A genome-wide approach identifies distinct but overlapping subsets of cellular mRNAs associated with Staufen1- and Staufen2-containing ribonucleoprotein complexes

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ABSTRACT

Messenger RNAs are associated with multiple RNA-binding proteins to form ribonucleoprotein (mRNP) complexes. These proteins are important regulators of the fate of their target mRNAs. In human cells, Staufen1 and Staufen2 proteins, coded by two different genes, are double-stranded RNA-binding proteins involved in several cellular functions including mRNA localization, translation, and decay. Although 51% identical, these proteins are nevertheless found in different RNA particles. In addition, differential splicing events generate Staufen2 isoforms that only differ at their N-terminal extremities. In this paper, we used a genome-wide approach to identify and compare the mRNA targets of mammalian Staufen proteins. The mRNA content of Staufen mRNPs was identified by probing DNA microarrays with probes derived from mRNAs isolated from immunopurified Staufen-containing complexes following transfection of HEK293T cells with Stau1⁵⁵-HA, Stau2⁵⁹-HA, or Stau2⁶²-HA expressors. Our results indicate that 7% and 11% of the cellular RNAs expressed in HEK293T cells are found in Stau1- and in Stau2-containing mRNPs, respectively. A comparison of Stau1- and Stau2-containing mRNAs identifies a relatively low percentage of common mRNAs; the percentage of common mRNAs highly increases when mRNAs in Stau2⁵⁹-HA- and Stau2⁶²-containing mRNPs are compared. There is a predominance of mRNAs involved in cell metabolism, transport, transcription, regulation of cell processes, and catalytic activity. All these subsets of mRNAs are mostly distinct from those associated with FMRP or IMP, although some mRNAs overlap. Consistent with a model of post-transcriptional gene regulation, our results show that Stau1- and Stau2-mRNPs associate with distinct but overlapping sets of cellular mRNAs.

Keywords: mRNA localization; RNA–protein interaction; ribonucleoproteins; Staufen; microarray

INTRODUCTION

The regulation of gene expression at the post-transcriptional level is a mechanism widely used by cells to control protein synthesis with acute precision. In the majority of cases, a sequence or a structure in the mRNA (*cis* element) is recognized by one or more proteins (*trans* factors) that assemble together in mRNPs and mediate an effect on mRNA metabolism. This interaction scheme has been shown to be involved in the intracellular localization of

mRNA (Bashirullah et al. 1998), in translational activation (Vagner et al. 2001) or repression (Chekulaeva et al. 2006), and also in mRNA stabilization and decay (Brennan and Steitz 2001; Chen et al. 2001). Many RNA-binding proteins that are *trans* factors can each associate with more than one mRNA species in the cell, and it has been postulated that the networks of mRNAs bound by these proteins can serve as post-transcriptional operons (Keene and Tenenbaum 2002).

Staufen is an RNA-binding protein first discovered in *Drosophila*, where it plays a role in oocyte patterning by localizing oskar and bicoid transcripts to the posterior and anterior poles, respectively (Riechmann and Ephrussi 2001). Staufen is also implicated in the translational derepression of oskar once localized at the posterior pole (Micklem et al. 2000). There are two mammalian homologs of Staufen, termed Stau1 and Stau2, and they both possess

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four copies of the double-stranded RNA-binding domain (dsRBD). Both Stau1 and Stau2 are expressed in the somato-dendritic compartment of neurons, where they form distinct RNA granules visible as puncta in dendrites. Stau1 and Stau2 are also involved in mRNA transport along the microtubule tracts in neuronal dendrites (Kohrmann et al. 1999; Tang et al. 2001; Duchaine et al. 2002; Kanai et al. 2004). More recently, Stau1 was identified as a central component of a new mRNA decay mechanism termed Staufen-mediated decay (Kim et al. 2005). In addition to functions in RNA localization and decay, Stau1 can also stimulate translation of repressed messengers containing structured RNA elements in their 5' UTR (Dugre-Brisson et al. 2005).

Both genes produce multiple isoforms that are generated by alternative splicing. The *Stau1* gene generates at least two isoforms of 55 kDa (Stau1⁵⁵) and 63 kDa (Stau1⁶³) and one isoform that does not bind mRNA (Stau1ⁱ). This latter isoform may be a regulator of Stau1 functions. Stau1 is found to be mainly associated with tubulo-vesicular structures in the cell as observed by microscopy and also is found to cofractionate with ribosomes and the rough endoplasmic reticulum in a sucrose gradient (Wickham et al. 1999; Duchaine et al. 2002). Similarly, the *Stau2* gene generates isoforms of 62 kDa (Stau2⁶²), 59 kDa (Stau2⁵⁹), and 52 kDa (Stau2⁵²) (Buchner et al. 1999; Duchaine et al. 2002). In a sucrose gradient of neuron extracts, Stau2⁶² is found in ribosome-free light complexes, whereas Stau2⁵⁹ and Stau2⁵² cofractionate with ribosomes.

Overall, Stau1 and Stau2 share 51% of amino acid sequence identity. When domains involved in mRNA binding (dsRBD3) are compared, the sequence identity between these proteins jumps to 77% (Fig. 1). The question is whether these highly similar proteins bind overlapping populations of mRNAs or whether they associate with

different subsets of mRNAs. The question is even more relevant for alternatively spliced Stau isoforms that are perfectly identical over domains involved in RNA binding. Answering these questions is fundamental for our understanding of Staufen functions in the cells and how they regulate redundant or specific pathways. To date, little is known about the RNA content of Staufen mRNPs. The present work was undertaken in an effort to identify the various mRNAs that are associated with Stau1 and Stau2 in mammalian cells and with differentially spliced Stau2 isoforms. The use of a genome-wide screen using microarrays to identify mRNA subsets associated with RNA-binding proteins is a proven approach that was successfully applied in many studies. This technique, termed RIP-Chip, has been helpful to uncover the identity of the Fragile X mental retardation protein (FMRP) (Brown et al. 2001), YB-1 (Evdokimova et al. 2006), U2AF⁶⁵ (Gama-Carvalho et al. 2006), Insulin-like growth factor II mRNA-binding protein (IMP1) (Jonson et al. 2007), and SLBP (Townley-Tilson et al. 2006) copurifying RNAs.

