Protein−Protein Interactions between the Testis Brain RNA-Binding Protein and the Transitional Endoplasmic Reticulum ATPase, a Cytoskeletal γ Actin and Trax in Male Germ Cells and the Brain†

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ABSTRACT: Numerous functions have been proposed for the testis brain RNA-binding protein (TB-RBP) and its human homologue, Translin, ranging from mRNA transport and translational regulation to DNA rearrangement and repair. To gain insight into the likely functions of this 26 kDa protein, immunoprecipitation was used to identify proteins that interact with TB-RBP in mouse cytosolic extracts. Three proteins, the transitional endoplasmic reticulum ATPase, a cytoskeletal γ actin, and Trax, were specifically immunoprecipitated with an affinity-purified antibody to recombinant mouse TB-RBP. In vitro binding assays with recombinant proteins and EM immunocytochemistry confirm that TB-RBP interacts with the TER ATPase in vitro and in vivo. Confocal microscopy has demonstrated that TB-RBP colocalizes with actin in the cytoplasm of male germ cells. The immunoprecipitation of Trax with TB-RBP confirms a published report demonstrating protein interactions between the two proteins in a yeast two-hybrid assay. These data support the hypothesis that TB-RBP serves as a link in attaching specific mRNAs to cytoskeletal structures and suggests an involvement for the ubiquitously expressed TER ATPase in intracellular and/or intercellular mRNA transport.

The localization and movement of mRNAs in cells is a complex process requiring protein recognition of specific mRNA sequences or secondary structures and interactions with additional protein complexes that drive the energy-dependent process of mRNA movement along cytoskeletal structures. Members of the kinesin and/or dynein protein families are often proposed to function as motor proteins which mediate mRNA movement in cellular compartments such as in the dendritic processes of neural cells. A growing number of cytoskeletal-bound RNA-binding proteins have been implicated in mRNA movement and localization (reviewed in refs *1*−*3*).

The DNA- and RNA-binding protein, testis brain RNA-binding protein (TB-RBP), has been shown to bind to specific conserved sequences in stored testicular and transported brain mRNAs (*4*−*7*). In vitro reconstitution studies have demonstrated that TB-RBP can serve as a linker protein to attach specific mRNAs containing the conserved Y and H sequences to microtubules (*6*). In the testis as germ cells differentiate, TB-RBP is detected in changing cellular locations. In meiotic pachytene spermatocytes, TB-RBP is primarily present in nuclei, whereas in the post-meiotic round spermatids, it is primarily a cytoplasmic protein (*8*). The abundance of TB-RBP in the cytoplasmic bridges that connect germ cells in a syncytium suggests that TB-RBP could facilitate the distribution of equal amounts of mRNA in haploid male germ cells. Morphological studies reveal large numbers of actin filaments in and near the intercellular bridges of male germ cells (*9*). When actin polymerization is inhibited by cytochalasin D, the intercellular bridge structures between cells are lost (*10*).

TB-RBP is the mouse homologue of Translin, a human protein identified as a single-stranded DNA-binding protein that recognizes DNA at recombination hot spots associated with chromosomal translocations (*11*−*13*). In the brain, Translin has been identified in two different nucleic acid−protein complexes (*14*−*16*). One Translin complex, containing sequence-specific single-stranded DNA and the Translin-like protein Trax (*17*), has been purified from rat cerebellum extracts, suggesting a nuclear role for Translin (*14*). The nuclear localization of TB-RBP in many but not all neurons of mouse brain has also been established by immunocytochemical staining (*15*). A cytoplasmic localization for Translin in brain ribonucleoprotein particles containing BC1 RNA and a 37 kDa protein has been reported (*16*). The BC1 RNA contains the Y and H element sequences originally identified as TB-RBP binding sites in the translationally repressed testicular protamine mRNAs (*5*). Cytoplasmic staining extending into dendrites was also seen in pyramidal neurons of the cortex and magnocellular neurons of the

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hypothalamus (*15*). These data demonstrate that TB-RBP−Translin forms complexes with either DNA or RNA in association with additional proteins.

