell et al., 1998) with the following modifications. Briefly, cerebral cortex was dissected from embryonic day 17-18 Sprague Dawley rats and digested with 0.25% trypsin. Neurons were plated at high density (80×10^6 per 150 mm plate) on poly-L-lysine- (10 μg/ml, overnight) and laminin-coated (2 ng/ml for 4 hours) 150 mm tissue culture dishes. After neurons had attached to the substrate (4 hours) in MEM+10% horse serum, cultures were allowed to differentiate in glutamate-free MEM with N2 supplements, including transferrin (100 μg/ml), insulin (5 μg/ml), progesterone (20 nM), putrescine (100 μM) and selenium dioxide (30 nM). In addition, extra glucose (600 mg/l), sodium pyruvate (1 mM), ovalbumin (0.1%) and 5% FBS were added to the media. Cells were allowed to achieve polarity for 5 days prior to extraction. For IF, primary hippocampal neurons derived from E17 rat embryos were cultured at low density according to the standard protocol (Goslin and Banker, 1991) as described previously (Kiebler et al., 1999). B27 supplements (Life Technologies, Karlsruhe, Germany) was used instead of the N2 supplement.

Immunocytochemistry

Immunocytochemistry was carried out essentially as described previously (Kiebler et al., 1999). The following primary antibodies (incubation overnight at 4°C) were used: rabbit anti-calnexin antibodies (dilution 1:400; a generous gift of A. Helenius, ETH, Zurich, Switzerland), mouse monoclonal anti-α-tubulin antibodies (dilution 10,000; Sigma, Munich, Germany), rabbit anti-Staufen1 antibodies (dilution 1:400, a generous gift of J. Ortin, Madrid, Spain) (Kiebler et al., 1999; Marión et al., 1999) and mouse anti-Staufen2 antibodies (dilution 1:400 from the glycerol stock, see above). The rabbit anti-Stau1 antibodies (Juan Ortín, Madrid) recognize Stau1 but not Stau2 (data not shown). As secondary antibodies, Texas-Red-conjugated affinipure goat anti-rabbit IgG Fab fragment (dilution 1:800; Dianova, Hamburg, Germany), Alexa Fluor 488 goat anti-rabbit IgG (H+L) conjugate (dilution 1:500; Molecular Probes, Leiden, Holland), biotin-conjugated sheep anti-mouse IgG (dilution 1:500; Roche Diagnostics, Mannheim, Germany), FITC-conjugated streptavidine (dilution 1:2,000; Amersham Pharmacia, Freiburg, Germany), Cy3-conjugated affinipure goat anti-mouse IgG (H+L) (dilution 1:800; Dianova, Hamburg, Germany) and Cy2-conjugated affinipure goat anti-mouse IgG (H+L) (dilution 1:800; Dianova, Hamburg, Germany) were used. Fluorescence and confocal microscopy were performed essentially as described previously (Kiebler et al., 1999). Neurons were also stained with monoclonal anti-MAP2 (dilution 1,000; Sigma, Munich, Germany) and anti-tau-1 (dilution 10,000; Chemicon International, Temecula, CA) antibodies, two markers of dendrites (Caceres et al., 1984) and axons (Binder et al., 1985), respectively. In the case of Fig. 6B, the Stau2 immunofluorescence signal was amplified using the biotin-streptavidine cascade.

Cytoplasmic extract preparation and crude cell fractionation

Cytoplasmic extracts were prepared from high-density cultures of cortical neurons. Neurons were washed in cold PBS (pH 7.5), then in isotonic buffer (110 mM KOAc, 2 mM MgOAC, 1 mM DTT, 10 mM HEPES, pH 7.5) and recovered with a rubber policeman in hypotonic buffer (10 mM KOAc, 2 mM MgOAc, 1 mM DTT, 5 mM HEPES pH 7.5) supplemented with 1 U/ml RNase inhibitors and EDTA-free COMPLETE protease inhibitor cocktail (Roche Diagnostics, Laval, QC, Canada). Cells were broken by two sets of 20 strokes in a 23-gauge syringe followed by centrifugation at 1,500 ***g*** for 10 minutes. Supernatants were adjusted to 100 mM KCl and allowed to stand on ice for 30 minutes. The S100-P100 fractionation was performed by centrifugation at 100,000 ***g*** for 1 hour in the Sorvall SW50.1Ti rotor as described previously (Siomi et al., 1996). Treatments of the cytoplasmic extracts before generating the S100/P100 fractions include 25 mM EDTA; 300 U/ml Micrococcal nuclease for 15 minutes prior to addition of 5 mM EGTA; 0.5% Nonidet P40; 0.5 M KCl. Incubations were carried out on ice for at least 30 minutes.

Sucrose gradient analysis

We used a 10-step discontinuous gradient ranging from 20 to 60% sucrose and containing 100 mM KCl, 10 mM KOAc, 2 mM MgOAc, 1 mM DTT and 5 mM HEPES, pH 7.5 as described previously (Luo et al., 2002). Gradients were centrifuged at 175,000 ***g*** in a SW41Ti rotor for 3 hours and fractions of 0.8 ml were collected. Proteins were recovered by acetone precipitation and analyzed by western blotting. Images were obtained on a BioRad Fluor-S MAX Multi-Imager.

For ribosome analysis, cytoplasmic extracts were left on ice for 30 minutes and centrifuged on a continuous 10 to 40% sucrose gradient containing 100 mM KCl, 10 mM KOAc, 25 mM EDTA, 1 mM DTT and 5 mM HEPES pH 7.5 for 4 hours at 250,000 ***g*** in a SW41 rotor. Fractions of 0.8 ml were recovered, and the RNA sedimentation profile was monitored with a spectrophotometer set at 254 nm. Proteins were recovered and analyzed as described above.

Co-immunoprecipitation

Neurons were washed in isotonic buffer, and cytoplasmic extracts were prepared as described above. Immunoprecipitation was carried out with either the anti-ribosomal P protein human antiserum as previously described (Siomi et al., 1996) or with the rabbit polyclonal anti-Stau2 antibodies. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and revealed by western blotting. Immunoprecipitated RNAs were purified, separated on a formaldehyde agarose gel, transferred to nylon membranes and hybridized with a [^{32}P]18S ribosomal RNA.