Here, we report the first high-throughput identification of Staufen-associated mRNAs in human HEK293T cells. HA-tagged versions of Stau1⁵⁵, Stau2⁵⁹, and Stau2⁶² were transfected in HEK293T cells. After immunopurification, the RNA content of Staufen mRNPs was isolated and used to hybridize Affymetrix human gene chips. Hybridizations identified several probe sets with each immunopurified mRNP. A high percentage of them are unique for Stau1 or Stau2 mRNPs. Enriched mRNAs were classified according to their cellular functions. Gene ontology (GO) annotations suggest that Staufen proteins are mainly involved in the regulation of cellular metabolism and physiological processes.

RESULTS

Stau1- and Stau2-containing RNP complexes copurify with RNA

The aim of this study was to identify and compare mRNAs in different populations of Staufen-containing mRNPs as a means to unveil the role(s) of these complexes in the cell regulatory pathways. Since Stau1 and Stau2 were described in different mRNP complexes, they may fulfill differential function by binding to alternative subsets of mRNAs (Duchaine et al. 2002; Thomas et al. 2005). The same may be true for two Stau2 isoforms that were shown to be differentially distributed in cells (Duchaine et al. 2002). Therefore, human embryonic kidney (HEK) 293T cells were transfected with plasmids coding for either Stau1⁵⁵-HA, Stau2⁵⁹-HA, or Stau2⁶²-HA, and Staufen-containing mRNPs were immunoprecipitated using a monoclonal anti-HA antibody for immunoprecipitation (IP). Coimmunoprecipitated mRNAs were isolated from each mRNP extract and used to generate probes in order to hybridize

FIGURE 1. Comparison of the Staufen paralogs. (Top) Schematic representation of Stau1⁵⁵ (P55), Stau2⁵⁹ (P59), and Stau2⁶² (P62). (Black boxes) Major RNA-binding domains, (gray boxes) minor RNA-binding domains, (white boxes) domains that do not bind RNA even if they show sequence similarity to RNA-binding domains, (hatched boxes) tubulin-binding domain. (RBD) RNA-binding domain, (Chr) chromosome. (Bottom) Sequence comparison of Stau1 and Stau2 dsRBD3. Amino acids are indicated by the one-letter code.

microarrays. As a control, immunoprecipitations with anti-HA antibodies were done on mock transfected cells. Western blot analysis showed that each Staufen-HA protein and isoform was efficiently immunoprecipitated (Fig. 2A). To determine whether RNA copurified with Stau1 and Stau2 complexes, RNA was isolated from the immunoprecipitates and visualized using RNA pico chips (Fig. 2B). A clear enrichment of mRNA was observed in samples of immunopurified Staufen-containing RNPs as compared with controls in which a very low amount of copurifying RNA was detected. As expected, mainly two peaks corresponding to 18S and 28S ribosomal RNA were seen from HEK293T cells total RNA. These data showed that subpopulations of RNA coimmunoprecipitate with each Staufen isoform.

Microarray analysis of Staufen-associated mRNAs

RNAs isolated from Staufen-HA and mock transfected cells were used as a source of probes to hybridize commercially available human DNA oligonucleotide microarrays from

Affymetrix. This experiment allowed us to perform a genome-wide identification of Stau1- and Stau2-associated mRNAs. To identify Staufen-HA-associated mRNAs, we performed a statistical (unpaired) analysis on mean-scale normalized MAS5 data with a *P*-value threshold of 0.05. We then kept genes for which the hybridization signal was at least 2.5 times stronger with probes derived from Staufen-HA-expressing cells than with probes made from their respective controls. Similarly, total RNA from untransfected HEK293T cells was used to generate probes for microarray hybridization.

First, arrays hybridized with probes synthesized from HEK293T cell total RNA showed that 19,491 probe sets corresponding to 12,188 unique genes are expressed in these cells. This corresponds to 37.3% of the total probe sets on the microarray. Then, in order to identify Stau1⁵⁵-HA-associated mRNAs, four independent immunoprecipitations were performed from extracts of Stau1⁵⁵-HA transfected cells and mock transfected control cells. Hybridization results identified 1384 probe sets (7% of expressed HEK293T mRNAs) that were enriched by more than 2.5-fold over control IP (Supplemental Table S1). Similarly, two independent hybridization experiments performed with mRNA isolated from Stau2⁵⁹-HA transfected cells and controls identified 2357 specific probe sets (12% of expressed HEK293T mRNAs) (Supplemental Table S2). Using mRNA isolated from Stau2⁶²-HA transfected cells and controls, 2008 (10% of expressed HEK293T mRNAs) probe sets were detected (Supplemental Table S3).

FIGURE 2. Stau1 and Stau2 copurify with RNA. (A) Immunoprecipitation of Staufen-HA constructs. HEK293T cells were either transfected with one of the plasmids expressing Stau1⁵⁵-HA, Stau2⁵⁹-HA, or Stau2⁶²-HA or mock transfected to control for nonspecific immunoprecipitation. Two days post-transfection, cells were lysed, and a fraction of the cell lysates was immunopurified using a mouse monoclonal anti-HA antibody. Stau1⁵⁵-HA (lane 1), Stau2⁵⁹-HA (lane 2), and Stau2⁶²-HA (lane 3) were identified on Western blotting using a rabbit polyclonal anti-HA antibody. (Lane 4) IP of mock transfected cells. (B) Analysis of the RNA content of Staufen-containing mRNPs. From the immunoprecipitates described in A, RNA was extracted and run on a Bioanalyser RNA pico chip (Agilent) as indicated. Total RNA was also extracted from HEK293T cells. Migration profile of HEK293T cells' total RNA sample shows mainly two peaks corresponding to 18S and 28S ribosomal RNAs. Note that Bioanalyser profiles are not all plotted on the same absorbance scale.