Numerous studies have reported the microtubule-mediated movement of mRNAs (reviewed in refs *1*−*3*). In neurons, microtubules are proposed to facilitate cellular targeting of mRNAs encoding proteins such as MAP 2 and α CAMK II and to limit the movement of tau mRNAs to the axon hillock (*18*). In hippocampal neurons, TB-RBP and kinesin motors act as trans acting proteins that are essential for the cellular distribution of dendritically localized mRNAs, but not for somata-restricted mRNAs (*19*). Recently, the mRNA encoding a *Xenopus* growth factor, Vg1, has been demonstrated to bind a protein named Vera or VG1 RBP, which mediates interactions between the 3′ untranslated region of VG1 mRNA and a subcompartment of the endoplasmic reticulum (*20*, *21*). A homologous protein in chicken somatic cells transports β actin mRNAs in association with microfilaments (*22*). Growing evidence suggests that individual RNA-binding proteins can modulate mRNA localization in different cell types and species in association with different cytoskeletal requirements. In most cases, mRNAs are transported as large complexes, indicative of multiple protein components.

To begin to define the proteins that specifically interact with TB-RBP in the testis and brain, mouse cytosolic extracts were immunoprecipitated with an affinity-purified antibody to recombinant mouse TB-RBP. Three proteins, the transitional endoplasmic reticulum ATPase, cytoskeletal γ actin, and Trax, specifically coprecipitated with TB-RBP. The TER ATPase is a principal ATPase involved in the fusion of intracellular transport vesicles, including endoplasmic reticulum and Golgi (*23*−*25*).

EXPERIMENTAL PROCEDURES

Preparation of Cytosolic Extracts. Cytosolic extracts from mouse testes and brain were prepared with minor modifications from sexually mature male CD-1 mice as previously described (*5*, *6*). Briefly, decapsulated testes and brain were homogenized in hypotonic buffer A [10 mM Hepes (pH 7.6), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT, containing 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 10 μg/mL aprotinin]. The homogenates were centrifuged for 15 min at 10000*g* and decanted, and the supernatants were recentrifuged at 100000*g* for 30 min. KCl and glycerol were added to the supernatants to final concentrations of 50 mM and 10%, respectively, and the extracts were stored at −80 °C.

Expression of Recombinant TB-RBP and GST−TER ATPase. Recombinant TB-RBP was expressed in *Escherichia coli* and purified as previously described (*13*). To express the GST−TER ATPase fusion protein, a cDNA encoding the murine VCPmyc cDNA (VCP and TER ATPase are the same protein) was cloned into the *Bam*HI−*Sma*I sites of the expression vector pGEX-3X (*25*). The GST−TER ATPase was expressed in *E. coli* DH5α cells and purified as reported previously (*25*).

Antibody Preparation. The antibody against recombinant mouse TB-RBP (α TB-RBP) is an affinity-purified polyclonal rabbit antibody (*13*). The polyclonal antibody, α KNDS, was produced by using a TB-RBP peptide (KNDSLRKRYDGLKYDV) as an immunogen (*13*). α KNDS can detect TB-RBP in Western blots, but does not immunoprecipitate TB-RBP (*13*). An antibody to TER ATPase (821) was kindly provided by R. Trible (National Institutes of Health, Bethesda, MD). A monoclonal anti-actin antibody, C4, was generously provided by J. Lessard (Children's Hospital, Cincinnati, OH). Biotinylated goat anti-mouse Ig antibody, rhodamine-conjugated streptavidin, and FITC-conjugated goat anti-rabbit Ig antibody were kindly provided by P. Bannerman (CHOP, University of Pennsylvania). Protein A linked with horseradish peroxidase was purchased from Amersham. Goat anti-mouse Ig linked with horseradish peroxidase was purchased from Boehringer Mannheim.

Western Blotting. The proteins from testicular and brain extracts (30 μg), prepared as described above, or immunoprecipitated from the extracts, were resolved on a 10% SDS−polyacrylamide gel and transferred onto nitrocellulose membranes. The membranes were incubated with Tris-buffered saline (TBS) containing 5% nonfat milk overnight at 4 °C, and then incubated with primary antibody (1:1000 dilution for TER ATPase antibody 821 or actin antibody C4) in TBS containing 0.25% nonfat milk for 1 h at room temperature. The membranes were washed with TBS three times and then incubated with horseradish peroxidase-conjugated protein A (1:5000 dilution for antibody 821) or anti-mouse Ig (1:3000 dilution for C4) for 1 h at room temperature. After being washed three times with TBS, the proteins were detected with the ECL protocol of Amersham.