Ribosome pull down on 1.5 M sucrose cushion

Cortical neurons were cultured for 4 days at high density. Neuronal cultures were either serum starved for 4 hours or starved for 4 hours and refed with 5% FBS for 2 hours before lysis. Untreated cells were refed with fresh 5% FBS two hours before cytoplasmic extraction. Other cultures were treated with either cycloheximide (100 μg/ml) or puromycin (100 μg/ml) for 30 minutes, or with pactamycin (2 μg/ml) or rapamycin (20 ng/ml) for 1 hour before cytoplasmic extraction. Neurons were then recovered, and cytoplasmic extracts were prepared in 0.5 ml KCl and Nonidet P40, which were adjusted to 0.11 M and 0.5%, respectively. Extracts were left on ice for 30 minutes and then placed on a 1 ml sucrose cushion (1.5 M) containing 110 mM KOAc, 2 mM MgOAc, 1 mM DTT, 0.05% Nonidet P40 and 10 mM HEPES-K pH 7.4. Tubes were centrifuged at 300,000 ***g*** for 3 hours in a

Beckman TLA 100.3 microfuge. After centrifugation, the pellets (ribosome enriched fraction) were resuspended in 1 vol Laemmli sample loading buffer. Supernatant/cushions were recovered, the proteins TCA-precipitated and recovered in 1 vol Laemmli sample loading buffer. Proteins were resolved on a 8.5% SDS-polyacrylamide gel and analyzed by western blotting. All experiments were performed in duplicate.

Results

Stau2 isoforms are generated by differential splicing

A screen of a fetal mouse cDNA library and 5′RACE experiments revealed that the *Stau2* gene expresses two transcripts; both diverge at their 5′end sequence, with an insert of 131 nucleotides in transcript T1 compared with T2. A splicing consensus sequence flanks this additional sequence, demonstrating that the two transcripts are produced by differential splicing (Fig. 1A). The additional exon contains an ATG initiation codon that begins the synthesis of a 570 amino acid isoform (named Stau2^{62}). Stop codons are present upstream from this ATG codon. By contrast, the T2 transcript codes for a 538 amino acid isoform (named Stau2^{59}) that is initiated from an ATG codon located in the upstream exon. The two isoforms only differ in their N-terminal extremities (Fig. 1C). A third isoform of 479 amino acids (named Stau2^{52}), with an additional deletion of the C-terminal region, has been described before (Buchner et al., 1999). Similar results were obtained from human brain RNA (GenBank accession numbers AF459097 and AF459098). Human and mouse proteins share 92% amino-acid sequence identity. Western analysis of brain extracts with anti-Stau2 antibodies indicated that Stau2^{62}, Stau2^{59} and Stau2^{52} were all expressed in neurons (Fig. 2A). Stau2^{59} and Stau2^{52} are highly expressed, whereas Stau2^{62} is barely detectable. The three