A large percentage of mRNA is specific for either Stau1⁵⁵- or Stau2-associated mRNPs

Then, the population of mRNAs associated with Stau1⁵⁵-HA mRNPs was compared with the mRNAs associated with Stau2-HA mRNPs (Fig. 3). Our data indicated that 63% and 58% of the probe sets identified with the Stau1⁵⁵-HA-derived probes were not found in Stau2⁶²-HA or Stau2⁵⁹-HA mRNPs, respectively, and 49% are specific to Stau1-containing mRNPs. The reverse is also true, since 75% and 74% of the probe sets identified with Stau2⁵⁹-HA- or Stau2⁶²-HA-associated mRNAs, respectively, were not found among the Stau1⁵⁵-associated mRNA. These results are consistent with the previous reports that Stau1 and Stau2⁵⁹ and Stau2⁶² define different populations of RNA granules (Duchaine et al. 2002; Thomas et al. 2005). Nevertheless, 368 probe sets were common to all Staufen-containing mRNPs.

Stau2⁶²- and Stau2⁵⁹-associated mRNPs share common mRNAs

Analyses of the Staufen2-associated mRNAs revealed that a greater proportion of probe sets are shared by the two Staufen2 isoforms as compared with what we described

FIGURE 3. Venn diagrams summarizing microarray analysis results. The RNA content isolated from Stau1⁵⁵-HA, Stau2⁵⁹-HA, and Stau2⁶²-HA IP (Fig. 2) was used to synthesize cRNA probes in order to hybridize Affymetrix HG_U133_plus_2 microarrays. For each hybridization, probe sets showing an enrichment of at least 2.5-fold over the control IPs were identified. The numbers of probe sets that are either specific or common to Stau1 and Stau2 isoforms are plotted in Venn diagrams.

above for Stau1 versus Stau2. Indeed, only 33% of the probe sets identified with probes derived from Stau2⁶²-HA mRNPs were uniquely found in these mRNPs, and 43% of the probe sets identified with probes derived from Stau2⁵⁹-HA mRNPs were specific for these mRNPs (Fig. 3). A total of 1338 probe sets were common to both Stau2-containing mRNPs. These results revealed that, although found in different mRNP complexes, these differentially spliced

Stau2 isoforms share several common mRNAs, suggesting that they play overlapping and/or complementary roles that finely regulate similar cellular pathways.

RT-PCR validation of enriched mRNAs

According to the microarray analyses, we chose several mRNAs identified as being specifically enriched in Stau1⁵⁵-HA, Stau2⁵⁹-HA, or Stau2⁶²-HA mRNPs to confirm their differential association with mRNPs (Table 1). First, cells were transfected with plasmids coding for either Stau1⁵⁵-HA, Stau2⁶²-HA, or Stau2⁵⁹-HA, and the resulting proteins were immunoprecipitated with anti-HA antibodies. Immunoprecipitates from mock transfected cells were used as control. Western blot showed that the HA-tagged proteins have been immunoprecipitated in equal amounts (Fig. 4A). Their expression was then compared with that of the endogenous proteins (Fig. 4B,C). Coimmunoprecipitated mRNAs were isolated and RT-PCR amplified with specific primers to validate the microarray results. As seen in Figure 5A, RNAs coding for FMN2 and IRF2BP2 were enriched in Stau1⁵⁵-HA-containing mRNPs, whereas RNAs coding for UQCRC2 and LTA4H and for MLLT11 and PPARGC1 were enriched in Stau2⁶²-HA- and Stau2⁵⁹-HA-containing mRNPs, respectively, as expected from the microarray analyses. Other mRNAs such as those coding for EIF5 and ZDHHC21 were common to Stau2 isoforms but not

TABLE 1. List of genes differentially found in Stau1 or Stau2 mRNPs and used for RT-PCR amplification

Gene name	Symbol	Probe set	Fold increase in RNP as compared with controls		
			Stau1 ⁵⁵	Stau2 ⁶²	Stau2 ⁵⁹
Probe sets enriched in Stau1 ⁵⁵ mRNPs					
Formin 2	FMN2	223618_at	3.4	0.8	1.0
Interferon regulatory factor 2 binding protein 2	IRF2BP2	224570_s_at	4.2	0.9	1.1
Zinc finger protein 236	ZNF236	222227_at	17.7	0.3	0.6
Probe sets enriched in Stau2 ⁶² mRNPs					
Leukotriene A4 hydrolase	LTA4H	208771_s_at	1.1	2.7	0.8
Ubiquinol-cytochrome c reductase core protein II	UQCRC2	212600_s_at	1.3	3.5	1.1
Probe sets enriched in Stau2 ⁵⁹ mRNPs					
Myeloid/lymphoid or mixed-lineage leukemia; translocated 11	MLLT11	211071_s_at	2.1	2.1	5.5
Peroxisome proliferative activated receptor gamma coactivator 1	PPARGC1	219195_at	1.0	1.6	3.0
Probe sets enriched in both Stau2 ⁵⁹ and Stau2 ⁶² mRNPs					
Eukaryotic translation initiation factor 5A	EIF5	208707_at	1.3	4.9	6.8
Zinc finger DHHC domain containing 21	ZDHHC21	243835_at	0.7	3.7	5.7
Thioredoxin domain containing 9	TXNDC9	203008_x_at	1.3	7.0	4.0
Probe sets enriched in both Stau1 ⁵⁵ and Stau2 ⁶² mRNPs					
Acyl-Coenzyme A oxidase 1 palmitoyl	Acox1	227962_at	8.7	8.1	6.7
3'(2') 5'-bisphosphate nucleotidase 1	BPNT1	232103_at	9.8	8.6	4.8
Control probe sets					
Ubiquinol-cytochrome c reductase binding protein	UQCRCB	209065_at	0.3	0.2	0.2
Likely ortholog of immediate early response erythropoietin 4	LEREPO4	201595_s_at	0.3	0.06	0.08
Growth and transformation-dependent protein	E2IG5	221533_at	2.4	2.1	1.3

FIGURE 4. Expression of Staufen-tagged proteins. HEK293T cells were transfected with plasmids coding for either Stau1⁵⁵-HA, Stau2⁶²-HA, or Stau2⁵⁹-HA. Mock-transfected cells were used as controls. Forty-eight hours post-transfection, cells were lysed, and Staufen-containing mRNPs were immunoprecipitated using anti-HA antibodies. Proteins in the immunoprecipitates were analyzed by Western blot analysis using anti(α)-HA (A), anti-Stau1 (B), or anti-Stau2 (C) antibodies. (NS) Nonspecific band.

