Proteomic Characterization of Messenger Ribonucleoprotein Complexes Bound to Nontranslated or Translated Poly(A) mRNAs in the Rat Cerebral Cortex*

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Receptor-triggered control of local postsynaptic protein synthesis plays a crucial role for enabling long lasting changes in synaptic functions, but signaling pathways that link receptor stimulation with translational control remain poorly known. Among the putative regulatory factors are mRNA-binding proteins (messenger ribonucleoprotein, mRNP), which control the fate of cytosolic localized mRNAs. Based on the assumption that a subset of mRNA is maintained in an inactive state, mRNP-mRNA complexes were separated into polysome-bound (translated) and polysome-free (nontranslated) fractions by sucrose density centrifugation. Poly(A) mRNA-mRNP complexes were purified from a postmitochondrial extract of rat cerebral cortex by oligo(dT)-cellulose affinity chromatography. The mRNA processing proteins were characterized, from solution, by a nanoflow reverse phase-high pressure liquid chromatography-μ-electrospray ionization mass spectrometry. The majority of detected mRNA-binding proteins was found in both fractions. However, a small number of proteins appeared to be fraction-specific. This subset of proteins is by far the most interesting because the proteins are potentially involved in controlling an activity-dependent onset of translation. They include transducfer proteins, kinases, and anchor proteins. This study of the mRNP proteome is the first step in allowing future experimentation to characterize individual proteins responsible for mRNA processing and translation in dendrites.

From the point of their transcription, the fate of every mRNA is controlled by RNA-binding proteins. Inside the nucleus these proteins enable proper transcription, splicing, processing, and their export into the cytosol. Once outside the nucleus, mRNA-binding proteins, also described as messenger ribonucleoproteins (mRNPs),1 are involved in processes controlling intracellular transport, subcellular localization, translation initiation, translational silencing (“masking”), stability, and degradation of poly(A) mRNAs. Only a subset of mRNAs is transported into distal parts of dendrites (1–3), and it is conceivable that these mRNAs in particular must be protected from premature translation during their transport through the cell body and dendrites. Furthermore, to ensure localized translation of these mRNAs at activated synapses, the masked mRNAs should become unmasked by receptor-triggered signaling mechanisms, implying dissociation of some mRNPs from the transport complex at the time of translation. Specific mRNA-binding proteins that bind preferentially to either nontranslated or translated poly(A) mRNAs have not been well characterized in neurons. To search for such putative neuronal poly(A) mRNA masking proteins and proteins involved in mechanisms of receptor-stimulated protein translation, we separated and enriched mRNP complexes associated with nontranslated poly(A) mRNAs and those associated with translated poly(A) mRNAs, and we determined the composition of co-purified proteins by mass spectrometry. Here we report that although most proteins were identified in both fractions, there were several examples of proteins only present in silent mRNP complexes and proteins found only in translated mRNP complexes. UNR, STRAP, the RNA-binding protein EWS, RNA, export factor-binding protein 1, and hnRNP-H1 are either specifically or highly enriched in small, <40 S, mRNP complexes, which are associated with nontranslated mRNAs. Based on their specific localization, these proteins are candidates to play a crucial role in masking poly(A) mRNAs in neurons. On the other hand, proteins such as FMRF, regulator of nonsense transcript 1, protein similar to protein C9orf10, BC010304, protein similar to ubiquitin C-terminal hydrolase-related polypeptide, protein similar to RNA helicase A, and RACK1, which were identified as part of the translated mRNA-mRNP complexes, are potentially involved in processes of translational control.