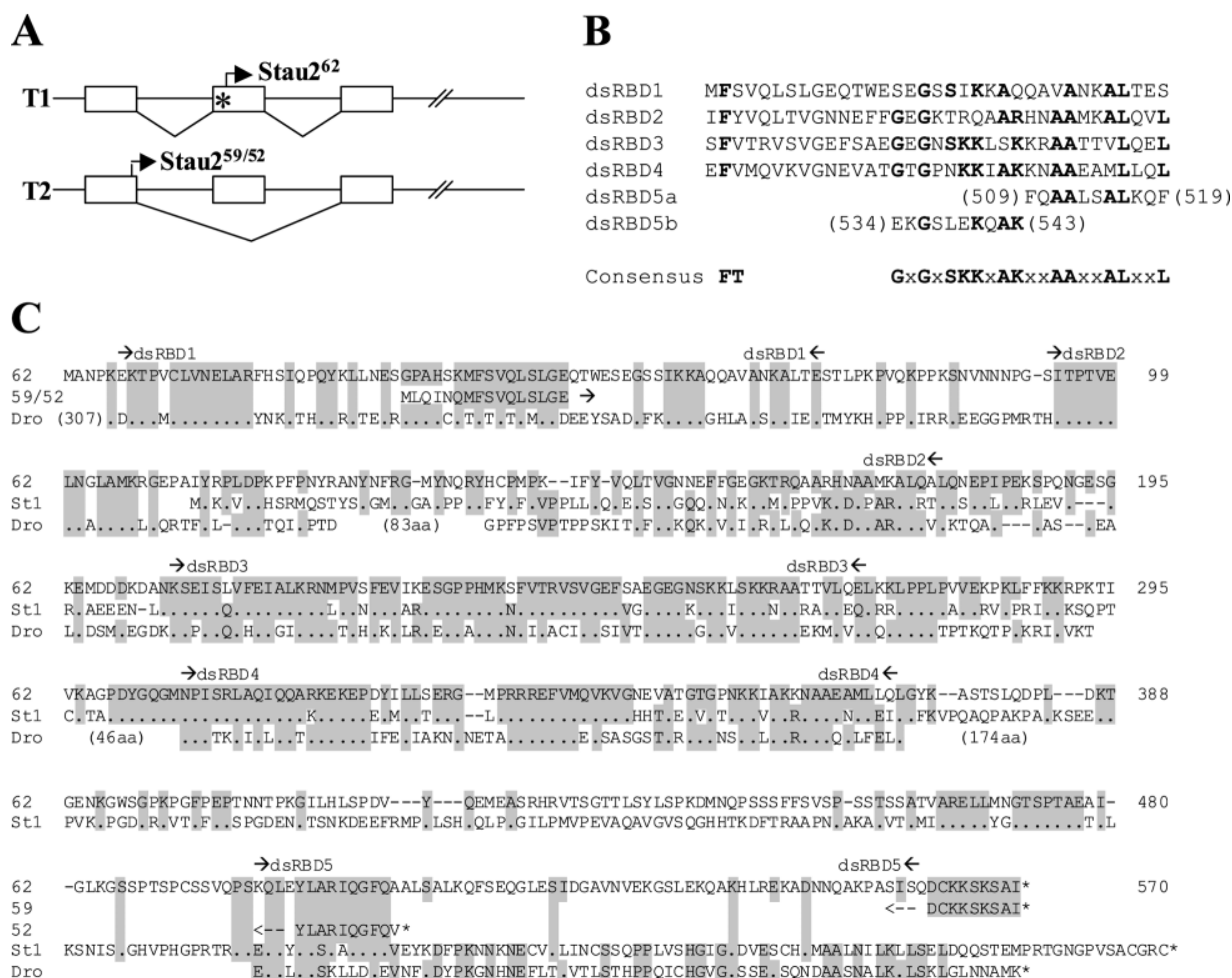

Fig. 1. Molecular analysis of Stau2 isoforms. (A) Alternative splicing in the 5′ end of the *Stau2* gene. Alternative splicing generates two transcripts, T1 and T2, that differ by the insertion of 131 nucleotides in T1 compared with T2. Arrows indicate the first in frame ATG initiation codon for translation of Stau2^{62} and Stau2$^{59/52}$, respectively. Note that the two ATG are not in frame in the T1 transcript and therefore translation initiation of Stau2^{62} starts at the second ATG. * indicates the presence of stop codons in the three open reading frames. (B) Amino-acid sequence alignment of Stau2 dsRBDs and the consensus dsRBD sequence. (C) Alignment of the amino-acid sequence of mouse Stau2^{62} (62), Stau2^{59} (59), Stau2^{52} (52), mouse Stau1^{55} (St1) and *Drosophila* Staufen (Dro). The sequence of Stau2^{52} is from Buchner et al. (Buchner et al., 1999). The position of dsRBDs (dsRBD1 to dsRBD5) is indicated above the sequence. Dots indicate amino-acid residues identical to those of Stau2. All identical amino-acid residues are boxed. Dashes represent gaps in the amino-acid sequences. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AF459099 and AF459100.

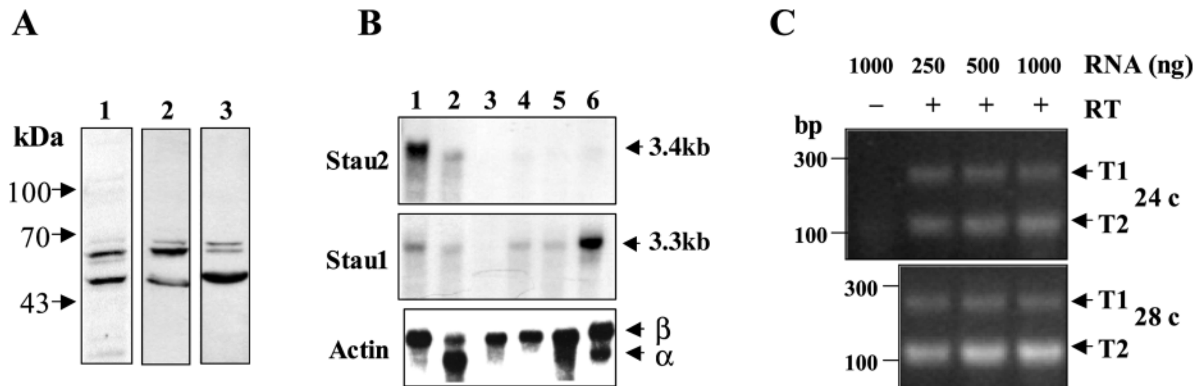

Fig. 2. Tissue distribution of Stau2 proteins and transcripts. (A) Western blot analysis of Stau2 in neurons. Brain extracts were separated by SDS-PAGE and stained with anti-Stau2 antibodies. Lane 1, mouse monoclonal; lane 2, rabbit polyclonal; lane 3, mouse polyclonal. (B) Northern blot analysis of *Stau2* expression in mouse tissues. mRNAs isolated from multiple tissues were transferred to a nylon membrane and hybridized with either a mouse Stau2 cDNA probe (top panel), a mouse Stau1 cDNA probe (middle panel) or an actin cDNA probe (lower panel). Lane 1, brain; lane 2, heart; lane 3, spleen; lane 4, kidney; lane 5, testis; lane 6, ovary. Blots were exposed for 16 hours. (C) RT-PCR analysis of the *Stau2* transcripts in brain. Total RNA was isolated from mouse brain and RT-PCR amplified with primers flanking the alternatively spliced exon. PCR amplification was performed with different amounts of starting RNA and cycle numbers. Control RNA (1 μg, no reverse transcription step) was amplified for 32 cycles.

bands were cleared when the antibodies were first incubated in the presence of the C-terminal antigen before western blotting (data not shown).

The predicted structure of Stau2 is similar to that of the *Drosophila* Staufen based on the existence of the different RBDs. In contrast to mammalian Stau1, Stau2 contains the corresponding *Drosophila* dsRBD1. Whereas $Stau2^{62}$ has a full-length dsRBD1 domain, $Stau2^{59}$ contains only the C-terminal half of the domain (Fig. 1C). Sequence analysis also revealed the presence of domains that correspond to the *Drosophila* split domain (dsRBD2) and the major (dsRBD3) and minor (dsRBD4) RNA-binding domains. A region of low sequence identity with the microtubule-binding domain of MAP1B is also present downstream of dsRBD4, as observed before in mammalian Stau1 (Wickham et al., 1999). Finally, only the C-terminal part of a putative RBD5 is found – in a rearranged form – in Stau2 (Fig. 1B).

Stau2 is mainly expressed in brain

Northern blot analysis with RNAs isolated from mouse tissues revealed that Stau2 is mainly expressed in brain and heart (Fig. 2B). Longer exposure of the blot allowed us to detect transcripts in the kidney, testis and ovary (data not shown). For comparison, a Stau1-specific probe hybridized to RNA in most tissues (Fig. 2B), as observed before in humans and rats (Marión et al., 1999; Wickham et al., 1999; Monshausen et al., 2001). Therefore, there are clear tissue-specific differences in the expression of the two genes. RT-PCR amplification of brain RNA with primers located on each side of the differentially spliced exon of 131 nucleotides demonstrated that the two transcripts are expressed in the brain. Consistent with the western blots, transcript T2 encoding $Stau2^{59}$ is more abundant than T1 (Fig. 2C). Varying concentrations of brain RNA and multiple numbers of cycles assured that PCR amplification was kept in non-saturable conditions.

Mapping of functional domains in vitro

To identify the functional RNA-binding domain(s) in Stau2, we used a Northwestern assay as previously described for Stau1 (Wickham et al., 1999). A full-length $Stau2^{59}$-MBP fusion protein was first produced in bacteria and tested in the dsRNA-binding assay. This protein bound [^{32}P]*bicoid* 3′UTR mRNA (Fig. 3A). None of the overexpressed MBP, BSA or the bacterial proteins in the extracts bound the probe, demonstrating the specificity of the RNA-binding assay. We then fused individual domains to GST (Fig. 3C), expressed them in bacteria and tested their capacity to bind the probe (Fig. 3A). In these conditions, GST-3 strongly bound the probe, whereas GST-4 bound the probe only very weakly. By contrast, GST-1, GST-2 and GST-5 did not bind at all. Fig. 3B shows that equivalent amounts of the fused proteins were loaded. These results demonstrate that dsRBD3 is the major dsRNA-binding determinant, as determined previously for the other Staufen homologues.

