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The Microtubule-associated Protein Tumor Overexpressed Gene Binds to the RNA Trafficking Protein Heterogeneous Nuclear Ribonucleoprotein A2[D]

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In neural cells, such as oligodendrocytes and neurons, transport of certain RNAs along microtubules is mediated by the *cis*-acting heterogeneous nuclear ribonucleoprotein A2 response element (A2RE) trafficking element and the cognate *trans*-acting heterogeneous nuclear ribonucleoprotein (hnRNP) A2 trafficking factor. Using a yeast two-hybrid screen, we have identified a microtubule-associated protein, *t*umor *o*verexpressed *g*ene (TOG)2, as an hnRNP A2 binding partner. The C-terminal third of TOG2 is sufficient for hnRNP A2 binding. TOG2, the large protein isoform of TOG, is the only isoform detected in oligodendrocytes in culture. TOG coimmunoprecipitates with hnRNP A2 present in the cytoskeleton (CSK) fraction of neural cells, and both coprecipitate with microtubule stabilized pellets. Staining with anti-TOG reveals puncta that are localized in proximity to microtubules, often at the plus ends. TOG is colocalized with hnRNP A2 and A2RE-mRNA in trafficking granules that remain associated with CSK-insoluble tissue. These data suggest that TOG mediates the association of hnRNP A2-positive granules with microtubules during transport and/or localization.

INTRODUCTION

The heterogeneous nuclear ribonucleoprotein (hnRNP) A2 is implicated in RNA processing, RNA transport, and translation regulation (Dreyfuss *et al*., 1993; Siomi and Dreyfuss, 1995; Hamilton *et al*., 1999; Kwon *et al*., 1999). HnRNP A2 binds to a specific *cis*-acting element, hnRNP A2 response element (A2RE), found in several dendritically localized mRNAs (Ainger *et al*., 1997; Munro *et al*., 1999). This interaction is necessary for transport of A2RE-mRNAs to the periphery of neural cells (Munro *et al*., 1999; Shan *et al*., 2003).

A2RE-mRNAs, hnRNP A2, and other components form complexes that look by light microscopy like granules that are transported to the cell periphery by a microtubule- and kinesin-based mechanism. In cultured oligodendrocytes and neurons, granules containing A2RE-mRNAs and hnRNP A2 are associated with the cytoskeleton (CSK) (Carson *et al*., 1997). HnRNP A2 and some A2RE-mRNAs copurify with the CSK from rat brain (Hoek *et al*., 1998; Boccaccio *et al*., 1999).

To identify molecular partners of hnRNP A2, we performed yeast two-hybrid analysis. One of the components identified is the *t*umor *o*verexpressed *g*ene (TOG) protein, a microtubule-associated protein (MAP) ubiquitously expressed in human tissues. TOG is a single copy gene that generates two mRNA splice variants (Nagase *et al*., 1995; Charrasse *et al*., 1998) encoding proteins that differ by a 60-aa insert near the C terminus. In dividing cells, TOG localizes with centrosome and spindle microtubules and may be necessary for microtubule rearrangements and spindle assembly (Charrasse *et al*., 1998; Lee *et al*., 2001). Orthologues of *c*olonic and *h*epatic *t*umor *o*verexpressed *g*ene (ch-TOG) (*Homo sapiens*), Dis1p, Stu2p, XMAP215, ZYG-9, and mini spindles, in fission yeast, budding yeast, *Xenopus laevis*, *Caenorhabditis elegans*, and *Drosophila melanogaster*, respectively, have similar functions. TOG and its orthologues all contain multiple HEAT repeats that may serve as a scaffold for other components to assemble into complexes (Neuwald and Hirano, 2000). In normal brain, TOG may function in cell morphogenesis and establishment of cellular polarity (Charrasse *et al*., 1995, 1998; Brunner and Nurse, 2000). It also has been suggested that proteins of the TOG family interact with the cell cortex to mediate motility (Schroer, 2001). However, depletion of TOG in human somatic cells does not have a detectable effect on microtubules stability in interphase (Holmfeldt *et al*., 2004).

TOG belongs to a class of MAPs, dubbed *plus*-end-*t*rack*i*ng *p*roteins (+TIPs) that interact preferentially with the plus ends of growing microtubules. The microtubule binding activity is contained within the N-terminal half of the protein (residues 368–910), whereas the C-terminal half of the protein contains tubulin dimer binding activity (Spittle *et al*.,

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Abbreviations used: AD, activation domain; A2RE, heterogeneous nuclear ribonucleoprotein A2 response element; BD, binding domain; CSK, cytoskeleton; HA, hemagglutinin; hnRNP, heterogeneous nuclear ribonucleoprotein; MAP, microtubule-associated protein; PCR, polymerase chain reaction; TOG, tumor overexpressed gene.

2000). A model has been proposed whereby TOG mediates polar elongation of microtubules by binding to the plus end of the microtubule and discharging a tubulin dimer (Spittle *et al*., 2000).

The role of TOG in nondividing cells, particularly in postmitotic neural cells, has not been established. It is postulated that elaboration of axons and dendrites, which requires frequent microtubule reorganization, involves TOG (Charrasse *et al*., 1998). It also has been speculated that proteins of the TOG family are part of a transport mechanism that delivers proteins to the cell periphery (Schuyler and Pellman, 2001). In this study, we examine the role of TOG in RNA trafficking in neural cells.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen

hnRNP A2 bait plasmid [GAL4BD (binding domain)-hnRNPA2 fusion] was constructed using a full-length copy of mouse hnRNP A2 cDNA prepared by polymerase chain reaction (PCR) of pHJ1-hnRNPA2 (Brumwell *et al*., 2002), by using primers LK2BDA2 and LK3BDA2 (Table 1 in Supplementary Material). The PCR product was cleaved with *Eco*RI and *Pst*I and ligated into pGBKT7 (Table 2 in Supplementary Material). The ligation mixture was used to transform XL-1 Blue MR Supercompetent cells (Stratagene, La Jolla, CA), and positive transformants were selected by kanamycin resistance. PCR of the insert, restriction endonuclease digestion of the product, and DNA sequencing of both strands confirmed that the plasmid, pBD-hnRNPA2, contains the full-length sequence of mouse hnRNPA2 (GenBank AF 406651; Brumwell *et al*., 2002). To determine whether pBD-hnRNP A2 expresses a fusion protein (GAL4-BD/hnRNP A2), whole cell extracts of AH109/pBD-hnRNPA2 were analyzed by PAGE. Western blotting (Figure S1) detected a band present in the bait sample, with a molecular mass between 50 and 60 kDa, which is the appropriate size for a fusion of GAL4 BD (21 kDa) and hnRNP A2 (36 kDa).

pBD-hnRNPA2 was transformed into *Saccharomyces cerevisiae* AH109 by using the Yeastmaker yeast transformation system (BD Biosciences Clontech/BD Biosciences, Franklin Lakes, NJ), and transformants were selected by tryptophan heterotrophy ($TRP1^+$). Viability of the transformed strain was determined by comparing the number of colony-forming units per milliliter to the number of cells per milliliter visible in a hemocytometer.