EXPERIMENTAL PROCEDURES

Preparation of mRNPs—Cerebral cortices of 15–19-day-old Long Evans rats were homogenized in buffer A containing 125 mM NaCl, 100 mM sucrose, 50 mM HEPES, 2 mM potassium acetate, and 40 units/ml of an RNase inhibitor (RNasin, Promega) and centrifuged for 2 min at 4000 × g (postnuclear supernatant) followed by a 10-min spin at 14,000 × g (post-mitochondrial supernatant). The supernatant was treated with buffer B (to get a final concentration of 50 mM Tris/HCl, pH 7.4, 10 mM MgCl2, 0.5 mM DTT, 0.5 mM spermine, 0.5 mM spermidine, 0.1 mM EDTA, and 0.1% Triton X-100). Next, the sample was passed through a 100-μm filter, and the debris was discarded. The filtrate was loaded on a 26/S anion exchange column (HiTrap Q HP, Amersham Biosciences) equilibrated with buffer B. After washing with buffer B, the column was washed with ten column volumes of buffer C (buffer B containing 0.2 M NaCl). After washing, the column was eluted with column volumes of buffer C containing 1.0 M NaCl. Fractions were collected and monitored for bound mRNA by measuring the absorbance at 260 nm. Fractions with the highest absorbance were pooled to yield the polysome-bound mRNA fraction (translated mRNPs). Fractions with the lowest absorbance were pooled to yield the nontranslated mRNA fraction (nontranslated mRNPs). The polysome-bound mRNA fraction was analyzed by reverse phase high pressure liquid chromatography (HPLC; see above). The nontranslated mRNA fraction was precipitated by addition of 2 vol of 10% perchloric acid. The precipitate was recovered by centrifugation and redissolved in a small volume of buffer C. This sample was analyzed by reverse phase HPLC, using the low pressure gradient (0–60% buffer D [25 mM ammonium bicarbonate, 30% acetonitrile]) to reduce the analysis time of the sample.

hmRNP, heterogeneous nuclear ribonucleoprotein; PABP, poly(A)-binding protein; DTT, dithiothreitol.
Composition of mRNP Complexes

7.5, 1% Nonidet P-40, 50 mM NaCl, 4 mM MgCl₂, 45 μM/ml cycloheximide) and layered on a discontinuous sucrose gradient (4.5 ml of 12% and 4.5 ml of 33.5% sucrose in 20 ml Tris/HCl, pH 9.0, 90 mM NaCl, 3 mM MgCl₂, and 0.2 mM β-mercaptoethanol). The tubes were centrifuged for 90 min at 130,000 × g in an SW41 rotor (Beckman LB-70 M centrifuge). To dissociate ribosomes/polysomes and to release poly(A)mRNP complexes, the resulting pellet (monosomes and polyribosomes, see Fig. 1A) was resuspended in a solution (pellet buffer) containing 30 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris/HCl, pH 7.5, and kept on ice for 10 min. The interfaces were recovered and centrifuged again for 20 min at 400,000 × g in a Beckman TL-100 ultracentrifuge. The resulting pellets, which contained mRNPs, free 40 S, and 60 S ribosomal subunits, and a small subfraction of monosomes (Fig. 1A), were resuspended in the pellet buffer and kept for 10 min at 4 °C. The suspension was centrifuged for 2 min at 14,000 × g, and the supernatant was used for the oligo(dT)-cellulose binding assay. The KCl concentration was adjusted to 200 mM, then 40 μl of pre-filter oligo(dT)-cellulose (100 μg/ml Sigma) was added. After 90 min of constant rotation at 4 °C, the cellulose was washed three times with 1 ml wash buffer (20 mM Tris/HCl, pH 7.5, 100 mM KCl, 5 mM MgCl₂). Poly(A) mRNAs and attached mRNPs were eluted with 200 μl of 10 mM Tris/HCl, pH 7.5, at 65 °C. Either 5 μl of the eluate was used for mass spectrometry analysis (see below) or separated in a one- (5–20% acrylamide) or two-dimensional SDS-PAGE (pI 3–10, 8–18% acrylamide) or two-dimensional SDS-PAGE (pI 3–10, 8–18% acrylamide). In a parallel preparation, the three fractions were used to prepare poly(A)mRNAs (micoPoly(A)Pure™, Ambion Inc., Austin TX), and the amount in each fraction was estimated (DNA DipStick™, Invitrogen).