Stau1 mRNPs. Some of the precipitated mRNAs expected to be unique to one mRNP according to microarray analyses were nevertheless slightly enriched in the other mRNPs as compared with controls. It is possible that the overexpression of the tagged proteins has facilitated the formation of a relatively low number of heterogeneous granules with the endogenous proteins. Finally, mRNAs coding for BPNT1 and Acox1 that were enriched in each mRNP according to the microarray data are also common to mRNPs in the RT-PCR assay. RT-PCR amplification of serial dilutions of mRNAs commonly enriched in the three mRNPs indicated that the amplifications were in the linear range (Fig. 5B,C). Consistent with the microarray data, these results show that specific mRNA can be differentially enriched in different mRNPs, whereas others are common to both isoforms.

Then, we tested whether transcripts are also enriched in endogenous Stau1- and Stau2-containing mRNPs. Endogenous Stau1- and Stau2-containing mRNPs were immunopurified using anti-Stau1 and anti-Stau2 antibodies, respectively. Nonimmune sera were used as controls. Coimmunoprecipitated mRNAs were isolated and RT-PCR amplified with specific primers. Consistent with the microarray data, mRNAs coding for IRF2BP2, FMN2, and ZNF236 were enriched in the Stau1 mRNP extracts as compared with their respective controls (Fig. 6A). Except for IRF2BP2 mRNA, they were not enriched in Stau2-containing mRNPs (Fig. 6B). Similarly, mRNAs coding for

EIF5, LTA4H, and TXNDC9 were enriched in Stau2-containing mRNPs as compared with controls (Fig. 6B). However, an enrichment was also seen in Stau1 immunoprecipitates as compared with controls (Fig. 6A), suggesting either that the anti-Stau1 antibody is not as specific as observed by Western blotting or that the endogenous Stau1-containing granules are more heterogeneous than expected. If so, the number of mRNAs common to Stau1- and Stau2-containing mRNPs may well be slightly higher than that seen by microarrays. Other mRNAs that were not enriched

FIGURE 5. RT-PCR amplification of selected mRNA following mRNPs immunoprecipitation. HEK293T cells were transfected with plasmids coding for either Stau1⁵⁵-HA (St1⁵⁵), Stau2⁶²-HA (St2⁶²), or Stau2⁵⁹-HA (St2⁵⁹). Mock transfected cells were used as controls (Ctl). Forty-eight hours post-transfection, cells were lysed, and Staufen-containing mRNPs were immunoprecipitated (IP) using anti-HA antibodies. Co-immunoprecipitated mRNAs were purified and RT-PCR amplified with pairs of oligonucleotides specific for the indicated genes (Supplemental Table S7). (A) Genes were chosen according to their differential enrichment in Stau1⁵⁵-HA, Stau2⁵⁹-HA, and Stau2⁶²-HA mRNPs according to the microarray data. Serial dilutions of mRNA in the immunoprecipitates (B) or in cell extracts (C) were used to indicate that RT-PCR amplifications were in the linear range. (FMN2) forming 2, (IRF2BP2) interferon regulatory factor 2 binding protein 2, (LTA4H) leukotriene A4 hydrolase, (EIF5) eukaryotic translation initiation factor 5A, (Acox1) acyl-Coenzyme A oxidase 1 palmitoyl, (BPNT1) 3'-(2') 5'-biphosphate nucleotidase 1, (MLLT11) ALL1-fused gene from chromosome 1q, (PPARGC1) peroxisome proliferative activated receptor gamma coactivator 1, (UQCRC2) ubiquinol-cytochrome c reductase core protein II, (ZDHHC21) zinc finger DHHC domain containing 21.

FIGURE 6. RT-PCR amplification of selected endogenous mRNA following Stau1 or Stau2 immunoprecipitation. HEK293T cells were lysed, and Stau1- (A) or Stau2-containing (B) mRNPs were immunoprecipitated (IP) with mouse monoclonal anti-Stau1 (St1) or rabbit polyclonal anti-Stau2 (St2) antibodies, respectively. Immunoprecipitation with preimmune sera was used as controls (Ctl). Coimmunoprecipitated mRNAs were isolated and RT-PCR amplified with pairs of oligonucleotides specific for the indicated genes (Supplemental Table S7). (IRF2BP2) interferon regulatory factor 2 binding protein 2, (FMN2) formin 2, (ZNF236) zinc finger protein 236, (EIF5) eukaryotic translation initiation factor 5A, (LTA4H) leukotriene A4 hydrolase, (TXNDC9) thioredoxin domain containing 9, (UQCRB) ubiquinol-cytochrome c reductase binding protein, (LEREPO4) likely ortholog of mouse immediate early response erythropoietin 4, (E2IG5) growth and transformation-dependent protein.

in the mRNPs over the controls in the microarray analyses also showed no enrichment by RT-PCR analysis, being found in both mRNP and controls. Altogether, these results confirm that specific mRNAs are preferentially found in Stau1- or Stau2-containing mRNPs as revealed by the microarray analyses, but the number of common mRNAs may be slightly underestimated.

Enriched mRNAs are involved in diverse cellular functions

We next tried to identify pathways in which Stau1 and Stau2 are involved as a means to understanding the biological functions of Staufen. Probe set lists have been analyzed with the DAVID functional annotation tool (Dennis et al. 2003) to assign cellular functions to mRNAs that were enriched in Staufen-containing RNPs. The result from GO classification reveals that mRNAs associated with Stau1- and Stau2-containing RNPs are distributed roughly in the same functional and molecular categories (Table 2; Supplemental Table S4). These include mRNAs coding for genes involved in cellular metabolism and cellular physiological processes, localization and transport, transcription, alternative splicing, metal-ion binding, and catalytic activ-

ities. Similarly, the subcellular distribution of the proteins encoded by the probe sets is quite similar between Stau1- and Stau2-associated mRNAs. Probe sets were then analyzed with the Babelomics software (Al-Shahrour et al. 2005) to identify common probe sets in each GO category. Interestingly, despite the fact that Stau1- and Stau2-derived probe sets are distributed essentially in the same functional categories, the nature of the genes encoded by these probe sets is quite different in each category (Supplemental Table S5), only ~30% of them being common. These results suggest that Stau1 and Stau2 play complementary roles that nevertheless fulfill similar functions.