A Matchmaker pretransformed cDNA library from a human, 37-year-old male brain was purchased from BD Biosciences Clontech. The library, herein called pAD-cDNA (GAL4 activation domain-cDNA), was amplified once in *E. coli* before transforming into *S. cerevisiae* Y187 to yield Y187/pAD-cDNA ($LEU2^+$). AH109/pBD-hnRNPA2 was mated with Y187/pAD-cDNA according to the BD Biosciences Clontech protocol (#PT3183-1, version #PR7 × 247). To identify diploid $TRP1^+$ $LEU2^+$ colonies positive for two-hybrid interaction, the mated cells were plated at high stringency to select for both HIS3 and ADE2 expression. Leaky expression of the HIS3 allele was suppressed by adding 2.5 mM 3-amino-1,2,4-triazole to the agar plates. Selected colonies were replated at high stringency, and those with reproducible phenotype were tested for *lac* Z expression in a colony lift-filter assay with 5-bromo-4-chloro-3-indolyl-β-D-galactoside as a substrate.

To distinguish between proteins that bind specifically or nonspecifically to hnRNP A2, plasmids from clones A5, C1, D14, D4, E3, B29, and C22 were used to transform AH109 containing one of four different plasmids: bait, vector alone, or false baits, and the number of $ADE2^+HIS3^+$ cotransformants was scored. The false baits are GAL4-BD fused to either a frameshift mutation of hnRNPA2 (pBD-ΔA2) or laminin (pGBKT7-lam) (see Tables 2 and 3 in Supplementary Material).

Each $LEU2^+TRP1^+ADE2^+HIS3^+$ Lac Z^+ clone was grown in liquid medium, and DNA extracted using Yeastmaker yeast plasmid isolation kit (BD Biosciences Clontech) was used to transform XL-1 Blue MR supercompetent cells. Ampicillin resistance identified pAD-cDNA clones. Clones were maintained as *E. coli* transformants and also as pure DNA.

Characterization of Genes Selected by the Yeast Two-Hybrid System

Plasmid DNA from positive clones was amplified by PCR with primers flanking the insertion site (LK3, LK4; Table 1 in Supplementary Material). Restriction endonuclease (*Hae*III, *Hin*fI, *Xba*I) digestion patterns of the PCR products were used to identify duplicate clones. DNA sequencing was performed by the University of Connecticut Health Center Molecular Core Facility or commercially (acgt, Wheeling, IL) by using LK1 primer. The 5′ terminal sequences were used to identify genes by a BLAST search of GenBank. Clones of interest were sequenced to their 3′ ends. Contigs were assembled and sequences analyzed using the GCG package of programs (Accelrys, San Diego, CA).

In Vitro Protein Synthesis

Labeled proteins ([^{35}S]methionine, Redivue in vitro translation grade; Amersham Biosciences UK, Little Chalfont, Buckinghamshire, United Kingdom) were synthesized by in vitro transcription-translation by using a rabbit reticulocyte lysate. Because pGBKT7 contains a T7 promoter downstream of the GAL4 BD and upstream of a c-*myc* epitope and the hnRNPA2 insert, pBD-hnRNPA2 could be used directly for in vitro synthesis. However, because pAD-cDNA clones do not contain a T7 promoter, each clone was copied by successive rounds of PCR to insert a T7 promoter upstream of the hemagglutinin (HA) epitope by using LKHA_A2B1 in the first round and LKT7HA2 (Table 1 in Supplementary Material) in the second round. The TOG clone was copied in one round of PCR by using LKT7HA3. PCR products were purified via spin columns (QIAGEN, Hilden, Germany). In vitro-translated products were analyzed by gel electrophoresis followed by autoradiography.

Coimmunoprecipitation of Proteins Synthesized In Vitro

Reticulocyte lysates containing in vitro synthesized proteins were combined in a volume ratio of 1:5 (^{35}S-labeled:unlabeled) and incubated for 10 min at 30°C. Protease inhibitor cocktail (Roche Complete; Roche Diagnostics, Indianapolis, IN) was added, and the protein mixture was diluted in binding buffer combined with protein A-agarose beads (immunoprecipitation kit [protein A]; Roche Diagnostics, Mannheim, Germany) to reduce nonspecific background. After incubation and centrifugation, the supernatant was immunoprecipitated with polyclonal goat anti-HA (H-6908; Sigma-Aldrich, MO), at 1:500 dilution, and additional protein A-agarose beads. Immunoprecipitates were collected, washed, solubilized as described in the manufacturer's protocol, and run on 4–12% polyacrylamide Tris-glycine gels that were subsequently exposed to x-ray films.

RT-PCR

RNA was isolated from cells in culture by using RNAgents total RNA isolation kit (Promega, Madison, WI). Total cell RNA was used as a template to make cDNA by using Im-PromII reverse transcription kit (Promega), with oligo (dT)15 as a primer. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with pairs of primers to distinguish ch-TOG mRNA and TOG2 mRNA (Figure 1), and the products were analyzed by gel electrophoresis. For semiquantitative RT-PCR, the number of cycles was reduced and products were compared with dilutions of mixtures of pBstog and pAD-E3 as templates. To better resolve the 102-base pair product from primers LKTOG_C and LKTOG_D (Table 1 in Supplementary Material), RT-PCR products were treated with 50 μg/ml DNase-free RNase at 37°C for 10 min (Maniatis *et al*., 1982) to reduce background staining of RNA.

Cell Culture, Cell Fractionation, and Cell Extraction

Primary glial cultures were prepared from the brains of rats at postnatal day 1–3. Oligodendrocytes were isolated from these cultures by the method of McCarthy and de Vellis (1980). Primary neuronal cultures were prepared from the brains of embryonic day 18 rats following the method of Goslin and Banker (1992). The B104 neuroblastoma cell line (Schubert *et al*., 1974) was grown in DMEM/Ham's F-12 containing 5% fetal calf serum. The U-2 OS human osteosarcoma cell line (Ponten and Saksela, 1967) was grown in McCoy's medium containing 10% fetal bovine serum. Procedures for animal use were approved by the Animal Care Committee of the University of Connecticut Health Center and followed federal guidelines for research with animals.

Procedure and reagents for CSK fractionation are described in Pigino *et al*. (2001). Cells were rinsed in microtubule-stabilizing buffer, and the insoluble fraction was harvested in cold radioimmunoprecipitation assay buffer containing a cocktail of proteases inhibitors (Roche Complete). Samples were concentrated to 100 μl by centrifugation in ultrafree centrifugal filter and tube (Millipore, Billerica, MA) before electrophoresis.

The protocol for cell extraction is essentially that of Biegel and Pachter (1991), with minor modifications. The cells were first washed with phosphate-buffered saline (PBS) followed by CSK buffer (0.3 M sucrose, 0.001 M EGTA, 0.01 M PIPES, pH 6.8, 0.025 M KCl, and 0.005 M $MgCl_2$). The cells were then treated with extraction buffer (CSK buffer containing 0.2% Triton X-100) at room temperature for 30–40 s and rinsed sequentially with CSK buffer and PBS. The remaining "extracted" cells were fixed at room temperature for 20 min in 4% phosphate-buffered formaldehyde.