Stau2 localizes in the somatodendritic domain of neurons but not in axons

We next determined the subcellular distribution of Stau2 in mature primary hippocampal neurons in culture (16 DIV), using a polyclonal antiserum. This antiserum is specific for Stau2 and does not recognize Stau1. Rat Stau2 localized in both the soma and neurites where it appeared as granules (Fig. 4A). In neurites, Stau2-containing particles appeared to be aligned on individual tracks. Consistently, our results show a significant colocalization of aligned Stau2 particles with α-tubulin (Fig. 4B), strongly suggesting that Stau2 is associated with polarized microtubular tracks. Stau2 immunoreactivity was found in the same compartment as MAP2 (Fig. 5A), clearly demonstrating that it is present in dendrites. By contrast, Stau2 was absent from axons as it did not colocalize with Tau (Fig. 5B), a marker of axons.

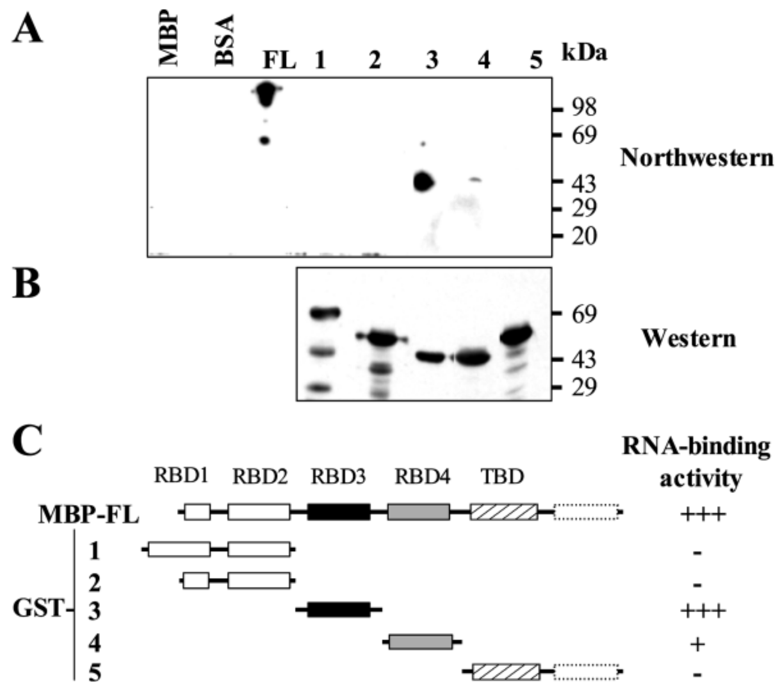

Fig. 3. RNA-binding activity of Stau2 dsRBDs. Full-length mouse Stau2 fused to MBP (FL) and different dsRBDs fused to GST (1 to 5) were expressed in bacteria. Crude protein extracts were resolved by SDS PAGE and transferred on nitrocellulose membranes. Filters were incubated with either [^{32}P]*bicoid* 3′UTR RNA (A) or anti-GST antibodies (B). MBP, overexpressed MBP protein; BSA, 5 μg BSA. (C) A schematic representation of the fusion proteins with a summary of their RNA-binding capacity. Black and grey boxes represent the major and minor RNA-binding domains, respectively, whereas white boxes represent regions with RNA-binding consensus sequence but lacking RNA-binding activity in vitro. The hatched box indicates the position of the region similar to the MAP1B microtubule-binding domain. Boxes with dotted lines represent the rearranged dsRBD5 domain.

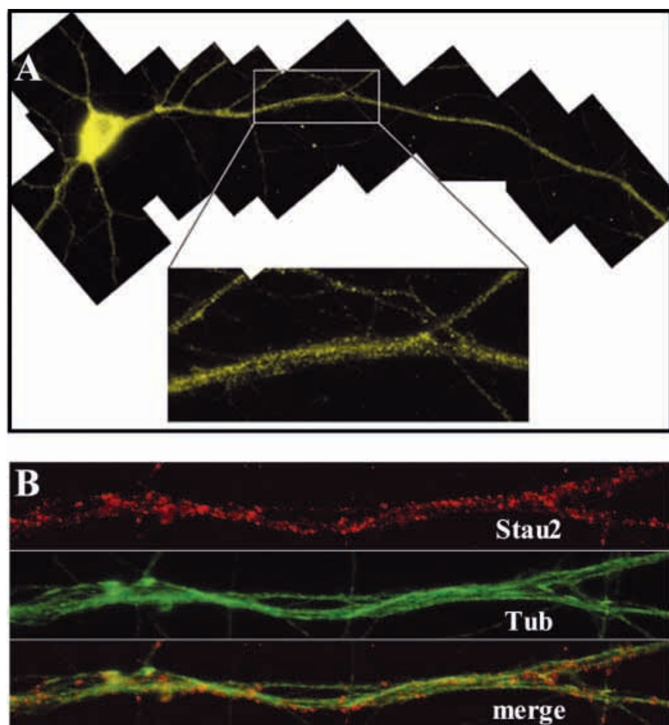

Fig. 4. Stau2 is found in the somatodendritic compartment of neurons and colocalizes with tubulin in dendrites. (A) Hippocampal neurons in culture were fixed and labeled with rabbit polyclonal anti-Stau2. Inset: high magnification of Stau2 in dendrites.
(B) Hippocampal neurons in culture were fixed and labeled with rabbit anti-Stau2 (red) or mouse monoclonal anti-tubulin (green) antibodies. The lower panel represents the superposition of both red and green signals. The average diameter of the cell body of a typical CA1 pyramidal neuron is between 8 and 12 μm.

Stau1 and Stau2 are components of distinct particles in dendrites

The distribution pattern of Stau2 is similar to that of Stau1, which was predominantly found in large granules both in the periphery of the cell body as well as in dendrites of mature hippocampal neurons (Kiebler et al., 1999). Therefore, we tested whether Stau1 and Stau2 colocalize in the same granules. We performed double-immunofluorescence microscopy of the same hippocampal neurons using specific Stau1 and Stau2 antibodies that do not cross-react with each other (data not shown). From these experiments it became clear that Stau2 is significantly more abundant in distal dendrites than Stau1 (Fig. 6A). At higher magnification in distal dendrites, the vast majority of both proteins did not colocalize (Fig. 6B), demonstrating that they are components of distinct particles.