GO classification was also done with mRNAs associated with Stau2⁶² and Stau2⁵⁹ mRNPs (Table 2; Supplemental Table S4). RNAs associated with these mRNPs are linked to the same GO categories. In addition, in each category, about 60% of the genes are common (Supplemental Table S6), suggesting that they play redundant functions.

DISCUSSION

Staufen-bound mRNAs

Staufen proteins are RNA-binding proteins ubiquitously expressed in animals that play several roles in mRNA transport, translation, and mRNA decay. Therefore, they are likely multifunctional proteins involved in several pathways and consequently should bind mRNAs that are not necessarily functionally related. In this paper, a genome-wide approach was used to identify mRNAs that are associated with either Stau1- or Stau2-containing particles. Independent RT-PCR analyses of several putative mRNA targets confirmed the reliability of this technique. Our results further indicate that these mRNAs are components of endogenous Stau1- and/or Stau2-containing mRNPs. The gene ontology distribution of these mRNAs allowed us to unveil the major pathways in which Stau1 and Stau2 proteins are involved. Our results with the microarray screens indicate that Stau1 and Stau2 associate with different but overlapping populations of mRNAs and that these mRNAs are nevertheless involved in common pathways. It is consistent with the high degree of sequence similarity between Stau1 and Stau2 that predicts that they may have conserved convergent functions and with the observation that they are distributed in distinct mRNP complexes in neurites. However, the more sensitive RT-PCR experiment suggests that the affinity of specific mRNAs for Stau1- or Stau2-containing mRNPs may not be all or none. Indeed, following overexpression of tagged proteins, mRNAs are enriched in specific mRNPs as predicted by the microarray analyses, but most of them are also enriched in the other IPs relative to the control, although to a lesser degree of enrichment. The same trend is observed with the endogenous mRNPs, especially in Stau1-containing complexes. Although it could be due to

TABLE 2. Summary of DAVID GO classification of target mRNA population

Gene ontology group	HEK293 (%)	Stau1 ⁵⁵ (%)	Stau2 ⁵⁹ (%)	Stau2 ⁶² (%)
Activator	1.95	2.20	2.2	2.3
Alternative splicing	18.52	18.04	18.0	18.2
Catalytic activity	25.77	22.44	24.6	25.4
Cellular metabolism	36.88	30.02	34.0	34.3
Cellular physiological process	48.72	41.70	46.8	46.6
Cellular protein metabolism	15.02	12.74	14.5	15.4
Establishment of protein localization	3.72	3.26	4.1	3.4
Glycoprotein metabolism		1.06	1.0	1.0
Ligase activity	2.98	3.03	3.4	4.1
Ligase activity, forming carbon–nitrogen bonds	2.21	2.58	2.9	3.5
Macromolecule metabolism	22.95	18.50	20.3	21.4
Magnesium ion binding	1.67	1.90	2.1	2.2
Metabolism	39.12	32.15	36.0	36.2
Metal binding	11.62	11.37	11.8	12.3
Mutagenesis site	5.45	5.84	5.8	6.0
Nucleic acid metabolism	17.99	14.18	15.8	15.5
Physiological process	51.69	44.20	49.4	49.1
Primary metabolism	35.98	29.64	33.6	33.6
Protein catabolism	1.26	1.29	1.7	1.8
Protein degradation	0.20	0.53	0.4	0.4
Protein localization	3.81	3.34	4.2	3.5
Protein metabolism	16.33	13.80	16.1	16.8
Protein modification	8.63	8.26	10.2	10.7
Protein transport	3.63	3.11	4.1	2.4
Protein ubiquitination	1.39	1.67	1.8	2.3
Regulation of cellular metabolism	11.94	10.46	11.2	11.2
Regulation of cellular physiological process	16.15	14.10	15.3	14.7
Regulation of metabolism	12.28	10.46	11.4	11.4
Regulation of nucleic acid metabolism	11.27	9.86	10.8	10.3
Regulation of transcription	11.06	9.70	10.6	10.1
Splice variant	18.42	18.35	18.0	18.2
Transcription	11.66	10.24	11.4	10.9
Transferase activity	9.00	8.34	9.1	8.4
Transition metal-ion binding	12.30	11.90	12.3	12.2
Ubiquitin cycle	3.14	3.11	4.2	4.7
Ubiquitin ligase complex	1.45	1.67	2.0	2.4
Ubiquitin–protein ligase activity	1.90	2.05	2.6	3.3
Ubl conjugation pathway	0.42	0.61	2.8	2.9
Vesicle-mediated transport	2.11	1.97	2.6	2.5
Zinc ion binding	10.52	10.01	10.4	10.6
Zinc finger	8.09	8.04	8.4	8.9
Cytoplasm	18.91	15.69	18.1	18.6
Endoplasmic reticulum	2.32	3.11	3.4	3.7
Golgi apparatus	2.78	3.71	3.7	3.5
Intracellular membrane-bound organelle	31.28	25.85	29.0	29.3
Intracellular organelle	35.15	28.28	31.6	32.0
Nuclear protein	18.26	14.56	15.8	15.9
Nucleus	21.57	17.21	18.8	19.1
Organelle	35.16	28.28	31.6	32.0
Organelle membrane	2.64	2.43	2.6	2.6

technical limitations (overexpression, specificity of antibodies, variation in the amounts of mRNA in the controls), the sequence similarity between Stau1 and Stau2 may also explain the fact that mRNA can bind to the two Staufen paralogs with differential affinity. It may allow more precise

regulation of the post-transcriptional expression of these mRNAs according to cell needs. Alternatively, it is possible that a certain percentage of heterogeneous granules may form in these cells, causing the immunoprecipitation of both paralogs in different proportions.