Microtubule Interaction

Preparation of extracts from B104 cells and polymerization of tubulin into microtubules were done according to Bulinsky and Borisy (1979) and Kelkar *et al*. (2004). Microtubules were stabilized by the addition of exogenous tubulin and taxol. PEM buffer (80 mM PIPES, pH 6.9, 1mM $MgCl_2$, 1 mM EGTA), guanosine triphosphate, tubulin, taxol, and glycerol cushion were purchased from Cytoskeleton (Denver, CO). The microtubule pellet was analyzed for the presence of TOG and hnRNP A2 by Western blotting.

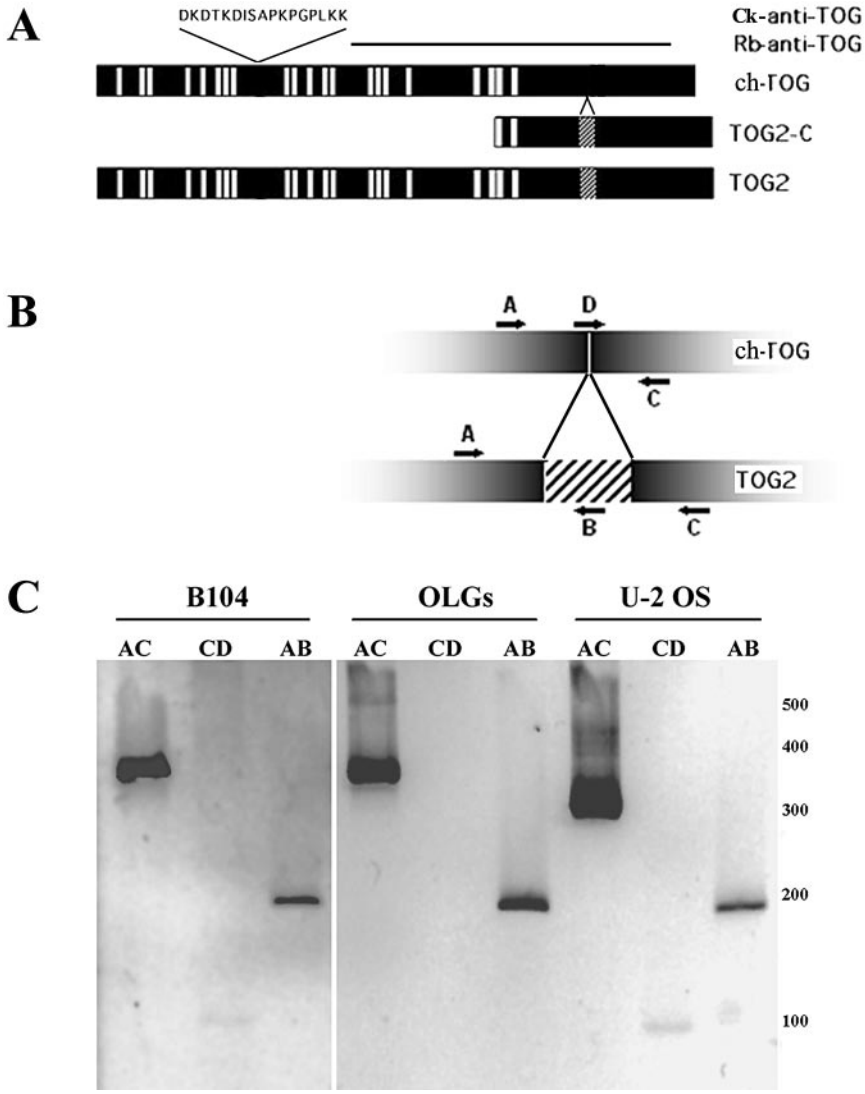

Figure 1. Identification of TOG isoforms. (A) The top black line represents the sequence (residues 829-1564. . . 1623–1957) used to raise antibodies in rabbits (Charrasse *et al*., 1998) (Rb-anti-TOG), whereas the displayed sequence to the left indicates the oligopeptide used to raise antibodies in chickens (Ck-anti-TOG) and its location (529–545). The top black bar, ch-TOG, is a map of the small isoform of TOG encoded by the TOG gene (GenBank accession no. D43948). The white boxes indicate the locations of HEAT repeats (Neuwald and Hirano, 2000; Andrade *et al*., 2001); the diagonally striped box represents the location of an exon encoding 60 residues (1564–1623) present in the large isoform of TOG, TOG2 (lowest black bar) but absent in ch-TOG. The middle black bar represents the C-terminal portion of TOG2, TOG2-C, that binds to hnRNP A2. (B) Positions and directions of primers (A–D, identified by arrows) designed to distinguish ch-TOG from TOG2 mRNA (see *Results*) based on the presence or absence of the exon (residues 1564–1623). (C) RT-PCR was performed using sets of primers (described above and in Table 1 in Supplementary Material) to identify both ch-TOG and TOG2 (AC) and to distinguish ch-TOG (CD) and TOG2 (AB) mRNAs in cells. mRNA from B104, oligodendrocytes (OLGs), and U-2 OS cells was amplified with LKTOG_A and LKTOG_C (AC), LKTOG_C and LKTOG_D (CD), or LKTOG_A and LKTOG_B (AB). The size of the bands is estimated from the pattern of a 100-base pair DNA ladder (right).

Immunofluorescence, Microinjection, and Image Acquisition and Analysis

Immunofluorescence procedures for cells grown on coverslips followed standard protocols. Mouse monoclonal antibody to hnRNP A2 (EF67) (used at 1:400 dilution) was a generous gift from Dr. William Rigby (Dartmouth Medical School, Lebanon, NH) (Nichols *et al*., 2000). A rabbit antibody produced against a fragment of TOG encompassing residues 829-1564 and 1623–1957 (Figure 1A) was a generous gift from Dr. Christian Larroque (Institut National de la Santé et de la Recherche Médicale, Montpellier, France) (Charrasse *et al*., 1998). Affinity-purified chicken anti-TOG (used at 1:50 dilution) was produced by Aves Labs (Tigard, OR) under contract by using the peptide encompassing residues 529–545 (Figure 1A). Thus, both antibodies to TOG were raised against epitopes present in both ch-TOG and TOG2. Antibody to tubulin was obtained commercially (Sigma-Aldrich). Antibody to CLIP-115 (#2221; Hoogenraad *et al*., 2000) was used at 1:50 dilution and was a generous gift from Dr. N. Galjart (Erasmus University, Rotterdam, The Netherlands). Antibody to hnRNP K (Van Seuningen *et al*., 1995) was used at 1:200 dilution and was a generous gift from Dr. K. Bomsztyk (University of Washington, Seattle, WA). Fluorescein-conjugated and Texas Red-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Microinjection of primary oligodendrocytes plated on glass bottom dishes (MatTek, Ashland, MA) was performed using an electronic microinjection system (Eppendorf, Hamburg, Germany). Alexa 488-conjugated UTP-labeled RNA was injected into the cytoplasmic compartment. The injected cells were examined by confocal laser scanning microscopy after fixation with 3.7% formaldehyde and immunostaining with antibodies to TOG.