Stau2 isoforms are found in the P100 fraction of cytoplasmic extracts

To better study the subcellular distribution of the Stau2 isoforms, we first used a crude cytoplasmic and nuclear fractionation approach starting from high-density primary cultures of rat cortical neurons. Western analysis of the fractions with the monoclonal antibody 1C6 indicated that Stau2^{62}, Stau2^{59} and Stau2^{52} were all present in the cytoplasmic fraction (Fig. 7A).

We then fractionated the cytoplasmic extracts into S100/P100 fractions by high-speed centrifugation. Under these conditions, soluble proteins are found in the supernatant (S100), whereas membrane-bound, heavy-complex-associated proteins and organelles sediment in the pellet (P100). As shown in Fig. 7B, the three Stau2 isoforms were consistently

found in the P100 fraction, as do calnexin (CNX), a marker of the RER and L7a, a ribosomal protein. This result showed that Stau2 isoforms are associated with dense particles/organelles. By contrast, tubulin (Tub) was found mainly in the S100 fraction. Pre-treatment of the cytoplasmic extracts with non-ionic detergent (Nonidet P40) before centrifugation did not abrogate the sedimentation of Stau2 proteins in P100, showing that solubilization of the membranes did not affect Stau2 association with dense particles/organelles. As a control, calnexin was now shifted to the S100. Similarly, pre-treatment with EDTA did not displace Stau2 from the dense particles. By contrast, treatment with 0.5 M KCl released roughly 50% of Stau2 in the S100 fraction. These observations demonstrate that all Stau2 isoforms are non-covalently associated with stable, EDTA- and detergent-resistant high-density particles/organelles. Treatment with a high concentration of RNase only released very low amounts of Stau2 isoforms in the S100 fraction, suggesting that their association with high-density particles may not be mediated mainly by RNA intermediates.

Stau2 splice isoforms are found in several complexes

To better characterize these Stau2-containing particles, we next fractionated cytoplasmic extracts on a 20–60% sucrose density gradient. Fractions were recovered and analyzed by western blotting with an anti-Stau2 monoclonal antibody, anti-calnexin and anti-L7a. Interestingly, more than 70% of both $Stau2^{59}$ and $Stau2^{52}$ co-fractionated with the major peak of ribosomes (Fig. 8A). Significant amounts (about 30%) of the two proteins were also found in fractions of higher density, which also contained low amounts of ribosomes. These proteins are not associated with membranes since detergent treatment of the cytoplasmic extract prior to sedimentation did not abolish the signal (data not shown). By contrast, $Stau2^{62}$ was found only in the lightest fractions of the gradient. These results demonstrate that $Stau2^{59}$ and $Stau2^{52}$ have the same distribution, that they are components of at least two complexes and that $Stau2^{62}$ has a distinct distribution compared to the two shorter isoforms.

To study this possible interaction between $Stau2^{59}$/$Stau2^{52}$ and the ribosomes, we first separated cytoplasmic extracts using a sharper sucrose gradient (10 to 40%) and a longer centrifugation time in the presence of EDTA to separate ribosome subunits. In these conditions, $Stau2^{59}$ and $Stau2^{52}$ still cofractioned with ribosome subunits and were found in the same fractions as both the 40S and 60S subunits (Fig. 8B). Second, we immunoprecipitated ribosomes with a human anti-ribosomal protein antiserum and detected Stau2 by western blotting with the anti-Stau2 antibody. A band corresponding to $Stau2^{59}$ was visible on the blot (Fig. 9A). The presence of a weak band in the immunoprecipitate is consistent with an association of Stau2 with only a small fraction of the total pool of ribosomes in the cells. Unfortunately, $Stau2^{52}$ comigrates with the remaining antibodies and therefore cannot be detected on the blot. A $Stau2^{59}$-specific band was not visible when a normal