This study also allows us to identify the level of divergence in the RNA content of two mRNPs that contain protein isoforms with identical RNA-binding domains. Stau2⁶² and Stau2⁵⁹ are differentially spliced isoforms that only differ at their N-terminal extremity. In neurons, they form distinct mRNPs that fractionate with ribosome-free RNPs and with ribosomes, respectively (Duchaine et al. 2002). Our results indicate that these mRNPs contain a high percentage of shared mRNAs, suggesting that these isoforms play redundant functions. However, about one-third of the associated mRNAs are unique to either Stau2⁶² or Stau2⁵⁹ mRNPs, indicating that the Stau2 RNA-binding domain is not the sole determinant for the selection of associated mRNAs. It is consistent with proteomic analyses that showed that RNA granules contain several RNA-binding proteins that may, in theory, bind specific mRNAs. RNA granules are likely heterogeneous particles composed of several RNA-binding proteins and their associated RNAs. Stau2 was indeed found in several RNA granules along with other RNA-binding proteins such as pur proteins, DeadBox helicases, FMRP, and hnRNPs (Ohashi et al. 2002; Elvira et al. 2006b). All of them may bind RNA and associate with Staufen to form RNA granules. Alternatively, the accessibility of specific RNAs and/or the composition of specific RNPs may be dictated by the availability of mRNA subpopulations in subcellular compartments allowing specific association with differentially distributed Stau2 isoforms.

GO distribution

RNAs associated with Stau1- and Stau2-containing mRNPs essentially fall into the same GO categories, suggesting that these paralogs have overlapping and/or complementary

functions. Although broadly distributed in several GO categories, these mRNAs code for proteins mainly involved in cell metabolism, cellular physiological processes, localization and transport, transcription, alternative splicing, metal-ion binding, and catalytic activities. These are cellular processes that need fine regulation to appropriately respond to cell needs and cell stimuli. Staufen-dependent post-transcriptional regulation of gene expression of a specific subpopulation of messengers would allow a cell to specifically and quickly respond to cell stimuli. Post-translational modifications of mRNP-associated proteins, such as phosphorylation, may transduce the external stimuli into functional responses. Indeed, phosphorylation of the zipcode-binding protein ZBP1 by the protein kinase Src results in the dissociation of repressed mRNAs from mRNPs and in their translation on polyribosomes (Huttelmaier et al. 2005).

Of interest is also the association between Stau1- and Stau2-bound mRNAs and localization and transport. It is well documented that one of the functions of both Stau1 and Stau2 is related to mRNA transport and localization. In polarized cells such as neurons, Stau1 and Stau2 are found in the form of RNA granules that move in dendrites (Kiebler et al. 1999; Kohrmann et al. 1999; Tang et al. 2001; Duchaine et al. 2002; Barbee et al. 2006; Kim and Kim 2006). Down-regulation of Stau1 using RNAi or impaired transport of Stau2 following expression of a dominant negative mutant both reduce the transport of mRNA in dendrites. Our results now suggest that Stau1 and Stau2 may also regulate their own function by controlling the post-transcriptional expression of proteins involved in the mechanics of transport and localization. Similarly, several mRNAs are linked to the ubiquitination process. To finely regulate signals induced by cell stimulation, it is important to quickly inactivate and/or eliminate signaling proteins. One efficient pathway relies on protein degradation by the proteasome, a process that is dependent on the ubiquitination of proteins to be degraded (Ciechanover et al. 2000). It is likely that mRNA transport and local translation is balanced by local protein degradation, allowing fine regulation of cell signaling. Components of the proteasome are found in dendrites, and the function of the proteasome is necessary for long-term potentiation (Hegde et al. 1997; Fonseca et al. 2006; Karpova et al. 2006).

Comparison to other RNPs

Our data indicate that there is an overlap between Stau1- and Stau2-associated mRNAs. We decided to look if there is also an overlap with other known mRNA populations associated with other RNA-binding proteins. FMRP is the product of the *Fmr1* gene. Absence or mutation of FMRP causes Fragile X syndrome, an X-linked form of mental retardation. It has been shown that FMRP is involved in translational repression and in synaptic plasticity (Zalfa et al.

2006). It was reported that FMRP copurifies with at least 432 different mRNAs following immunoprecipitation from mouse brain extracts (Brown et al. 2001). FMRP is also found in complex with Stau1 (Brendel et al. 2004; Villace et al. 2004), which suggests that there could be a common set of target mRNAs for both proteins. In order to test this possibility, we wanted to compare the 432 mRNAs previously identified as potential FMRP targets with the Stau1⁵⁵-HA-associated mRNA population identified in the present study. Unfortunately, it is hard to gain a global picture of the relationships that exist between the RNA content of different mRNPs that were characterized using the RIP-chip approach. Comparisons are difficult to make between datasets available in the literature because of high variations in (1) the array platforms that are used; (2) the tissue or cell type used as starting material; (3) the developmental stage at which the experiment was conducted; and (4) the species that were used to study mRNA content of mRNPs. Nevertheless, we first performed a search for probe sets corresponding to the human orthologs of mouse probe sets. From the 432 probe sets corresponding to FMRP-associated mRNAs, 375 probe sets possess a human ortholog. These 375 probe sets represent 355 unique genes. Human U133 gene chip contains 1363 probe sets that represent the orthologs of the 375 mouse probe sets. Of these 1363 probe sets, 58 (4.3%) are enriched in Stau1⁵⁵-HA purification. These 58 probe sets represent 52 unique genes, which leads to the conclusion that ~15% (52/355) of FMRP-associated mRNAs are also enriched in Stau1⁵⁵-HA purification (Table 3). Similarly, the population of mRNAs associated with Stau1 is distinct from that of mRNA bound to the human zipcode binding protein IMP. Indeed, only 26 probe sets (Table 4) are common among the 352 (7%) probe sets in IMP1 complexes and Stau1 (1.6%) complexes. These two RNA granules do not colocalize in HEK293T cells (Jonson et al. 2007).