Dual channel confocal images were collected using a Zeiss LSM 510 confocal laser scanning microscope with a 63× 1.4 numerical aperture oil immersion objective (Carl Zeiss, Thornwood, NY) by simultaneous recording in the 488λ and 568λ channels. The fluorescence intensity of individual granules was obtained using a specialized macro for the NIH Image 1.6 program.

In Vitro Transcription

Template DNA (RTS11) consisting of the coding region of green fluorescent protein (GFP) with the A2RE sequence (previously called RTS; Ainger *et al*., 1997) inserted into the 3′ untranslated region was transcribed in vitro in the presence of Alexa 488-conjugated UTP according to the manufacturer's protocol (Epicenter Technologies, Madison, WI). Transcripts were run on gels to estimate their size and concentration before microinjection.

Coimmunoprecipitation of Endogenous Proteins

The protocol is that recommended by eBioscience (http://www.bestprotocols.com/ebioscience/appls/IP.htm). The solubilized CSK pellet of B104 cells and oligodendrocytes was incubated with chicken anti-TOG antibodies or with the preimmune IgY fraction. Agarose beads conjugated with protein A that had been incubated with rabbit anti-chicken IgY antibodies were added to the mix. The agarose beads/immune complexes were spun down and washed. The antigen–antibody complexes were dissociated, separated by PAGE, and transferred to membrane for Western blot analysis by using mouse anti-hnRNP A2 antibodies.

Western Blotting

Proteins were run on 4–12% (Figures S1, in supplemental online material, and 3C), 12% (Figure 2D), or 6% (Figure 3A) polyacrylamide/Tris-glycine gels and transferred electrophoretically onto Immobilon-P membranes (Millipore). Immunodetection was done using a mouse anti-c-*myc* (4 μg/ml) (BD Biosciences

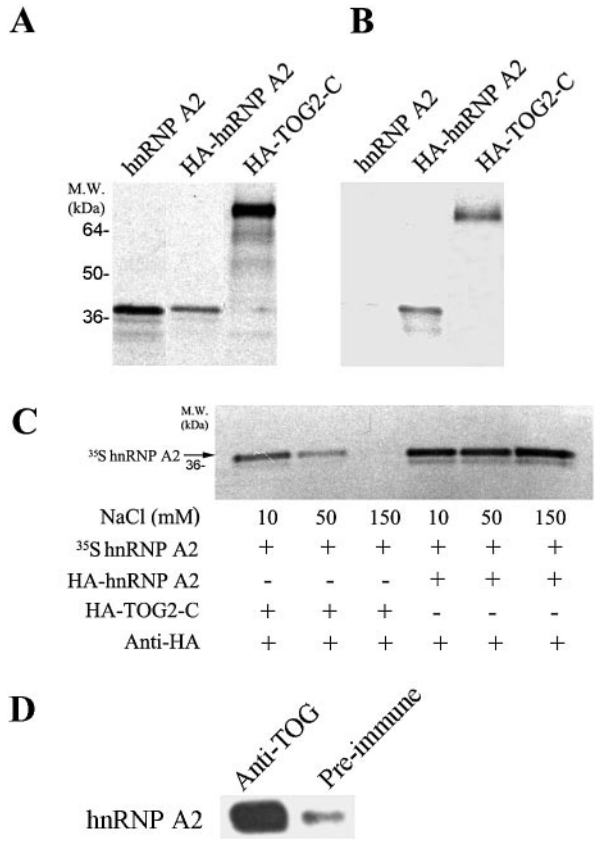

Figure 2. HnRNP A2/TOG interactions. (A) Autoradiogram of a PAGE of in vitro transcription-translation reactions: [^{35}S]methionine-labeled proteins encoded by the bait (pBD+hnRNPA2), hnRNP A2, and two pAD-cDNA positive clones, HA-hnRNP A2 and HA-TOG2-C. Molecular mass standards in kilodaltons are shown on the left. (B) Autoradiogram of the immune precipitates of the proteins described in A by using anti-HA antibodies (see *Materials and Methods*). (C) Coimmunoprecipitation of the [^{35}S]methionine-labeled hnRNP A2 described in A and cold HA-hnRNP A2 and HA-TOG2-C by using anti-HA antibodies. The reaction was carried out at various salt concentrations as indicated. (D) An equal amount of a B104 cellular extract was reacted with an anti-TOG IgY fraction or with a preimmune IgY fraction of the same concentration. Immune complexes were precipitated and analyzed as described in *Materials and Methods*. The same volume of the immune and preimmune reaction mixes was applied to the gel. The presence of hnRNP A2 in the immune complexes was revealed by Western blot by using a monoclonal anti-hnRNP A2 murine antibody (Nichols *et al*., 2000). The exposure time necessary to detect hnRNP A2 in the preimmune pellet results in a band of saturated intensity in the anti-TOG pellet.

Clontech/BD Biosciences), a mouse anti-hnRNP A2 (1:1000) (described above), a chicken or rabbit anti-TOG (1:1000) (described above), and a mouse anti-β-tubulin (1:1000) (Roche Diagnostics, Mannheim, Germany), and appropriate secondary antibodies conjugated to peroxidase. chemilumiscent substrate (Pierce Chemical, Rockford, IL) were used to detect positive reactions, and the membranes were exposed to x-ray films.

RESULTS

Identification of Proteins That Bind to hnRNP A2 in the Yeast Two-Hybrid Screen

Yeast two-hybrid analysis was performed by mating AH109/pBD-hnRNPA2 to Y187/pAD-cDNA containing the human brain cDNA library. The initial mating yielded 250 HIS3$^+$ clones, of which 50 were also ADE2$^+$. Thirty clones were also Lac Z$^+$, thus expressing all three gene reporters of interaction between the hnRNP A2 bait and proteins expressed from the cDNA library. These 30, which probably represent gene products with the highest affinity for hnRNP A2, were investigated further. Restriction endonuclease digestion of the PCR-amplified insert assigned 18 of these 30 clones to six groups. The remaining 12 clones had unique digestion patterns.

DNA sequencing identified group 1 as hnRNP A2 (5 clones) and group 2 as hnRNP B1 (2 clones). Each clone contains the complete coding sequence of the gene (NT_007819.11). HnRNP B1 and hnRNP A2 are splice isoforms of the same gene, and both proteins are expressed in oligodendrocytes (Kamma *et al*., 1999; Hatfield *et al*., 2002; Maggipinto *et al*., 2004). These results confirm those of Cartegni *et al*. (1996) showing that hnRNP A2 binds to itself and to hnRNP B1 by yeast two-hybrid methodology.

One of the unique clones, E3, contains the C-terminal third of a splice variant of ch-TOG, a protein previously identified as a MAP (X92474, gi 1045056, Charrasse *et al*., 1995) (Figure 1B). The cDNA of a variant ch-TOG has previously been identified (D43948, gi 603950, Nagase *et al*., 1995). To distinguish ch-TOG from the variant sequence identified by the yeast two-hybrid system, we will refer to the coding sequence of the splice variant as TOG2 and to the clone that binds to hnRNP A2 as TOG2-C (Figure 1A).

Yeast two-hybrid systems are notorious for identifying false positives (Hong, 2000; Serebriiskii *et al*., 2000). To distinguish between proteins that bind specifically and those that bind nonspecifically to hnRNP A2, a cotransformation assay was performed (see *Materials and Methods*). Clone E3 (TOG2-C) seems to be a genuine positive because cotransformants express the reporter genes with the bait better than with the vector or the false baits (Table 3 in Supplementary Material).