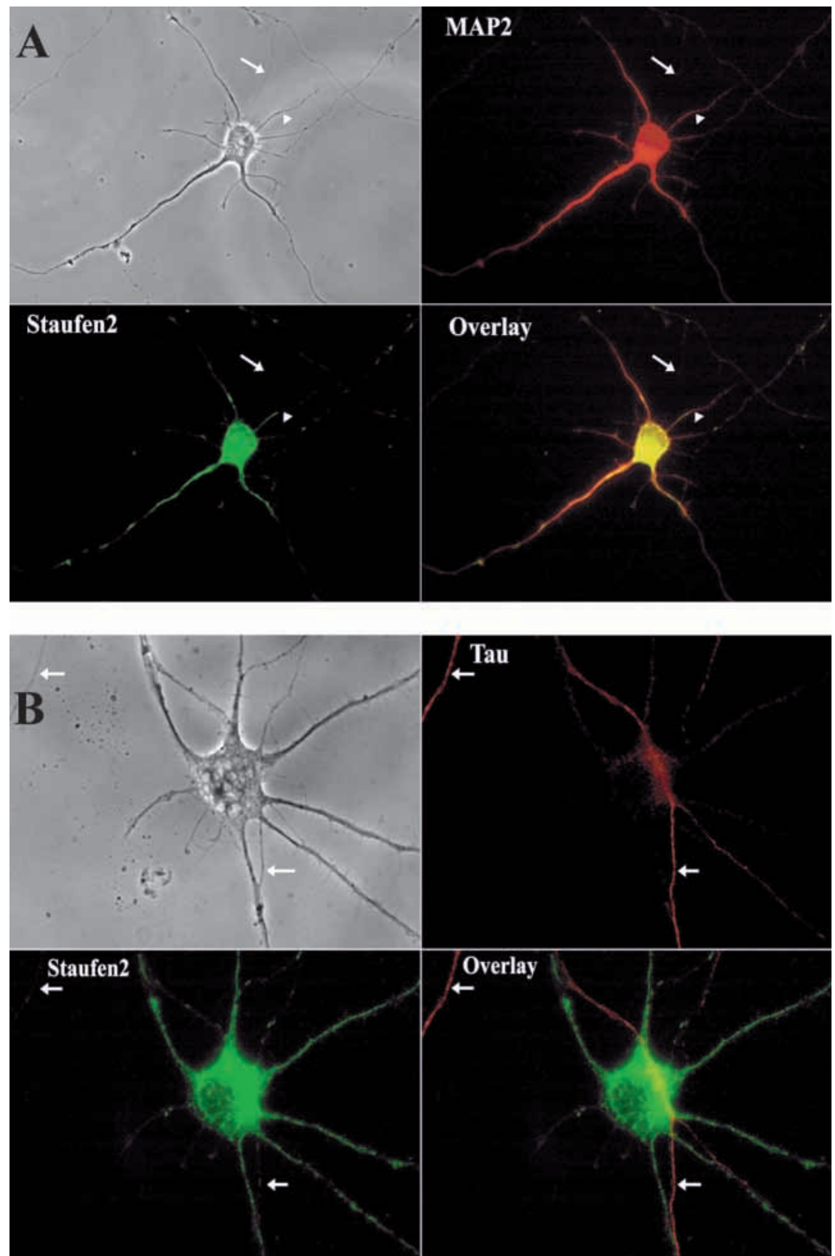

Fig. 5. Stau2 is found in dendrites but not in axons. (A) Hippocampal neurons in culture were fixed and labeled with rabbit anti-Stau2 (green) or mouse monoclonal anti-MAP2 (red) antibodies. Arrows indicate axons (negative in MAP2) with little or no staining for Stau2. The arrowhead indicates a very thin dendrite (MAP2 and Stau2 positive) comparable in size to the marked axon. (B) Hippocampal neurons in culture were fixed and labeled with rabbit anti-Stau2 (green) or mouse monoclonal anti-tau1 (red) antibodies. Arrows indicate axons (negative for Stau2). In (A,B), the upper left panels show phase contrast microscopy. The lower right panels show superposition of both red and green signals. The average diameter of the cell body of a typical CA1 pyramidal neuron is between 8 and 12 μm.

human serum was used for immunoprecipitation. Similarly, when Stau2 was immunoprecipitated with an anti-Stau2 antibody, the P ribosomal protein (Fig. 9B) and 18S rRNA (Fig. 9C) were detected in the immunoprecipitate. No signal was found when immunoprecipitation was done with the pre-immune serum. All these results are consistent with an interaction of two Stau2 isoforms with ribosomes, pointing to a very new role for Stau2 in translation.

$Stau2^{59}/Stau2^{52}$ association with ribosomes is independent of translation

Finally, we tested whether the translational activity of ribosomes modulates the association of Stau2 with ribosomes. Neurons were treated with pactamycine (an inhibitor of translation initiation). rapamycin (an inhibitor of the FRAP/TOR signaling pathway) or puromycine or cycloheximide (two inhibitors of translation elongation). In

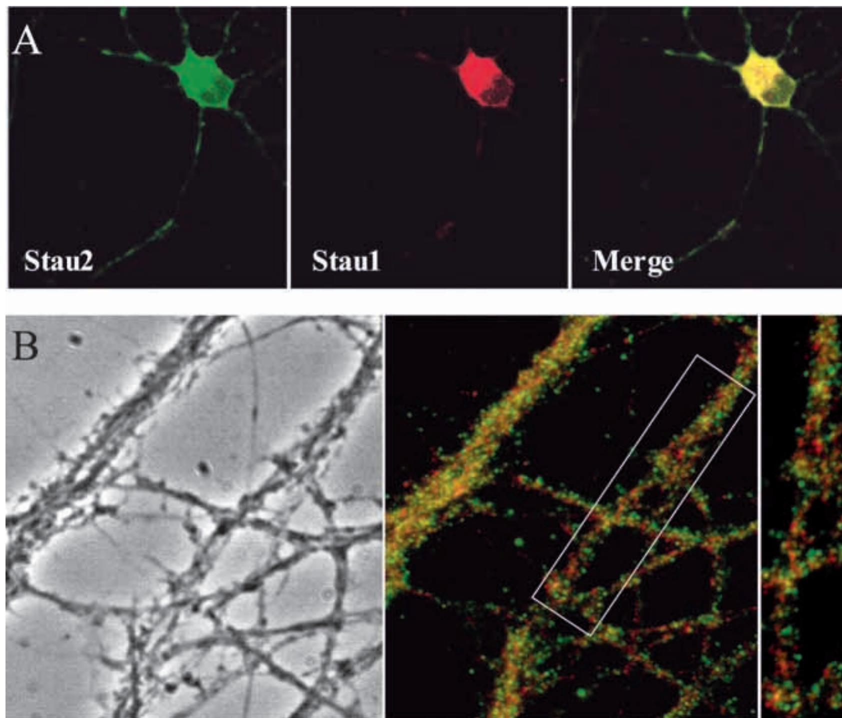

Fig. 6. Stau2 does not colocalize with Stau1 in distal dendrites. (A) Confocal images from fixed, mature hippocampal neurons in culture. Neurons were labeled with mouse polyclonal anti-Stau2 (green) and rabbit anti-Stau1 (red) antibodies. The third panel represents the superposition of both green and red signals. (B) Higher magnification of images taken with conventional fluorescence microscopy. Neurons were labeled with mouse polyclonal anti-Stau2 (green) and rabbit anti-Stau1 (red) antibodies. The middle and right panels represent the superposition of both green and red signals at different magnification. The average diameter of the cell body of a typical CA1 pyramidal neuron is between 8 and 12 μm.