Identification and Characterization of TB-RBP-Associated Proteins by Immunoprecipitation. Cytosolic extracts (8 mg), prepared from mouse testes, were preincubated with protein A agarose beads (100 μL) for 2 h at 4 °C, followed by centrifugation at 5000 rpm for 2 min. The extracts were then incubated in 1 mL of TBS containing 0.1% NP-40 and 60 μg of affinity-purified antibody to recombinant TB-RBP (α TB-RBP) or α KNDS (which cannot precipitate TB-RBP) (*13*) overnight at 4 °C. After centrifugation for 2 min at 2000 rpm, the beads were collected and washed four times with 1 mL of TBS containing 0.1% NP-40. The beads were resuspended in SDS loading buffer (40 μL) and boiled for 3 min. Proteins released from the beads were resolved on a 15% SDS−polyacrylamide gel and visualized by Coomassie staining. Protein bands, present in the immunoprecipitate but absent in controls, were cut from the gel, and protein identities were determined by peptide sequencing in the Department of Microchemistry of Harvard University (Cambridge, MA) or the Protein Chemistry Laboratory of the Worcester Foundation for Biomedical Research (Worcester, MA).

For analytical immunoprecipitations, preincubated extracts (1 mg) were incubated with 2 μg of antibodies and 10 μL of protein A agarose beads for 4 h at 4 °C. The beads were washed four times with 1 mL of TBS containing 0.1% NP-40 and boiled in SDS loading buffer (20 μL) for 3 min. Immunoprecipitated proteins were resolved on a 10% SDS−polyacrylamide gel and detected by Western blotting.

In Vivo Radiolabeling of Mouse Testicular Cells and Controls for Immunoprecipitation Specificity. Germ cells were prepared from mouse testes as described previously (*5*). The testes from an adult CD-1 mouse were decapsulated and incubated with 4 mL of PRMI 1600 medium containing 2.5 mg of collagenase for 12 min at 34 °C. The medium

was removed, and the cells were washed once with 4 mL of PRMI 1600 containing 2.5 mg of trypsin and 10 μg of DNase I for 20 min at 34 °C. Tubules released from the testes were pipetted to disperse. Trypsin inhibitor (3 mg) and DNase I (10 μg) were added. Cells were collected by centrifugation at 2000 rpm for 2 min and washed twice with PRMI medium lacking methionine. The cells were resuspended in 4 mL of PRMI medium lacking methionine and cultured in two 30 mm dishes at 34 °C with 5% CO_2 for 2 h. The cells were incubated with [^{35}S]methionine (200 μCi) for 4 h, collected by centrifugation as described above, and washed twice with 1 mL of PBS. The cells were homogenized in 200 μL of buffer A containing proteinase inhibitors, and the cytosolic extract was prepared as described above.

To determine the specificity of the immunoprecipitates, recombinant TB-RBP was used to block the antibody to TB-RBP. α TB-RBP (3 μg) was incubated with 5 μL of protein A agarose beads and 16 μg of BSA or 16 μg of recombinant TB-RBP for 1 h in 200 μL of TBS for 1 h at 4 °C. The antibody was mixed with the cytosolic extract (1 mg) prepared from the [^{35}S]methionine-labeled mouse germ cells in 500 μL of TBS and incubated for 4 h at 4 °C. The beads were collected by centrifugation and washed as described above. Proteins in the pellet were resolved in 15% SDS gels and visualized by autoradiography.

In Vitro Interactions between TB-RBP and the TER ATPase. Recombinant TB-RBP (2 μg) was incubated with 30 μg of GST−TER ATPase, BSA, or GST for 60 min at room temperature in 40 μL of buffer A [10 mM Hepes (pH 7.6), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT]. The proteins were then mixed with 5 μL of BSA-saturated glutathione−Sepharose 4B beads and 200 μL of TBS containing 0.1% NP-40. After being gently rocked for 60 min at room temperature, the beads were collected by centrifugation at 2000 rpm for 2 min. The pellets were washed four times with 200 μL of TBS containing 0.1% NP-40. The pellets were boiled in SDS loading buffer for 3 min, and the released proteins were resolved on a 10% SDS−polyacrylamide gel. TB-RBP was detected by Western blotting (*13*).