**Western Blot Assay**—For the detection or determination of the amount of different mRNPs, the appropriate fractions were loaded on a gel (5–20% acrylamide). After separation, the proteins were electrotransferred to nitrocellulose (0.45-μm pore size) in a transfer buffer (25 mM Tris, 192 mM glycine, 0.02% SDS, 20% methanol) for 90 min at room temperature. After a short wash with TBST, an antibody specific for PABP1 (mouse IgG, clone 1C3, 1:1000, Chemicon International Inc., Temecula CA), S6 ribosomal protein (mouse IgG, clone 1:2000, Transduction Laboratories, Lexington, KY), or FMRP (mouse IgG, clone 10E10, 1:1000, kindly provided by Dr. G. Dreyfuss, Howard Hughes Medical Institutes and Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia), STRAP (goat polyclonal antibody, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), EWS (goat polyclonal antibody, 1:500, Santa Cruz Biotechnology, Inc, Santa Cruz CA), hnRNP-H (rabbit polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz CA), RACK1 (mouse IgM, 1:2000, Transduction Laboratories, Lexington, KY), or FMRP (rabbit IgG, clone IC3, 1:1000, Chemicon International Inc., Temecula CA), S6 (rabbit polyclonal antibody, 1:2000, Cell Signaling Technology, Inc, Beverly, MA) was applied overnight at 4 °C. The nitrocellulose was washed with TBST, and the immunoreactivity was revealed using an appropriate peroxidase-conjugated anti-mouse IgG (Sigma, 1:10,000), anti-rabbit IgG (Sigma, 1:10,000), or anti-goat antibody (Sigma S15000) and the ECL system (Pierce).

**Northwestern Blot Assay**—Oligo(dT)-cellulose bound proteins were released with SDS sample buffer (4% SDS, 250 mM Tris, 50 mM DTT, 3 mM EDTA, 20% glycerol, pH 8.0), separated on a 5–20% polyacrylamide gel, and blotted onto a nitrocellulose membrane. Detection of mRNA-binding proteins was performed as described previously (4). The nitrocellulose membrane was rinsed twice with phosphate-buffered saline, and the proteins were renatured in incubation buffer A (10 mM Tris/HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1× Denhardt’s (0.2 mg/ml bovine serum albumin, 0.2 mg/ml Ficoll, 0.2 mg/ml polyvinylpyrrolidone) solution) for 3 minutes each, followed by a 5-min incubation in a hybridization solution (buffer A including 20 μg/ml RNA and 5 μg/ml heparin). Total poly(A) mRNAs were prepared from rat cortex according to the manufacturer’s protocol (Poly(A)-Pure™, Ambion, Austin, TX), labeled using [32P]7-ATP and poly(A) polymerase (U. S. Biochemical Corp.), precipitated with ethanol sodium acetate, washed, and finally resuspended in 10 μl Tris/HCl. Labelled poly(A) mRNA was added to the hybridization buffer (final concentration about 200,000 cpm/ml), and the nitrocellulose membrane was incubated in a probe for 120 min at room temperature. After a series of washes the blot was dried, and bound radioactivity was detected by PhosphorImaging (FUJIX-BAS1000).

**Mass Spectrometry Assay**—Approximately 7.5 μg (75 pmol; 100 kDa average molecular mass) of total protein from each prepared subcellular fraction was loaded (20 μl) onto a 150-μm column packed with 10% C18 and 90% C18 (Phenomenex). Fractions were eluted using the following gradient: 0-10% solvent A (95% acetonitrile, 5% water) in 15 min, 10-100% solvent A in 15 min, and then 100% solvent A for 15 min. The mass spectrometer (San Jose, Ca) was used to analyze the digests using a HPLC-MS interface and a survey scan over 500–3500 m/z. The resultant MS/MS spectra were acquired and analyzed using a short HPLC gradient on a ThermoFinnigan LCQ Deca mass spectrometer (San Jose, CA) to ensure complete digestion. A parallel preparation, the three fractions were used to prepare poly(A)mRNAs (micoPoly(A)Pure™, Ambion Inc., Austin TX), and the amount in each fraction was estimated (DNA DipStick™, Invitrogen).