The protein products of several of the positive clones were synthesized in vitro and labeled with [^{35}S]methionine. Two are shown in Figure 2A: the protein synthesized from one of the hnRNP A2 clones (A5), and the protein, TOG2-C, from the unique clone E3. These proteins contain an HA epitope, and their size corresponds to that expected for the fusion proteins, HA-human hnRNP A2 and HA-TOG2-C. The bait protein, mouse hnRNP A2, containing a c-*myc* epitope is also shown.

The presence of TOG cDNA in a library derived from human brain is consistent with the reported expression of the ch-TOG gene in various areas of the human brain where TOG expression seemed ubiquitous (Charrasse *et al*., 1996). We undertook to characterize TOG expression at the cellular level in differentiated oligodendrocytes and neurons, as well as in B104 neuroblastoma cells in culture.

TOG2 Expression in Oligodendrocytes in Culture

The yeast two-hybrid screen identified only one TOG clone from a human brain library. To identify the isoform(s) of TOG expressed in different neural cells, RT-PCR was used to amplify mRNA from oligodendrocytes, neurons, B104 neuroblastoma cells, and U-2 OS, a human osteosarcoma cell line, with primers specific for ch-TOG (LKTOG_D), TOG2 (LKTOG_B), or both (LKTOG_A, LKTOG_C) (see Table 1 in Supplementary Material for the primers sequence, and Figure 1B for their position).

RT-PCR with primers LKTOG_A and LKTOG_C yields a 194-base pair product from ch-TOG mRNA and a 374-base pair product from TOG2 mRNA. Primers LKTOG_C and LKTOG_D yield a 102-base pair product only with ch-TOG

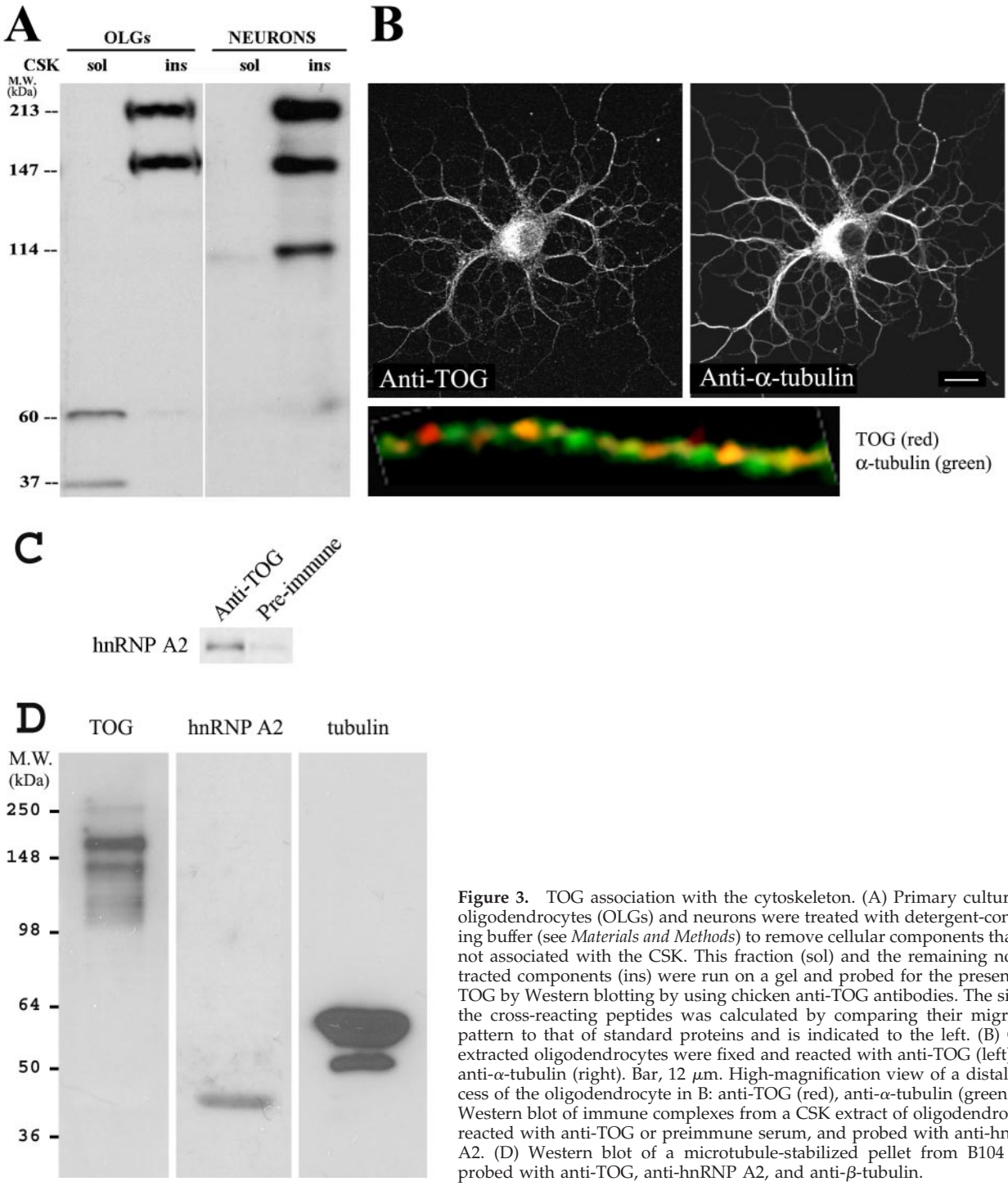

Figure 3. TOG association with the cytoskeleton. (A) Primary cultures of oligodendrocytes (OLGs) and neurons were treated with detergent-containing buffer (see *Materials and Methods*) to remove cellular components that are not associated with the CSK. This fraction (sol) and the remaining nonextracted components (ins) were run on a gel and probed for the presence of TOG by Western blotting by using chicken anti-TOG antibodies. The size of the cross-reacting peptides was calculated by comparing their migration pattern to that of standard proteins and is indicated to the left. (B) CSK-extracted oligodendrocytes were fixed and reacted with anti-TOG (left) and anti-α-tubulin (right). Bar, 12 μm. High-magnification view of a distal process of the oligodendrocyte in B: anti-TOG (red), anti-α-tubulin (green). (C) Western blot of immune complexes from a CSK extract of oligodendrocytes reacted with anti-TOG or preimmune serum, and probed with anti-hnRNP A2. (D) Western blot of a microtubule-stabilized pellet from B104 cells probed with anti-TOG, anti-hnRNP A2, and anti-β-tubulin.

mRNA, whereas primers LKTOG_A and LKTOG_B yield a 182-base pair product only from TOG2 mRNA. Because the sequence at the splice junction that distinguishes ch-TOG from TOG2 is different in human and rat genes, two different versions of primer D were used for human cells (U-2 OS) and rat cells (oligodendrocytes, neurons, B104).