Preparation of the TER Membrane Fraction. The TER membrane fraction was prepared from mouse testicular cytosolic extracts using the published protocol of Zhang et al. (*24*) with minor modification. Briefly, decapsulated testes were homogenized in 2 volumes of buffer B [37.5 mM Tris-maleate (pH 6.5), 0.5 M sucrose, 5 mM $MgCl_2$, and 1% dextran]. After centrifugation at 12000*g* for 15 min, the supernatants were layered onto discontinuous sucrose gradients containing 2.0, 1.5, and 1.3 M sucrose (sucrose dissolved in buffer B). After centrifugation at 85000*g* for 90 min, the membranes overlaying the 1.3 M sucrose were collected and pelleted by centrifugation at 70000*g* for 20 min. The supernatants were saved as the postmembrane cytosolic fractions. The pellets were washed twice with buffer B and resuspended in SDS loading buffer. The TER ATPase was detected by Western blotting using TER ATPase antibody 821.

Colocalization of TB-RBP and the TER ATPase by EM Immunocytochemistry. Adult male CD-1 mice were anesthetized with sodium pentobarbital, and the testes were fixed by perfusion with 0.5% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer containing 15 mM lysine at pH 7.4. The testicular tissues were trimmed into small blocks, kept in the same fixative for 2 h, washed several times with phosphate-buffered saline PBS [137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 6.5 mM $NaHPO_4$ (pH 7.4)], dehydrated in a graded series of methanol washes to 90%, and then embedded in Lowicryl K4M as described previously (*26*). Ultrathin sections were cut and mounted on 200 mesh Formvar-coated nickel grids.

Grids were first blocked with 10% normal goat serum in Tris-buffered saline (TBS) for 15 min. The sections were then incubated with anti-TB-RBP antibody (α TB-RBP) diluted to 1:50 with TBS for 1 h and extensively washed four times with 0.05% Tween-20 (TWBS) for 5 min each. The sections were then blocked with 10% normal goat serum, followed by an incubation with colloidal gold (10 nm) conjugated to goat anti-rabbit antibody (1:50) diluted in TBS for 1 h (EM.GAR 10, Cedarlane Laboratories Ltd., Hornby, ON), and washed four times with TWBS (5 min each). The grids were then incubated with anti-TER ATPase antibody diluted 1:50 with TBS for 1 h. The sections were subsequently washed four times with 0.05% Tween-20 (TWBS) for 5 min each, blocked with 10% normal goat serum, and incubated with colloidal gold (15 nm) conjugated to protein A (1:50) diluted in TBS for 1 h (EM.PAG 15, Cedarlane Laboratories Ltd.). The grids were finally washed four times with TWBS (5 min each) and three times in distilled water and counterstained with uranyl acetate for 2 min, followed by treatment with lead citrate for 30 s. Normal rabbit serum (1:20) was used as a negative control. Electron micrographs were taken on a Philips 400 electron microscope.

Detection of TB-RBP and Actin Associations by Confocal Microscopy. Germ cells were prepared from mouse testes as described previously (*8*). Briefly, the testis from an adult CD-1 mouse was decapsulated and incubated with 4 mL of RPMI 1640 culture medium containing 2.5 mg of collagenase for 12 min at 34 °C with gentle shaking. The culture medium was removed, and the seminiferous tubules were rinsed once with fresh culture medium. The seminiferous tubules were then incubated with 4 mL of RPMI 1640 containing 2.5 mg of trypsin and 10 μg of DNase I for 12 min at 34 °C. The tubules were pipeted to disperse the cells. After the addition of 3 μg of trypsin inhibitor and 10 μg of DNase I, the cells were passed through a nylon membrane to remove cellular debris. The cells were mixed with 10 mL of RPMI 1640 containing 0.5% BSA, collected by centrifugation for 10 min at 500 rpm, resuspended in 10 mL of RPMI 1640 containing 0.5% BSA, and centrifuged as described above. The cells were resuspended in 5 mL of RPMI 1640 (concentration of about 10^7 cells/mL) and fixed onto polylysine-coated coverslips by incubation with 4% paraformaldehyde (freshly prepared in PBS) for 10 min at room temperature. The coverslips were rinsed with PBS four times, covered with 50 μL of α TB-RBP antibody (10 μg/mL) and actin C4 antibody (1:100) in blocking solution [minimum essential medium (without bicarbonate) containing 15 mM Hepes (pH 7.5), 10% fetal bovine serum, 0.3% Triton X-100, and 0.02% sodium azide], and incubated overnight at 4 °C in a nondesiccating environment. After being rinsed four times in PBS, the coverslips were covered with 50 μL of blocking solution containing FITC-linked anti-rabbit IG antibody (species-specific, Jackson Immunoresearch Laboratory, 1:100) and biotinylated anti-mouse Ig antibody (species-specific,