**RESULTS**

To characterize protein complexes that are preferentially associated with nontranslated (“free”) or translated (“ribosome-bound”) mRNA, we first separated both pools of mRNAs by sucrose density centrifugation as described recently (5). Based on the presence or absence of ribosomal subunits and ribosomes (Fig. 1, A and B), mRNPs complexes enriched from the first interface (on top of the 12% sucrose; fraction 1) were considered to be associated with mRNAs not engaged in translation. The second interface (between 12 and 33.5% sucrose; fraction 2) contained a mixture of nontranslated mRNAs, mRNAs associated with the (pre)initiation complex as well as translated mRNAs, whereas the 33% sucrose pellet (fraction 3) contained mainly translated, polysome-bound mRNAs. From each fraction the poly(A) mRNA-mRNP complexes were bound to oligo(dT)-cellulose, washed, and eluted with 10 mM Tris/HCl (Fig. 1C, Fig. 2). All poly(A) mRNAs were covered by a number of proteins including the poly(A)-binding protein 1 (PABP1). To verify that despite the presence of the PABP1, poly(A) mRNA-mRNP complexes could be purified using oligo(dT)-cellulose, we first performed a Northwestern blot assay. Briefly, oligo(dT)-cellulose-bound proteins were eluted and separated on a 5–20% polyacrylamide gel, blotted on nitrocellulose, renatured, and finally incubated with 32P-labeled poly(A) mRNAs, which should bind to the mRNA-binding proteins present. A number of proteins (poly(A)) mRNA-binding proteins were detected, and the amount and variability of these poly(A) mRNA-binding proteins differed considerably between the three fractions studied (Fig. 2B). With this approach, most poly(A) mRNA-binding proteins were found in the fraction corresponding to translated mRNAs. This indicates that the majority of poly(A) mRNAs is already engaged in translation (Fig. 2A). One of the most abundant proteins in the preparation is the poly(A)-binding protein (PABP1), indicating that the association of this protein with the poly(A)-tail does not prevent binding of poly(A) mRNAs to oligo(dT). Pretreatment of the sample with RNase A (10 μg/ml) resulted in an almost complete loss of proteins that could be purified with the method used (Fig. 2C), strongly suggesting...
that poly(A) mRNA-associated proteins are purified with this method.

To identify the protein composition in each fraction, the eluted proteins were used directly for nano-HPLC-EI-MS analysis. Because fraction 2 contained a mixture of free and ribosome-bound mRNA-mRNP complexes, we focused our investigations on fraction 1 (free, nontranslated poly(A) mRNAs) and fraction 3 (ribosome associated, translated poly(A) mRNAs). As listed in Table I, most known poly(A) mRNA-binding proteins identified by mass spectrometry were detected in both fractions, indicating that the majority of mRNPs is associated with mRNAs before and during translation. Although a postmitochondrial supernatant was used as starting material, a number of proteins were detected that are presumed to be present only within the nucleus, such as various splicing factors, and proteins involved in polyadenylation (cleavage stimulation factor and hnRNP-H). Although the total amount of proteins associated with nontranslated poly(A) mRNAs is very small, some of them are specifically or highly enriched in this fraction (Table I). These include the UNR protein (upstream of N-Ras), STRAP (serine/threonine kinase receptor associated protein, also known as UNR-interacting protein), the RNA-binding protein EWS, and phosphofructokinase C. Similarly, calcium/calmodulin-dependent protein kinase II was found primarily in the fraction associated with nontranslated mRNAs. To confirm the finding that these proteins are predominantly associated with nontranslated mRNAs, a Western blot assay was performed. Again, STRAP was detectable only in the first fraction, whereas the EWS protein, hnRNP-H, and CamKII were highly enriched in the first fraction (Fig. 3).

As stated above, the majority of mRNA-binding proteins was present in fraction 3, purified from mRNAs co-localized with polysomes. Although a number of them appear to be specifically localized in this fraction (Table I), not all of them are necessarily associated with polysome-bound mRNAs. Thus, it is conceivable that this fraction also contains very large poly(A) mRNA-mRNP complexes (>80 S) as well as poly(A) mRNA-mRNP complexes that are very tightly bound to cytoskeletal elements. The complexes tightly associated to cytoskeletal elements may co-migrate with polysomes in the sucrose gradient, even though they are not linked to translated (polysome-bound) poly(A) mRNAs. Indications for this assumption come from our observation that after a complete dissociation of polysomes and ribosomes, which can be achieved by performing the entire preparation in the absence of magnesium, proteins such as Staufen or FMRP (fragile X mental retardation protein) were still detectable in fraction 3 (Fig. 4). Therefore, any protein we identified to be specifically localized in fraction 3 was not unambiguously a component of a mRNP particle linked to translated (ribosome-bound) poly(A) mRNAs only, but might also be a component of a large, nontranslated mRNP complex.