Staufen-binding site

To understand the precise role of Staufen in mammalian cells, it will be necessary to determine how Staufen regulates the fate of each of its associated mRNAs and therefore how it regulates the function of the encoded protein. First, it must be determined whether Stau1 or Stau2 directly bind RNP-associated RNAs or whether these RNA copurify by virtue of their binding to other RNA-binding proteins in the complexes. This is not a trivial task, since Stau1 binds any structured RNA in vitro (Ramos et al. 1999, 2000; Wickham et al. 1999). Second, the RNA structure of the Stau1- and Stau2-binding sites and their position relative to the 5' or 3' UTR of each mRNA must be determined. The position of the Stau1-binding site in a particular mRNA is of primary importance since it dictates whether the mRNA is targeted to polysomes for enhanced translation (Dugre-Brisson et al. 2005) or is degraded by

TABLE 3. mRNAs associated with both Stau1⁵⁵-HA (this study) and FMRP (Brown et al. 2001)

Stau1 ⁵⁵ -HA/FMRP-associated mRNAs	HG_U133	MG_U74v2
Adenylate cyclase 1 (brain)	213245_at	16691_AT
AP1 gamma subunit binding protein 1	239678_at	133560_AT
AT rich interactive domain 2 (ARID, RFX-like)	225486_at	103079_AT
ATM/ATR-substrate Chk2-interacting Zn ²⁺ finger protein	201854_s_at	130730_F_AT
ATPase, Class VI, type 11A	230875_s_at	103899_AT
Cdk5 and Abl enzyme substrate 1	225531_at	117085_AT
Chromosome 15 open reading frame 39	215087_at	104389_AT
Chromosome 6 open reading frame 107	226135_at	103773_AT
Chromosome 9 open reading frame 10	15555908_at	91488_AT
Chondroitin polymerizing factor	202175_at	97387_AT
DIP2 disco-interacting protein 2 homolog A (<i>Drosophila</i>)	1552677_a_at	114468_AT
Dipeptidyl-peptidase 9	230018_at	114469_AT
Dymeclin	223171_at	99988_AT
Eukaryotic translation initiation factor 4 gamma, 3	243149_at	96850_AT
Exostoses (multiple)-like 1	206329_at	115969_AT
Glucocorticoid receptor DNA binding factor 1	229394_s_at	96208_AT
Guanine nucleotide binding protein (G protein) alpha 1	221737_at	97226_AT
Hypothetical protein DKFZp76112123	54970_at	102271_AT
Jun D proto-oncogene	203752_s_at	1022364_AT
Kelch-like 21 (<i>Drosophila</i>)	203068_at	104293_AT
KIAA0226	212735_at	111540_AT
KIAA0284	213242_x_at	131216_F_AT
KIAA0317	202128_at	109944_AT
KIAA0515	212069_s_at	107033_AT
Latrophilin 1	219145_at	112497_AT
Leucine-rich repeat neuronal 6A	227933_at	139527_AT
MCM3 minichromosome maintenance deficient 3	212269_s_at	97433_AT
Meningioma (disrupted in balanced translocation) 1	205330_at	110774_AT
Nischarin	227392_at	94236_AT
Nuclear factor I/C (CCAAT-binding transcription factor)	226895_at	92241_AT
PH domain and leucine rich repeat protein phosphatase	212719_at	109399_AT
Phosphatidylinositol 4-kinase, catalytic, alpha polypeptide	207081_s_at	104208_AT
Protein phosphatase 1, regulatory subunit 9B	225124_at	112345_AT
PRP8 pre-mRNA processing factor 8 homolog	200000_s_at	98574_AT
RAB6 interacting protein 1	212561_at	104108_AT
Ral guanine nucleotide dissociation stimulator	20050_at	100530_AT
SET domain containing 1B	213153_at	129315_AT
Spectrin domain with coiled-coils 1	240765_at	102942_AT
Steril alpha motif domain containing 4B	227511_at	100889_AT
Synaptotagmin IX	1563658_a_at	107392_AT
TBC1 domain family, member 16	228488_at	110258_AT
TBC1 domain family, member 24	227908_at	97710_F_AT
Transmembrane protein 1	1555446_s_at	104202_AT
Tyrosine kinase, nonreceptor, 2	2033839_s_at	102850_AT
Ubiquitin protein ligase E3 component n-recognin 2	212760_at	111342_AT
Ubiquitin-specific peptidase 2	229337_at	92820_AT
Unc-51-like kinase 1 (<i>Caenorhabditis elegans</i>)	209333_at	102332_AT
WW domain containing E3 ubiquitin protein ligase 2	204022_at	103717_AT
WW, C2, and coiled-coil domain containing 1	241950_at	96518_AT
Zinc finger protein 236	222227_at	115194_AT
Zinc finger protein 42 (Myeloid-specific retinoic acid receptor)	40569_at	94101_AT
ZXD family zinc finger C	230209_at	117039_AT

the Stau1-mediated mRNA decay pathway (Kim et al. 2005). Although a gene-by-gene approach is required at least at the beginning to characterize the Stau1-binding site, an in silico high-throughput technique to assess where Stau1 binds to a given group of mRNAs would facilitate the

search. The classification presented here of Stau1- and Stau2-associated mRNAs in functional subclasses will be helpful in orienting further studies. We recently reported that Stau1 binds to a stem of 19 nucleotides (nt) in the 3' UTR of the Arf1 mRNA (Kim et al. 2007). However, the

TABLE 4. mRNAs associated with both Stau1⁵⁵-HA (this study) and IMP (Jonson et al. 2007)

Stau1 ⁵⁵ -HA/IMP-associated mRNAs	HG_U133
—	224752_at
Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 2	223266_at
ASF1 anti-silencing function 1 homolog B (<i>S. cerevisiae</i>)	218115_at
Chromosome 1 open reading frame 160	52078_at
Chromosome 22 open reading frame 16	224932_at
Coagulation factor VIII-associated (intronic transcript) 1	203274_at
Dymeclin	223171_at
Family with sequence similarity 89, member B	32209_at
Gem (nuclear organelle) associated protein 7	222821_s_at
Glucosamine-6-phosphate deaminase 1	202382_s_at
Golgi reassembly stacking protein 2, 55 kDa	208842_s_at
Hypothetical gene LOC283846	217948_at
Leucine zipper and CTNNBIP1 domain containing	212281_s_at
Opioid receptor, sigma 1	201692_at
OTU domain, ubiquitin aldehyde binding 1	38710_at
Phosphatidylinositol glycan anchor biosynthesis, class S	223148_at
Phosphoribosylaminoimidazole carboxylase	201013_s_at
PRA1 domain family, member 2	203456_at
Progestagen-associated endometrial protein	227511_at
Protein phosphatase 1, regulatory (inhibitor) subunit 11	1566303_s_at
Pterin-4 alpha-carbinolamine dehydratase	203557_s_at
Rhomboid domain containing 2	222995_s_at
Solute carrier family 38, member 6	214830_at
Splicing factor 3b, subunit 4, 49 kDa	209044_x_at
TBC1 domain family, member 22B	223375_at
Zinc finger protein 236	222227_at