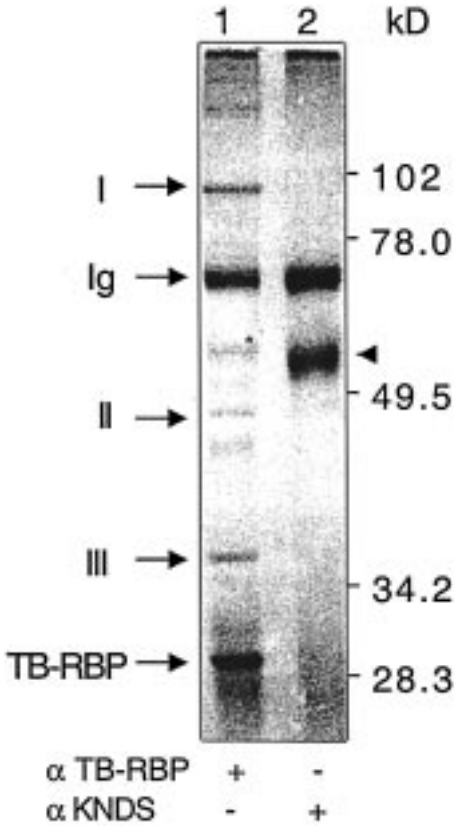

FIGURE 1: Proteins that co-immunoprecipitate with TB-RBP. Immunoprecipitations were carried out with cytoplasmic extracts from mouse testes and antibody to recombinant TB-RBP (α TB-RBP) (lane 1) or antibody to a peptide of TB-RBP (α KNDS) (lane 2). The immunoprecipitated proteins were resolved on 15% SDS−polyacrylymide gels and detected by Coomassie blue staining. The three protein bands that co-immunoprecipite with TB-RBP are denoted with arrows and the numbers I−III. Ig is denoted with an arrow. The identity of the protein denoted by an arrowhead in lane 2 is unknown.

Amersham International, 1:100) for 30 min at room temperature. After being washed four times in PBS, the coverslips were incubated with 50 μL of blocking solution containing streptavidin conjugated with rhodamine (1:100) for 20 min at room temperature. The coverslips were rinsed with PBS, fixed with cold methanol for 10 min, rinsed with PBS, and mounted onto slides. Immunolabeled cultures were sectioned optically using a computer-interfaced, laser scanning microscope (Leica TCS 4D) fitted with a 488 nm/568 nm/647 nm krypton argon laser. This allowed simultaneous analysis of fluorescein and rhodamine chromophores.

RESULTS

Proteins That Co-Immunoprecipitate with TB-RBP. To identify proteins that interact with TB-RBP, testicular extracts were immunoprecipitated with an affinity-purified antibody to recombinant TB-RBP (α TB-RBP) (Figure 1). Three proteins with estimated molecular masses of 97, 46, and 36 kDa (bands I−III, respectively) co-immunoprecipitated with TB-RBP (Figure 1, lane 1). These proteins and TB-RBP were not precipitated by a control antibody, α KNDS, which was raised against a peptide of TB-RBP (Figure 1, lane 2). α KNDS detects TB-RBP in Western blots, but cannot immunoprecipitate TB-RBP (*13*). Protein bands with similar estimated molecular masses were also detected when TB-RBP was immunoprecipitated from extracts of PC12 cells, 3T3 cells, liver, and spleen (data not shown). Protein bands I−III were cut off from gels and identified by peptide sequencing and mass mapping.