**Fig. 1.** Preparation of mRNPs associated with free or translated poly(A) mRNAs. A, postmitochondrial supernatant was lysed and layered on a discontinuous sucrose gradient (12/33.5%) and centrifuged at 41,000 rpm for 90 min. The resulting pellet and interfaces were resuspended and separated using a continuous sucrose gradient (12–45%). The presence and distribution of RNA (ribosomal RNA) within the sucrose gradient was detected by an ISCO spectrophotometer at a wavelength of 254 nm. In fraction 1 (on top of the 12% sucrose), we could not detect any RNA, indicating the absence of ribosomal subunits. Fraction 2 (interface between 12% and 33.5% sucrose) contained small and large ribosomal subunits (40 S and 60 S), monosomes (80 S), and 2 and 3 ribosomal complexes, whereas fraction 3 (pellet) contained polysomes and a small amount of the large ribosomal subunit. B, to confirm the presence or absence of the small ribosomal subunit (40 S) in these three fractions, proteins of each fraction were separated by SDS-PAGE and blotted onto nitrocellulose. The small ribosomal subunit protein S6 was then detected in fraction 2 and fraction 3 by immunostaining with a specific antibody (arrow). C, fractions 1 and 3 were used to purify poly(A) mRNA-mRNP complexes. After binding poly(A) mRNA to oligo(dT)-cellulose, the poly(A) mRNA-protein complexes were eluted with 10 mM Tris/HCl, pH 7.5. Thereafter RNA was digested with RNase A, and the remaining proteins were precipitated, solubilized, separated by two-dimensional-PAGE, and finally visualized by silver staining (arrow points to the added RNase A).
DISCUSSION

Rapid changes in protein synthesis after receptor stimulation, as observed after induction of long term potentiation, long term depression, and neurotrophin-induced synaptic plasticity (1, 6–11), could be controlled, at least partly, by a rapid increase in the accessibility of dormant mRNAs to the translation machinery. In addition to stimulus-triggered transport of newly transcribed mRNAs to activated synapses, as described for Arc-mRNA (6), other, already postsynaptically localized, mRNAs may become more readily available for translation initiation as a result of an enhanced polyadenylation (12–14) or an unmasking process. In particular, mRNAs that are transported into distal parts of dendrites are likely to be protected to avoid premature translation during their transport through the cell soma, where the majority of ribosomes is localized. To search for candidates that are involved in such a process of masking/unmasking and in receptor-triggered control of translation, we have begun to characterize the protein composition of mRNP complexes associated specifically with nontranslated poly(A) mRNAs and specifically associated with translated poly(A) mRNAs in rat cortical tissue. Our approach was intended to identify not only mRNA-binding proteins but also proteins that are part of the entire mRNP complex associated with poly(A) mRNAs. There exists no established method to purify or enrich poly(A) mRNA-mRNP complexes associated with nontranslated poly(A) mRNAs, probably because the size of these protein complexes can vary considerably, and therefore, they may co-migrate in a sucrose density gradient with a number of other large protein complexes. Furthermore, recent results point to a possible rearrangement of RNA-binding proteins and mRNAs subsequent to cell lysis (15), such that detected mRNA-mRNP complexes may be formed during the purification procedure. Therefore, we kept the time between homogenization and separation of nontranslated and translated (polysome-bound) mRNA within the discontinuous sucrose density gradient as short as possible (15 min). An alternative procedure would be to UV cross-link mRNA and the associated proteins immediately after cell lysis. However, because of the relatively low efficiency of this reaction (16), this
After sucrose density centrifugation, the fractions corresponding to nontranslated poly(A)-mRNA/mRNP complexes (fraction 1) and to polysome-bound poly(A)-mRNA/mRNP complexes (fraction 3) were resuspended and incubated in the presence of oligo(dT)-cellulose for 60 min at 4 °C. Poly(A)-mRNA/mRNP complexes were released from the oligo(dT)-cellulose by 10 mm Tris/HCl (pH 7.5), and the present proteins were identified by mass spectrometry analysis. Listed are the identified protein sequences with the highest \( x_{\text{corr}} \), and the number of different independent hits for the same protein.