When total cell RNA from oligodendrocytes and neurons was amplified, semiquantitatively, with primers LKTOG_A and LKTOG_C, the only visible product was a band of ~190 base pairs. This indicates that TOG2 mRNA is expressed in mouse neural cells but ch-TOG mRNA is not expressed (<100 molecules/cell) (our unpublished data).

To ensure that the experiment was not missing low levels of ch-TOG mRNA, RT-PCR was repeated on oligodendrocyte mRNA, B104 mRNA, and U-2 OS mRNA under conditions to maximize yield with three sets of primers as described above. Figure 1C shows the results of this experiment. All three mRNA preparations produce bands indicating the presence of TOG2 mRNA, but a 102-base pair band, with primers LKTOG_C and LKTOG_D, is present using B104 and U-2 OS mRNA as tem-

plates, but not when using oligodendrocyte mRNA as template, supporting the conclusion that oligodendrocytes express only TOG2 mRNA and not ch-TOG mRNA.

Coimmunoprecipitation of TOG2-C, TOG2, and hnRNP A2

If the bait hnRNP A2 binds to hnRNP A2 and TOC2-C expressed from the cDNA library, it will coimmunoprecipitate with antibodies specific for the HA-epitope present in these proteins expressed in vitro. Controls of [^{35}S]methionine-labeled hnRNP A2, HA-hnRNP A2, and HA-TOG2-C indicate that the HA/fusion proteins are immunoprecipitated with anti-HA but that hnRNP A2 alone is not (Figure 2B). To test the coimmunoprecipitation of hnRNP A2 with itself and with TOG2-C, ^{35}S-labeled hnRNP A2 was combined with unlabeled HA-hnRNP A2 and HA-TOG2-C and immunoprecipitated with anti-HA antibodies. Figure 2C shows coimmunoprecipitation at three salt concentrations. Binding of hnRNP A2 to itself (^{35}S-hnRNP A2 and cold HA-hnRNP A2) is relatively salt insensitive, but binding of hnRNP A2 to TOG2-C (^{35}S-hnRNP A2 and cold HA-TOG2-C) is detected at 10 mM NaCl, decreased at 50 mM NaCl, and eliminated at 150 mM NaCl. These results indicate that hnRNP A2 binds to TOG2-C but that binding is sensitive to ionic strength under the conditions of the assay.

Coimmunoprecipitation also was used to determine whether endogenous full-length TOG2 interacts with hnRNP A2 in vivo in neural cells. An extract of B104 neuroblastoma cells containing endogenous TOG2 and hnRNP A2 was mixed with chicken anti-TOG antibodies in buffer containing 150 mM NaCl, and the immune complex was precipitated. In addition to TOG2, the immune complex contains hnRNP A2 as revealed by Western blot analysis (Figure 2D). HnRNP A2 is 7 times more abundant in the precipitate obtained with anti-TOG than in the precipitate obtained with the preimmune IgY fraction as seen after overexposure of the blot. The specificity of coprecipitation of hnRNP A2 and TOG2 under these conditions suggests that TOG2 interacts with hnRNP A2 in neural cells. Although binding of recombinant C terminal fragment of TOG2 (TOG2-C) to hnRNP A2 in vitro is sensitive to ionic strength, binding of endogenous full length TOG2 to hnRNP A2 in vivo is not. Possible explanations for this discrepancy are considered in *Discussion*.

Subcellular Distribution of TOG2 in Oligodendrocytes and Neurons

TOG has been identified as a microtubule binding protein (Spittle *et al*., 2000). The association of TOG2 with the CSK in neural cells was investigated by cell fractionation. Western blot analysis using antibodies to two different epitopes of TOG (see *Materials and Methods* and Figure 1A) reveals the presence of a polypeptide of ~213 kDa (full-length TOG2 protein predicted molecular mass ~225 kDa) in the CSK-insoluble fraction of oligodendrocytes and neurons (Figure 3A) along with a smaller polypeptide of ~147 kDa. An additional polypeptide of ~114 kDa is detected in the CSK-insoluble fraction of neurons. These two lower molecular mass peptides are likely degradation products of TOG, because in the absence of protease inhibitors, these bands become more prominent, and the intensity of the 213-kDa band is correspondingly decreased. A similar electrophoretic pattern of ch-TOG and its putative degradation products was reported by the group of Larroque (Charrasse *et al*., 1998). Full-length TOG2 is not detected in the CSK-soluble fraction of either cell type, but small amounts of low molecular mass peptides (114, 60, and 37 kDa) are present.

The subcellular localization of TOG in oligodendrocytes was analyzed by immunocytochemistry. Oligodendrocytes were treated with a CSK extraction buffer to remove components not associated with the CSK, and immunostained with anti-TOG (left, red) and anti-α-tubulin (right, green). The most intense staining for TOG is in the form of puncta in the cell body, as well as in proximity to microtubules in processes emanating from oligodendrocytes (Figure 3B). A high-magnification view of a distal portion of one of the processes shows that some TOG puncta colocalize with α-tubulin staining as seen by their yellow coloration. TOG is similarly distributed in postmitotic neurons in culture (our unpublished data).

The distribution of TOG was compared with that of CLIP-115, a protein reported to be preferentially located at the plus end of microtubules (Hoogenraad *et al*., 2000). Oligodendrocytes were stained with anti-TOG and anti-CLIP-115, and dual channel images were recorded by fluorescence confocal microscopy. The fluorescence intensity of individual puncta in each channel (number of puncta analyzed [n] = 3647) (Figure 4, A–C) was measured. A small number of puncta contained only CLIP (2%), or only TOG (18%), but most puncta contained both CLIP and TOG (80%). Colocalization is seen as yellow (red superimposed on green) puncta in a magnified portion of a process (Figure 4B, inset). The correlation coefficient (r) between channels was 0.4 in the overall population of puncta, indicating that not only are TOG and CLIP 115 colocalized in a large proportion of puncta but also that the proportions of TOG and CLIP 115 in different puncta are comparable. In other words, puncta that contain large amounts of TOG contain large amounts of CLIP 115, whereas puncta that contain small amounts of TOG contain small amounts of CLIP 115. Covariation of TOG and CLIP115 suggests that TOG is colocalized with CLIP115 at plus ends of microtubules.

To determine where TOG interacts with hnRNP A2, we treated oligodendrocytes to obtain a CSK-insoluble fraction that was used for immunoprecipitation. The fraction was incubated with anti-TOG antibodies, and the immune complexes were isolated and analyzed by Western blotting. Figure 3C shows that the TOG immune pellet contains hnRNP A2. When the CSK fraction was incubated with preimmune serum, little hnRNP A2 was detected in the pellet, indicating the specificity of TOG-hnRNP A2 interaction. A microtubule-stabilized pellet obtained from B104 cells also contains TOG and hnRNP A2 (36 kDa), as seen in Figure 3D, indicating that the interaction of these two proteins may take place on or in proximity to microtubules in neural cells in vivo.