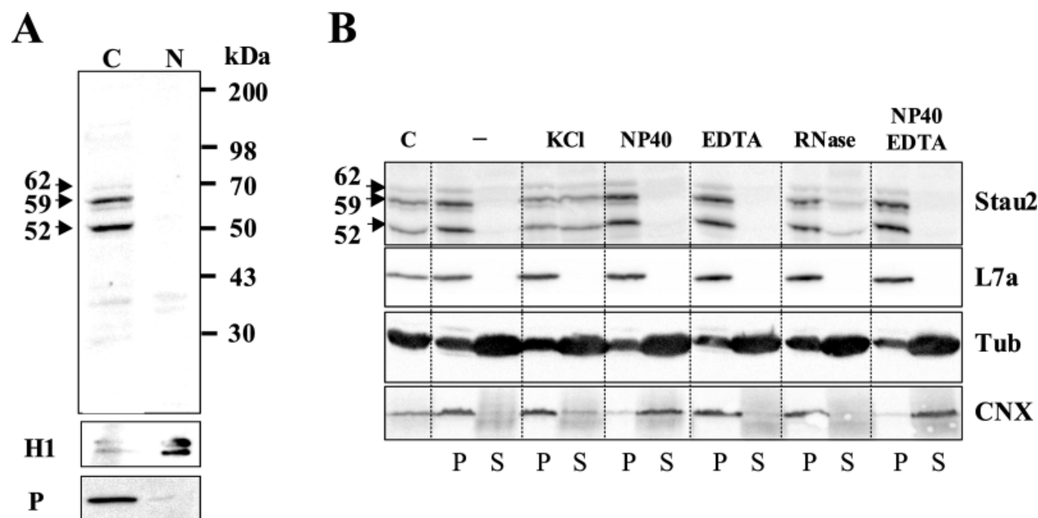

Fig. 7. Stau2 splice isoforms are found in high-density particles. (A) Rat cortical neurons (6 DIV) were lysed, homogenized and cytoplasmic and nuclear extracts were prepared by low-speed centrifugation. The supernatant (cytosolic fraction, C) and pellet (nuclear fraction, N) were recovered and analyzed by western blotting with monoclonal anti-Stau2 antibodies. The purity of each fraction was tested with antibodies directed against a nuclear (histone H1) and a cytosolic (ribosomal protein P) protein. (B) Western blot analysis of the S100 and P100 fractions following different treatments. Cytoplasmic extracts were either left untreated (–) or were treated for 30 minutes prior to centrifugation with 0.5 M KCl (KCl), 0.5% Nonidet P40 (NP40), 25 mM EDTA (EDTA), 300 U/ml micrococcal nuclease (RNase) and 0.5% Nonidet P40 and 25 mM EDTA (NP40+EDTA). S100/P100 fractions were analyzed with monoclonal anti-Stau2 (Stau2), anti-ribosomal protein L7a (L7a), anti-α-tubulin (Tub), and anti-calnexin (CNX) antibodies. The same results were obtained in two independent experiments.

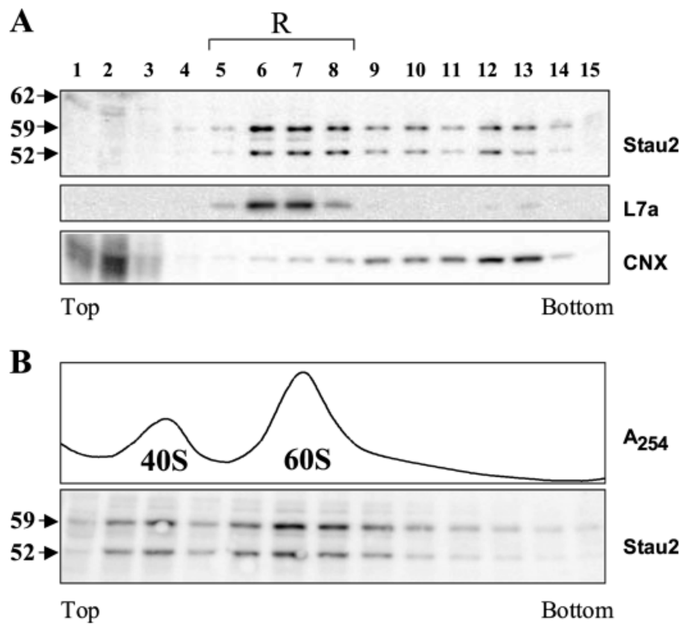

Fig. 8. $Stau2^{59}$ and $Stau2^{52}$ isoforms are associated with ribosomes. (A) Cytoplasmic extracts from rat cortical neurons in culture were prepared, placed on a 20-60% discontinuous sucrose gradient and centrifuged at 175,000 ***g*** for 3 hours. Fractions were recovered and analyzed by western blotting with monoclonal anti-Stau2 (Stau2), anti-L7a (L7a) and anti-calnexin (CNX) antibodies. The same results were obtained from three independent experiments. R, ribosomes. (B) Cytoplasmic extracts from neurons in culture were treated with 25 mM EDTA to dissociate ribosomal subunits and centrifuged at 250,000 ***g*** for 4 hours on a 10-40% continuous sucrose gradient. Fractions were recovered and analyzed by western blotting with monoclonal anti-Stau2 antibodies. Similar results were obtained with rabbit polyclonal anti-Stau2 antibodies (data not shown). The position of the 40S and 60S ribosomal subunits was determined with a spectrophotometer set at 254 nm.

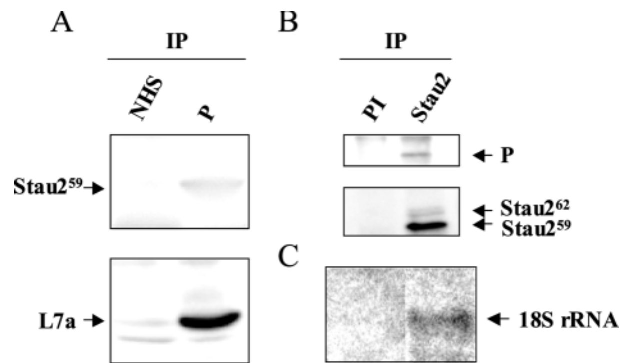

Fig. 9. Co-immunoprecipitation of $Stau2^{59}$ with ribosomes. Cytoplasmic extracts from rat cortical neurons in culture were prepared and immunoprecipitated with (A) normal human serum (NHS) and anti-ribosomal protein P (P) or (B) pre-immune serum (PI) and rabbit polyclonal anti-Stau2 (Stau2) antibodies. Immunoprecipitates were analyzed by western blotting with anti-L7a, monoclonal anti-Stau2 and anti-P as indicated. Results similar to those in (A) were obtained when the immunoprecipitation was carried out with the anti-L7a antibodies (data not shown). (C) The Stau2 immunoprecipitates (as in B) were also analyzed by northern blotting for the presence of 18S rRNA (small ribosomal subunit). These experiments were performed three times with reproducible results.

other experiments, cells were first incubated in a serum-free medium, and half of the cultures were re-exposed to normal serum concentrations. Cytoplasmic extracts were fractionated through a 1.5 M sucrose cushion in the presence of detergent. Supernatants and ribosome-enriched pellets were analyzed for the presence of Stau2. In untreated cells, $Stau2^{59}$ and $Stau2^{52}$ were found in the pellet as expected. In treated cells, they were also associated with the pellets and not released in the supernatant (data not shown). These results strongly suggest that translation does not influence association of both $Stau2^{59}$ and $Stau2^{52}$ with ribosomes.