### Composition of mRNP Complexes

| Fraction | mRNA stability | mRNA transport | mRNA localization | Translational control | Polyadenylation | Signal transducer | RNA processing |
|----------|----------------|----------------|-------------------|-----------------------|-----------------|------------------|---------------|
| 1        | g16758924      | g13348739      | g485813           | g16758924            | g18875338       | g470914          | g5031733      |
| 3        | g34867134      | g113307        | g6680948          | g7243023             | g5031733        | g470914          | g5031733      |
|          | g16758924      | g113307        | g346338           | g16758924            | g18875338       | g470914          | g5031733      |
|          | g34867134      | g113307        | g135432           | g7243023             | g5031733        | g470914          | g5031733      |
|          | g16758924      | g113307        | g135432           | g16758924            | g18875338       | g470914          | g5031733      |
|          | g34867134      | g113307        | g135432           | g7243023             | g5031733        | g470914          | g5031733      |

**TABLE I**

Summary of proteins detected by nanoflow HPLC-μESI-MS analysis

| mRNP stability | g16758924 | X | FUSE-binding protein 1 | 40 | 3 |
|----------------|-----------|---|------------------------|----|---|
| g34867134      | X         | Heat shock 70-kDa protein | 41 | 3 |
| g16758924      | X         | Unr protein              | 42 | 7 |
| g33516945      | X X       | hnRNP D                  | 43 | 5 |
| g45504715      | X X       | hnRNP L                  | 43 | 1 |
| g14114181      | X X       | hnRNP U                  | 44 | 1 |
| g31542602      | X X       | Hu-antigen A             | 43 | 1 |
| g11386163      | X X       | Hu-antigen D             | 3  |   |
| g6981248       | X X       | Nucleolin                | 45 | 7 |
| g45055756      | X X       | Poly(A)-binding protein, cytoplasmic 1 | 43 | 15 |
| g4504715       | X X       | Poly(A)-binding protein, cytoplasmic 4 (inducible form) | 2  |   |
| g9580989       | X X       | RNA-binding protein p42 AUF1 | 43 | 24 |
| g4758262       | X         | Hu-antigen B             | 4  |   |
| g1144009       | X         | Hu-antigen C             | 3  |   |
| g24234753      | X         | Interleukin enhancer-binding factor 3 | 46 | 4 |
| g79861628      | X         | PAI-1 mRNA-binding protein | 47 | 5 |
| g18375673      | X         | Regulator of nonsense transcripts 1 | 48 | 18 |
| g13348739      | X         | Dynein, cytoplasmic, heavy chain 1 | 2  |   |
| g33418258      | X X       | Syncrip (hnRNP Q)        | 49 | 6 |
| g485813        | X         | RNA-binding protein EWS  | 50 | 2 |
| g16758924      | X X       | Actin                    | 51 | 4 |
| g346338        | X X       | Purine-rich element-binding protein A | 52 | 7 |
| g135432        | X         | Tubulin α-chain          |    |   |
| g135493        | X         | Tubulin β-chain          |    |   |
| g16307507      | X         | Staufen 2 protein        | 54 | 11|
| g6756033       | X         | Y box protein 1           | 55 | 5 |
| g8335791       | X         | Y box protein 2           | 56 | 3 |
| g16758924      | X         | Unr protein              | 30 | 7 |
| g7243023       | X X       | 82-kDa FMRP-interacting Protein | 3  |   |
| g6680948       | X X       | Cold-inducible RNA-binding protein | 57 | 2 |
| g133262        | X         | hnRNP C                  | 58 | 2 |
| g45055756      | X X       | Poly(A)-binding protein, cytoplasmic 1 | 43 | 13 |
| g4504715       | X X       | Poly(A)-binding protein, cytoplasmic 4 (inducible form) | 2  |   |