Association of TOG2 with hnRNP A2/A2RE-RNA–containing Granules

In the cytoplasm, hnRNP A2 is associated with RNA transport granules containing A2RE-mRNAs (Ainger *et al*., 1997). Coimmunostaining of cultured oligodendrocytes with antibodies to TOG and to hnRNP A2 shows that TOG has a distribution in the cytoplasm similar to that of hnRNP A2 (Figure 4, D–F). Magnification of a cellular process reveals distinct granules that look yellow, indicating that they contain both TOG (green) and hnRNP A2 (red) (Figure 4E, inset). The area shown is representative of the areas selected for quantitative analysis (Figure 4F). Very little TOG is detected in the nucleus, especially compared with the amount of hnRNP A2 present there. Quantitative analysis of the images acquired by dual channel recording in fluorescence confocal microscopy reveals that TOG and hnRNP A2 are colocalized in 90% of the granules found in the medial and

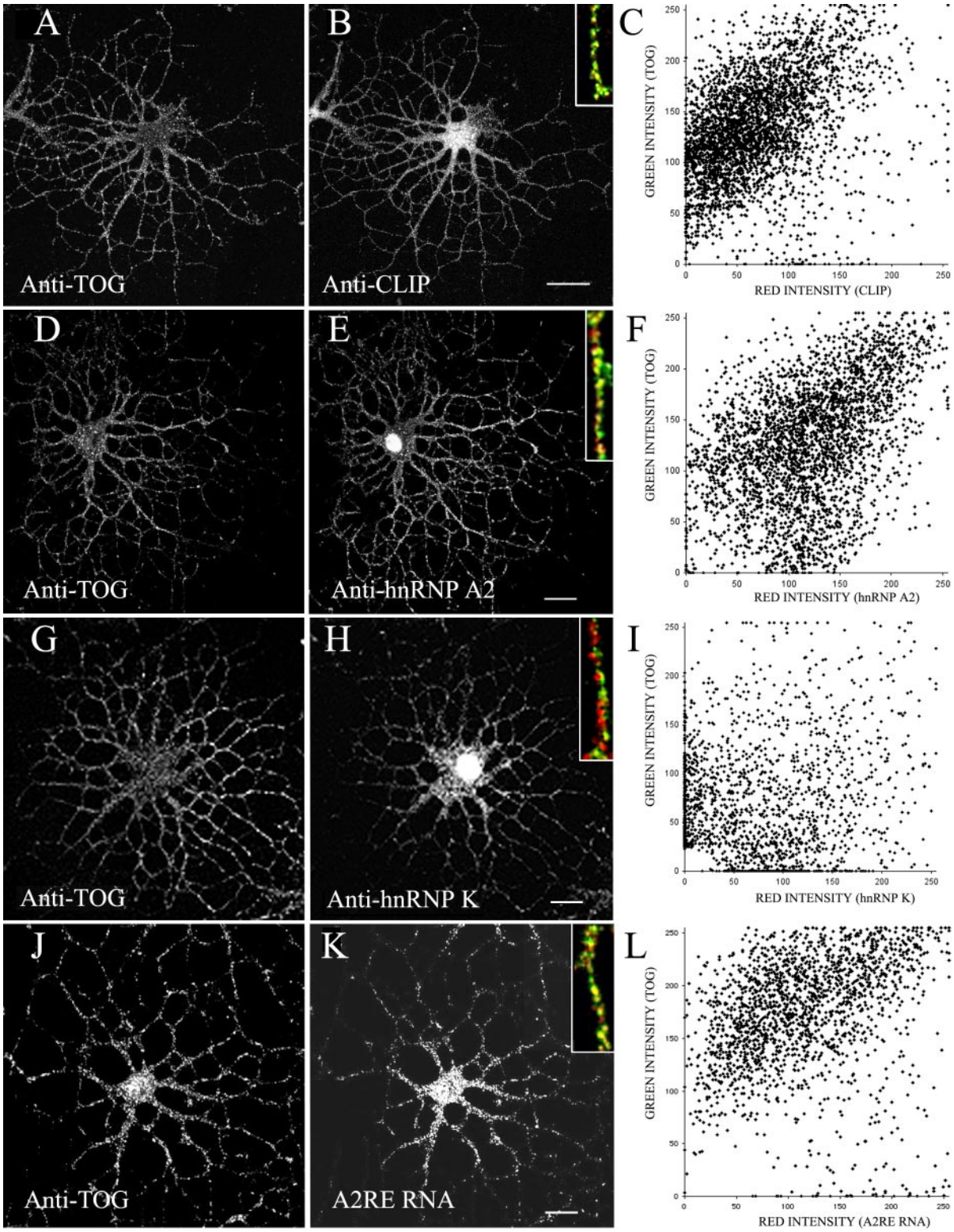

Figure 4. TOG association with subcellular components. Representative images of oligodendrocytes that were fixed, permeabilized, and immunostained for TOG (A, D, G, and J), CLIP 115 (B), hnRNP A2 (E), and hnRNP K (H) or injected with fluorescent UTP-labeled A2RE RNA (K). Green channel: A, D, G, and J and pairwise red channel: B, E, H, and K. TOG/CLIP (A–C), TOG/hnRNP A2 (D–F), TOG/hnRNP K (G–I), and TOG/A2RE RNA (J–L). Insets in B, E, H, and K: enlargements of a portion of cellular processes visualized as merged channels (A and B, D and E, G and H, and J and K, respectively). Yellow indicates spatial coincidence in the green and red channels. Bars, 20 μm (A and B), 15 μm (D and E), 12 μm (G and H), and 10 μm (J and K). Single granule ratiometric analysis (C, F, I, and L) was performed on cells treated as described above by integrating the intensities in the green channel (A, D, G, and J) and red channel (B, E, H, and K), respectively, for each granule. Only well resolved granules located in the medial and distal portions of the cellular processes were analyzed. Each dot represents the coordinates of one granule. Number (n) of granules analyzed: n = 3647 (C), n = 3217 (F), n = 1854 (I), and n = 1897 (L).

distal portions of the processes (number of granules analyzed [n] = 3217) (Figure 4F). The r value between channels is 0.5 for the overall population of granules, indicating that most of the granules contain comparable proportions of TOG and hnRNP A2. Covariation of TOG and hnRNP A2 suggests that the two proteins are present in defined stoichiometry in RNA transport granules.

In a control experiment, colocalization of TOG with another RNA binding protein, hnRNP K, which has not been implicated in RNA trafficking, was analyzed (Figure 4, G–I). Like hnRNP A2, hnRNP K is found in the nucleus and in the cytoplasm in the form of granules. However, hnRNP K granules are more abundant in the proximal portion of the processes and are not transported as far as hnRNP A2 in the distal cell processes. Colocalization analysis reveals that 50% of the granules present in the processes (n = 1854) contain both hnRNP K and TOG and that 30% contain only hnRNP K (Figure 4I); the latter population show as red granules in the magnified view of a process (Figure 4H, inset). The r value between channels for the overall granule population is 0.1, indicating that the majority of the granules do not have a defined TOG to hnRNP K ratio and suggesting that TOG and hnRNP K are not part of the same structure.

Fluorescently labeled A2RE-mRNA injected in oligodendrocytes also occurs in granular structures (Figure 4K). Most of the A2RE-mRNA–positive granules (94%) in the cellular processes coincide with endogenous TOG-containing puncta (n = 1897) (Figure 4L). This is seen in the inset of Figure 4K where colocalized TOG puncta (green) and A2RE granules (red) are yellow. The r value for the overall population is 0.3, indicating that a large proportion of granules contain TOG and A2RE-mRNA in comparable proportion. Together with the hnRNP A2 colocalization data, these results suggest that TOG is part of the RNA transport granules.