critical features that confer specific binding for Stau1 are still unclear, and adjacent uncharacterized region(s) are also needed for optimal binding. Indeed, in silico analyses of several Stau1 target mRNAs failed to identify a 19-nt stem in these mRNAs, indicating that the recognition element is probably more complex than a single stem. This is consistent with the Stau1-binding site in the *Drosophila* bicoid mRNA that included three stem-loops (Ferrandon et al. 1994). Similarly, other dsRNA-binding proteins were shown to bind complex RNA structures composed of stems, bulges, and loops (Herbert and Rich 2001; Hallegger et al. 2006). The nature of the Stau2-binding site is completely unknown.

We previously reported a partial list of Stau1-associated mRNAs using the 22,000 probe sets microarrays from Affymetrix (Kim et al. 2005). We now extend this list with four additional independent hybridizations of the 54,000 probe sets microarrays. Among the 23 previously identified Stau1-associated mRNAs, nine are present in the new Stau1⁵⁵-HA analysis. Notably, the probe set corresponding to Arf1 is not present in the list. However, Arf1 mRNA has been shown to be a real target of Stau1 (Kim et al. 2005), and its Stau1-binding site in the 3' UTR has been identified (Kim et al. 2007). Careful analysis of the novel hybridizations indicates that the probe set corresponding to Arf1 is enriched at least 2.5-fold over the controls in three of the

four hybridizations. This is also true for all other previously identified probe sets that are either enriched in the four new microarrays but were not retained in this study because of their *P*-values above the threshold (three probe sets), or are enriched in two (five probe sets) or three (five probe sets) of the four new hybridizations. Therefore, although not statistically significant, these probe sets are likely targets for Stau1. Indeed, if microarray data are analyzed using the robust multiarray average (RMA) data processing method, 19 out of the 23 previously identified mRNAs (including Arf1) are identified. These data suggest that more mRNAs than those in the list may be associated with Stau1.

We have for the first time showed, by an unbiased approach, that Stau1 and Stau2 associate with different although overlapping subsets of mRNAs in the cells. Interestingly, mRNAs associated with Stau1- and Stau2-containing mRNPs code for proteins that are sorted in similar GO categories, suggesting that the two paralogs play complementary roles in mammalian cells. It is possible that during evolution the *Stau*

gene has duplicated to finely regulate specific pathways. Differential splicing events in each gene then allow a further diversification of the repertoires of possible functions. Altogether our results are consistent with the post-transcriptional operon hypothesis (Keene and Tenenbaum 2002) for concerted expression of functionally related genes in specific pathways.

MATERIALS AND METHODS

Immunopurifications

HEK293T cells were propagated in DMEM medium supplemented with 10% cosmic calf serum (HyClone); 3×10^6 cells were plated in 150-mm Petri dishes, and transfections were performed 24 h after plating. Cells were transfected with one of the following plasmids: Stau1⁵⁵-HA₃ (Duchaine et al. 2000), Stau2⁵⁹-HA₃, or Stau2⁶²-HA₃ (Elvira et al. 2006a) using the calcium-phosphate coprecipitate technique. Mock cells transfected with untagged expressing vectors or the vector alone were used for control. More than 80% of the cells were transfected. Cells were collected and lysed 48 h after transfection. Immunopurification, using anti-HA antibody, was performed as previously described (Duchaine et al. 2000) except that *Escherichia coli* tRNA (Sigma-Aldrich) was added to a final concentration of 200 µg/mL during the incubation with Protein-A-Sepharose (GE healthcare). After extensive washing, the resin was incubated 5 min at 95°C in

1× Laemmli buffer. One-tenth of the eluate was kept for Western blot analysis; the remainder of the sample was used for RNA extraction. A rabbit polyclonal anti-HA antibody (Sigma) and Western blotting were used to detect transfected Stau1-HA and Stau2-HA proteins.

Microarray analysis

RNA was extracted with Trizol reagent (Invitrogen). The RNA pellet was solubilized in water, and residual contaminants were removed using an RNA nanoprep column (Stratagene). Biotinylated cRNA probes were synthesized by the two-cycle target labeling assay following the Affymetrix procedure. Affymetrix human genome arrays (HG-U133_Plus_2: comprising 52,245 probe sets that correspond to 29,555 unique genes) were used for hybridization according to Affymetrix guidelines. Hybridized chips were scanned using an Affymetrix GeneChip 3000 Scanner. Results were recorded using the GeneChip Operating Software (GCOS) platform, which includes the GeneChip Scanner 3000 high-resolution scanning patch that enables feature extraction (Affymetrix). Notably, the Affymetrix Gene Expression Assay identifies changes that are greater than twofold with 98% accuracy (Wodicka et al. 1997).

To identify mRNAs that specifically copurify with Stau1⁵⁵-HA, Stau2⁵⁹-HA, and Stau2⁶²-HA, signal intensities obtained for specific IPs were compared with the baseline signal intensities of control IPs using statistical analysis on mean-scale normalized MAS5 data with a *P*-value limit of 0.05. The same methodology was used to compare microarrays hybridized with cRNAs synthesized from specific IPs to cRNAs synthesized from the total RNA content of HEK293T cells. Each probe set presenting a fold enrichment over control of more than 2.5 was scored as a potential Stau1-associated mRNA. Only the probe sets showing a difference of more than 200 arbitrary units of intensity between the IP and control were kept for further analysis. Complete microarray analysis results can be found in the Supplemental Data. Microarray data have been deposited in the GEO database and are available through the series accession numbers GSE8437 and GSE8438.

RT-PCR amplification

RT-PCR was performed as previously described (Kim et al. 2005). Primer pairs are listed in Supplemental Table S7.

SUPPLEMENTAL DATA

Supplemental material can be found online at www.rnajournal.org.

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