| g346338        | X X       | Purine-rich element-binding protein A | 59 | 16 |
| g1518669       | X         | RNA-binding protein, FMRP | 60 | 2 |
| g6756033       | X         | Y box protein 1           | 55 | 5 |
| g5031733       | X         | Hu-antigen A             | 43 | 1 |
| g18875338      | X         | Cleavage stimulation factor subunit 2 | 62 | 2 |
| g5031733       | X         | hnRNP H1                 | 43 | 4 |
| g12804941      | X         | U2 small nuclear RNA auxiliary factor 1 | 63 | 2 |
| g19526824      | X         | U2 small nuclear ribonucleoprotein auxiliary factor 2 | 63 | 3 |
| g470914        | X         | Phosphofructokinase C     | 5  |   |
| g6756033       | X         | Serine/threonine kinase receptor-associated protein | 4  |   |
| g488513        | X         | RNA-binding protein EWS  | 33 | 64 |
| g11120682      | X X       | Calcium/calmodulin-dependent protein kinase type II | 2  |   |
| g31982757      | X X       | G3BP2a                   | 35 | 13|
| g4234753       | X         | Interleukin enhancer binding factor 3 | 65 | 4 |
| g4505581       | X         | Protein activator of the interferon-induced protein kinase | 39 | 2 |
| g5174447       | X         | RACK1                    | 7  |   |
| g5031733       | X         | hnRNP H1                 | 43 | 4 |
| g488513        | X         | RNA-binding protein EWS  | 66 | 2 |
| g38014635      | X         | Splicing factor proline/glutamine-rich | 43 | 1 |
| g92559         | X         | Nucleolin                | 45 | 7 |
| g6912654       | X X       | Splicing factor 3b, subunit 1, 155 kDa | 3  |   |
| g34868816      | X         | Similar to splicing coactivator subunit Srm300 | 6  |   |
| g34852270      | X         | Similar to splicing factor, arginine-serine-rich 3 | 6  |   |
| g32498587      | X         | RNA-binding region containing protein 2 isoform c | 3  |   |
| g19526824      | X         | U2 small nuclear ribonucleoprotein auxiliary factor 2 | 63 | 3 |
| g6756033       | X         | Y box protein 1           | 55 | 5 |
approach seems only advisable when one interaction partner is known. To minimize the problem of potential contamination by other protein complexes, we first purified poly(A) mRNAs by oligo(dT) affinity chromatography and then eluted the bound protein complexes. Although the effect of a pretreatment with RNase A points to a specific enrichment of RNP complexes (Fig. 2), we also co-purified a number of cytoskeletal proteins such as actin and tubulin. This confirms the previously described close association of actin/tubulin with poly(A) RNAs (17) and may also explain the abundant presence of cytoskeletal associated proteins like the MAP2b and MAP1b in our preparation. On the other hand, no indications for the presence of the highly abundant axonal localized microtubule-associated protein tau were found in our preparation. This may indicate that the dentritically localized MAP2b could also be involved in mechanisms of targeting mRNP complexes within the dendritic compartment.

In addition to proteins containing a known RNA-binding domain, such as RNA recognition motif, double-stranded RNA-binding motif, K homology RNA-binding domain, and cold shock domain, we identified a number of proteins without any known RNA-binding domains. This may point to the fact that we in fact purified complex mRNP particles rather than simply mRNA-binding proteins. This is supported by the fact that among the identified proteins, three proteins (RACK1, STRAP, and cleavage stimulation factor subunit 1) were present that consist almost entirely of WD repeat (Trp-Asp) domains that are thought to mediate various protein-protein interactions.