Discussion

Staufen paralogues share conserved features

This study reports the molecular characterization and the subcellular localization of three splice isoforms of mouse Stau2, a new member of the Staufen family in mammals. We also compare Stau2 distribution to that of the paralogue Stau1. At the molecular level, mouse Stau2 has the same structural organization as Staufen in *Drosophila* and *C. elegans*, suggesting that they are derived from a common ancestor gene. In *Drosophila*, Staufen is involved in at least three distinct mRNA localization events (St Johnston, 1995). During oogenesis, Staufen is necessary to anchor *bicoid (bcd)* transcripts to the anterior pole of the oocytes and to localize *oskar (osk)* mRNA to the posterior pole. In the latter pathway, Staufen is further involved in the derepression of translation of *oskar* mRNA when it is localized at the posterior pole (Kim-Ha et al., 1995). Later, during neurogenesis, Staufen asymmetrically localizes *prospero (pros)* mRNA to the apical crescent of dividing neuroblasts (Li et al., 1997; Broadus et al., 1998). Therefore, *Drosophila* Staufen is involved in both RNA transport and translational regulation of at least some transcripts. The recent implication of mammalian Staufen with RNA transport in neurons (Köhrmann et al., 1999; Tang et al., 2001) and their biochemical association with ribosomes (Marión et al., 1999; Luo et al., 2002) (this paper) suggest that members of the Staufen family share evolutionarily conserved functions.

Mouse Stau2 also shares high amino-acid sequence identity with the paralogous protein Stau1. As observed previously with Stau1 (Kiebler et al., 1999; Köhrmann et al., 1999), Stau2 is found in the somatodendritic compartment of neurons and colocalizes with microtubules in dendrites. Consistently, drugs that disrupt microtubule organization impair both Stau1 (Kiebler et al., 1999; Köhrmann et al., 1999) and Stau2 (Tang et al., 2001) distribution in dendrites. Nevertheless, double immunofluorescence microscopy demonstrates that Stau1 and Stau2 are components of distinct particles. This conclusion is further supported by experiments with co-immunoprecipitation (T.F.D. and L.DG., unpublished) and biochemical cell fractionation (A.D. and M.A.K., upublished). All these results suggest that Stau1 and Stau2 may have similar and/or complementary function(s) in neurons but that they are components of distinct population of complexes.

Evolutionary conserved features of Stau2

Conservation of the overall structure and the primary sequence of corresponding dsRBDs among Staufen homologues in different species suggests that these domains have kept their unique functional features throughout evolution. In all species, dsRBD3 is the major RNA-binding domain (St Johnston et al., 1992; Micklem et al., 2000). By contrast, dsRBD2 and dsRBD5 do not bind to RNA in vitro. Nevertheless there are requirements for dsRBD2 for localization of *oskar* RNA at the posterior pole and dsRBD5 for the derepression of translation of *oskar* mRNA once localized at the posterior pole (Micklem et al., 2000). dsRBD5 is also involved in *bicoid* and *prospero* mRNA localization in oocytes and neuroblasts, respectively (Micklem et al., 2000). Most probably, dsRBD5 establishes protein-protein interaction as it was shown to bind to Miranda in neuroblasts (Schuldt et al., 1998). The rearrangement/ absence of dsRBD5 in the mouse Stau2 isoforms may be used to modulate these functions. The role of dsRBD1 and dsRBD4 is still unknown. In contrast to *Drosophila* dsRBD1, mouse Stau2 dsRBD1 does not bind to dsRNA in our in vitro conditions. Whether it binds to RNA in vivo is still an open question. Interestingly, whereas Stau1 lacks dsRBD1, Stau2 lacks a bona fide dsRBD5, suggesting that during evolution some functions of *Drosophila* Staufen were split between the two paralogues.

Stau2-containing particles

In the cell fractionation assay, Stau2 isoforms are consistently found in the P100 fraction, demonstrating that they are components of rather large complexes. These Stau2-containing complexes share common properties. First, they seem remarkably stable. They are resistant to EDTA and to relatively high concentration of KCl. Second, RNAse treatment only releases very small amounts of Stau2 isoforms in the supernatant, in contrast to the poly(A)-binding protein (PABP), which is completely released in the supernatant (data not shown). Third, Stau2 association with complexes appears to be independent of translation. Drugs that inhibit translation or serum starvation do not affect these associations.

Our results with sucrose gradients further demonstrate that Stau2 isoforms are distributed in several complexes. $Stau2^{62}$ fractionates in fractions of low density. These fractions also stain for calnexin but are devoid of ribosomes. This calnexin-positive material has been observed in neurons (Villa et al., 1992) but not in other cell lines such as COS7, HEK293 or HeLa and may represent the smooth ER elements that extend into both axons and dendrites (Krijnse-Locker et al., 1995). However, the comparative behaviors of $Stau2^{62}$ and calnexin in the S100/P100 fractionation experiments suggest that $Stau2^{62}$ may not be physically linked to these vesicles.

$Stau2^{59}$ and $Stau2^{52}$ have a more complex subcellular distribution than $Stau2^{62}$. They are found in at least two complexes of different density across the sucrose gradient. The first complex is found in fractions of high density. These complexes are not associated with the RER since they are not solubilized with detergent. Whether they are associated with ribosomes and/or represent large clusters of ribosomes, as those recently observed in neurons and shown to contain Stau1 (Krichevski and Kosik, 2001) do, need to be resolved. The second complex is associated with ribosomes. Sucrose gradients, co-immunoprecipitation and ribosome pull-down assays are all consistent with this observation. However, it is possible that this small population of Stau2-containing ribosomes is also bound to the RER in vivo. Indeed, confocal microscopy shows colocalization of Stau2 and calnexin in vivo (data not shown). Nevertheless, the harsh lysis conditions used in our experiments establish that the primary binding site of $Stau2^{59}$ and $Stau2^{52}$ is likely to be the ribosomes and not the membranes themselves.

Molecular characterization of Staufen's functions in *Drosophila* demonstrated that both Staufen-RNA and Staufen-protein interactions occur during localization (Ferrandon et al., 1994; Li et al., 1997; Schuldt et al., 1998; Micklem et al., 2000). The heterogeneity of Stau2- and Stau1-containing particles/organelles suggests that they are part of a dynamic and multi-step process that modulates the composition of RNA/protein complexes along the pathway. This is essential for proper localization and/or translational regulation of mRNAs. We continue to be challenged to determine the subcellular localization and the roles of each isoform in neurons in relation to these complex processes. Since new tools to address these problems are now available, we can anticipate answers to these questions.

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