DISCUSSION

A variety of proteins have been identified that are isolated in complexes with hnRNP A2 (Kamma *et al*., 1999). These include other hnRNP proteins as well as proteins that can posttranslationally modify hnRNP A2 such as casein kinase 2 (Pancetti *et al*., 1999). Because hnRNP A2 shuttles in and out of the nucleus and may perform multiple functions in the cell, it is likely to interact with several partners. Here, we show that hnRNP A2 binds to itself, and to TOG2, the larger isoform of the alternatively spliced TOG gene. The binding of hnRNP A2 to itself reproduces a previous finding (Cartegni *et al*., 1996). Further studies will be necessary to elucidate the subcellular site of hnRNP A2 dimer formation and its significance with regard to granule assembly and RNA trafficking. The smaller isoform of the TOG gene, ch-TOG, previously identified by Charrasse *et al*. (1995, 1998) in human hepatomas and colonic tumors, was not detected in our screen. This could mean that ch-TOG does not bind to hnRNP A2, or it may mean that it is not expressed, or expressed at very low levels, in the normal human adult brain, the source of our cDNA library. Our finding that TOG2 is the main or only splice variant detected in differentiated oligodendrocytes in culture favors the latter explanation.

The molecular mechanism by which TOG interacts with hnRNP A2 is unknown. It is possible that TOG HEAT motifs are involved in its binding to hnRNP A2. TOG contains 20 HEAT repeats, one of which is present in the TOG fragment that interacts with hnRNP A2 in the yeast two-hybrid screen (Neuwald and Hirano, 2000). HEAT repeats in other proteins have been shown to mediate protein–protein interactions (Neuwald and Hirano, 2000; Andrade *et al*., 2001). HnRNP A2 is known to interact with another HEAT repeat-containing protein, transportin, during import into the nucleus (Siomi *et al*., 1997). This interaction is mediated by a C-terminal nuclear localization sequence of hnRNP A2, called the M9 domain (Izaurralde *et al*., 1997). HnRNP A2 also contains a glycine-rich domain that has been implicated in protein–protein interactions (Mayeda *et al*., 1994). Our yeast two-hybrid screen indicates that the C-terminal portion of TOG is sufficient for binding to hnRNP A2. The C-terminal portion of TOG contains two predicted phosphorylation target sites, one by MARK and one by CDK1, which could modulate these protein–protein interactions (Becker and Gard, 2000).

The C-terminal portion of TOG2 (TOG2-C) translated in vitro binds to hnRNP A2 at low ionic strength but not at 150 mM NaCl, whereas endogenous full-length TOG2 in cell extract does bind hnRNP A2 in 150 mM NaCl. One possible explanation for this discrepancy is that full-length TOG2 contains multiple HEAT motifs, whereas TOG2-C only contains one. The additional HEAT repeats in full-length TOG2 may provide more binding sites for hnRNP A2 that allow for interaction at high salt concentrations. A second possible explanation is that posttranslational modification(s) of TOG2 occur in vivo but not in recombinant TOG2-C in vitro. Such posttranslational modification(s) may enhance binding of TOG2 to hnRNP A2. A third possible explanation is that cell extract contains other proteins that associate with TOG2 and/or hnRNPA2 to enhance binding. Such proteins may be present in cells in vivo but not in vitro. These different explanations are currently being investigated.

The site of TOG2 interaction with hnRNP A2 in oligodendrocytes is primarily in the cytoplasm, on or in proximity to microtubules. There is little TOG2 immunoreactivity in the nucleus (Figures 3 and 4; Charrasse *et al*., 1998) but coincident reactivity in puncta decorating the microtubules in the cellular processes. Both hnRNP A2 and TOG2 also fractionate with the CSK and are present in the microtubule-stabilized pellet of neural cells. Thus, the subcellular distribution of TOG2 reported here is consistent with previous studies that have characterized TOG as an MAP. Although association of TOG and its *Xenopus* homologue XMAP215 with mitotic spindles has been emphasized, Charrasse *et al*. (1998) have reported that immunostaining for TOG showed a punctate pattern in the perinuclear cytoplasm of some mammalian cell lines.

Our data suggest that TOG2 puncta are A2RE-mRNA granules. However, the mode of association of TOG with these granules is unknown. TOG may be present in clusters at the microtubule plus ends where moving A2RE-mRNA granules are captured when hnRNP A2 interacts with TOG. Alternatively, TOG may be an intrinsic component of A2RE-mRNA granules that move along microtubules and get captured at the plus end of microtubules. The latter model is supported by the finding that an orthologue of TOG, *alp14*, has been visualized as particles moving along microtubules in fission yeast (Ohkura *et al*., 2001) and that other microtubule-associated proteins such as MAP 1A in PC12 cells (DeFranco *et al*., 1998), and the fibronectin ARE-dependent RNA binding protein, which is itself an MAP (MAP 1 light chain 3) (Zhou *et al*., 1997), have been shown to be part of RNA or ribonucleoprotein complexes.

In many systems, association of mRNA with the CSK is thought to be necessary for targeted protein synthesis (Hesketh, 1994; Condeelis, 1995; Han *et al*., 1995; Antic and Keene, 1998). The A2RE-containing mRNA for myelin basic

protein is associated with microtubules (Boccaccio *et al*., 1999) and translated in the myelin compartment (Barbarese *et al*., 1999; Gould *et al*., 1999). This suggests that hnRNP A2-TOG–microtubule interactions may be necessary for capture and immobilization of A2RE-mRNA granules on microtubules and translation of the transported A2RE-mRNAs.

TOG also may affect the direction of RNA trafficking. A2RE-mRNA granules are transported toward the plus ends of microtubules in the distal dendrites in oligodendrocytes and neurons, whereas non-A2RE-mRNA granules are retained near the minus ends of microtubules in the perikaryon. Both A2RE and nonA2RE-mRNA granules contain both plus end (conventional kinesin) and minus end (cytoplasmic dynein) motors. Therefore, the different directionality of movement along microtubules for A2RE- and non-A2RE-mRNA granules must reflect differences in the activity of the plus end and minus end motors in individual granules. This implies that the presence of the A2RE in the sequence of the RNA cargo of each granule somehow regulates the balance of power between the motors associated with that granule in favor of kinesin (Carson *et al*., 2001). Because the A2RE sequence is recognized by hnRNP A2, it is possible that regulation of the balance of power between motor activities in A2RE RNA granules is mediated by hnRNP A2. In other systems, TOG orthologues interact with members of the kinesin family to regulate kinesin activity (Ohkura *et al*., 2001). Therefore, it is possible that in A2RE RNA granules, the binding of hnRNP A2 to TOG alters the balance of power between kinesin and dynein in favor of kinesin to facilitate granule transport to the distal dendrites.

In summary, we have shown that TOG2 protein, which has been previously characterized as a mitotic spindle protein (XMAP215, ch-TOG), binds to hnRNP A2 and is associated with A2RE-mRNA granules in neural cells, suggesting a new function for TOG protein in regulating the A2RE trafficking pathway.

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