### Table I—continued

| Fraction | Ref. no. | No. of hits |
|----------|----------|-------------|
| 1        |          |             |
| 2        |          |             |
| 3        |          |             |

* FUSE, far upstream element.
(18, 19). Furthermore, we identified a number of so far uncharacterized proteins, such as BC010304, protein similar KIAA1096, protein similar to C9orf10, protein similar to expressed sequence AI256361, protein similar to ubiquitin C-terminal hydrolase, and caprin-1. Caprin-1, formerly known by the misleading name glycosylphosphatidylinositol-anchored protein p137, appears to be very abundant in the mRNP fraction. Caprin-1, a protein that is highly conserved throughout vertebrate evolution, is a cytosolic localized phosphoprotein and has potential sites for binding of SH2 and phosphotyrosine-binding domains (20). This protein, although it contained no RNA-binding domains, was also found to be part of a protein complex that linked the receptor for activated C kinase (RACK1) to poly(A) mRNAs (5) and to change the degree of phosphorylation during mitosis in Xenopus embryos (21); thus it becomes tempting to speculate that Caprin-1, as part of an mRNP complex, is able to link second messenger-dependent phosphorylation systems with the mechanism of translational control. Of special interest is also the repeated detection of protein similar to ubiquitin C-terminal hydrolase within mRNP complexes, which is likely involved in the processes of de-ubiquitination. Whether this protein is part of a specific signaling pathway or an element controlling the ubiquitination and function of ribosomal proteins, such as L40, S27a P1, translation factors, or AUF1 (22–25), remains to be investigated.

One of the aims of this study was to identify those proteins within the mRNP complex that may have served as a link between the receptor-triggered signaling pathway and translational control. Based on our findings the following proteins are candidates for such a function. (i) For the STRAP-UNR complex, both proteins interact with each other in an mRNA-independent manner (25), and although UNR binds internal ribosomal entry site-containing mRNAs (30), STRAP can interact with the serine/threonine receptor kinase, transforming growth factor-β (31), and thus this complex may be involved in mechanisms of receptor-triggered translation of internal ribosomal entry site-containing mRNAs. (ii) For the RNA-binding protein EWS (preferentially bound to nontranslated mRNAs), this protein contains an IQ domain, known as a calmodulin-binding domain and, most interestingly, can be phosphorylated by protein kinase C (32, 33). Because phosphorylation by protein kinase C reduces the ability of EWS to bind mRNAs (33), a hypothetical masking effect of EWS might be reduced by activated protein kinase C. (iii) For calcium/calmodulin-dependent protein kinase II, this protein was the only protein kinase we could identify among the proteins associated with poly(A) mRNAs. However, in order to describe calcium/calmodulin-dependent protein kinase II unambiguously as an mRNP-associated kinase, the interacting protein(s) that link the kinase to poly(A) mRNAs must be identified. (iv) For G3BP2a, a protein that contains several SH3 domain-binding consensus sequences and an endoribonuclease activity is controlled by phosphorylation (34, 35). (v) For RACK1, a receptor for activated protein kinase C has recently been described in the mechanisms of translational control (5, 36–38). (vi) The protein activator of the interferon-induced protein kinase (PACT) is the only known cellular protein that binds and thereby activates protein kinase R (39). This in turn inhibits translation. The binding of PACT to protein kinase R is again controlled by the phosphorylation degree of PACT (39), and thus a possible receptor-mediated phosphorylation of PACT may control the translation efficiency.

In summary, our proteomic approach to characterize proteins associated with poly(A) mRNAs led to the identification of UNR/STRAP and the RNA-binding protein EWS, both components of complex signaling pathways, as putative members of the mRNA-masking machinery, based upon their selective association with nontranslated mRNAs (versus those undergoing translation). To what extent these proteins are also involved in processes of receptor-triggered unmasking of dendritic mRNAs can begin to be examined when the mRNAs bound to these proteins are identified. Although the UNR-STRAP complex has properties compatible with control of translation initiation of mRNAs with an internal ribosomal entry site, no subset of mRNAs has yet been described that binds specifically to the EWS protein. Furthermore, the identification of a protein likely involved in processes of de-ubiquitination (protein similar to ubiquitin C-terminal hydrolase) may point to an additional regulatory mechanism for translational control besides protein phosphorylation. The characterization of the so far unknown proteins, such as KIAA1096, similar to expressed sequence AI256361, similar to protein C9orf10, and caprin 1 may reveal additional control mechanisms for mRNA metabolism in the brain.

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