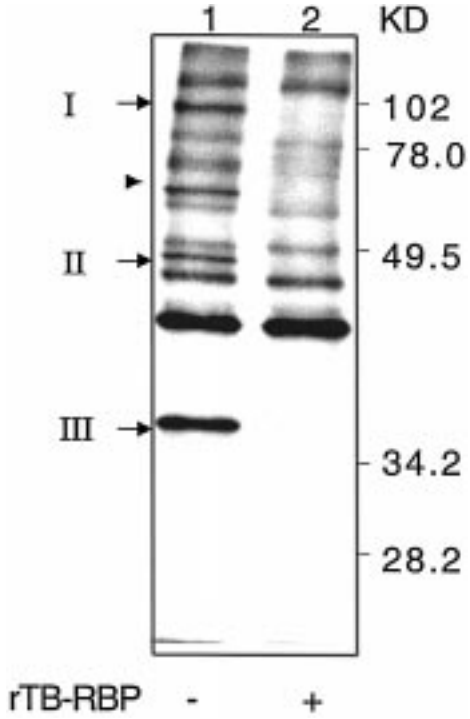

FIGURE 2: Immunoprecipitation of ^{35}S-labeled testicular extract with anti-TB-RBP antibody. The testicular cytosolic extract was prepared from mouse germ cells previously labeled with [^{35}S]methionine. Immunoprecipitation was performed with 1 mg of extract and 3 μg of α TB-RBP preincubated with 16 μg of bovine serum albumin (lane 1) or 16 μg of recombinant TB-RBP (rTB-RBP, lane 2). The precipitated proteins were resolved on a 15% SDS−polyacrylamide gel and detected by autoradiography.

To demonstrate the specificity of the immunoprecipitation, a blocking competition experiment was performed with α TB-RBP and [^{35}S]methionine-labeled testicular extracts. Excess recombinant TB-RBP or bovine serum albumin was added to the testicular extracts with the precipitating antibody α TB-RBP (Figure 2). In the presence of bovine serum albumin, bands I−III were immunoprecipitated from the extract (Figure 2, lane 2), whereas no immunoprecipitation of bands I−III was seen when an equal amount of recombinant TB-RBP had been added (Figure 2, lane 1). TB-RBP only transiently contains methionine at its N-terminus and is therefore not detected in this autoradiogram. Other minor bands shown to be specifically recognized by α TB-RBP were not identified, because of the difficulty in obtaining sufficient amounts for microsequencing.

A search of the database revealed that a proteolytic fragment from band I is identical to amino acids 639−651 of the mouse transitional endoplasmic reticulum ATPase (*24*), also known as valosin-containing protein (*25*) (Figure 3). The specific co-immunoprecipitation of the TER ATPase with TB-RBP was confirmed by Western blotting the solubilized precipitate with an antibody specific to the TER ATPase (Figure 4). In both testis and brain extracts, TER ATPase was detected at its predicted electrophoretic mobility of 97 kDa (panels A and B of Figure 4, lane 1). A fraction of the total cellular TER ATPase was detected in immunoprecipitates from testicular extracts (Figure 4A, lane 3) and

Band	Peptide sequence	Protein
I	LDQLIYIPLPDEK 639 LDQLIYIPLPDEK 651	Mouse TER ATPase
II	VAPEXHPVLLTEAPLNPK 89 VAPEEHPVLLTEAPLNPK 106	Mouse γ-actin
III	SFQQELDAR DITVESK 41 SFQQELDAR 49 63 DITVESK 69	Human TRAX

FIGURE 3: Amino acid sequences of proteolytic fragments obtained from protein bands I−III shown in Figure 1. The peptide sequences are shown above the underlined sequences from the database. The positions of the peptides in the proteins are denoted with numbers.

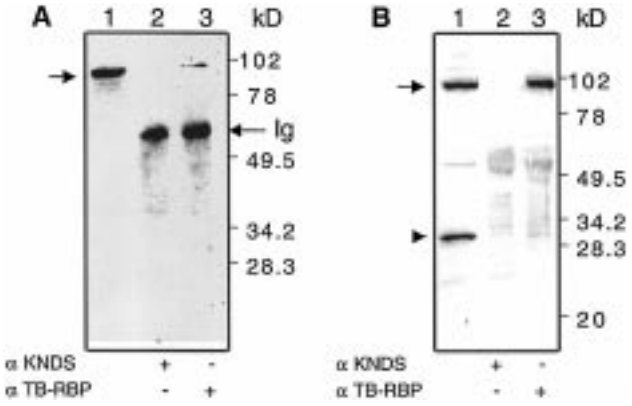

FIGURE 4: TER ATPase co-immunoprecipitates with TB-RBP. Cytosolic extracts (lanes 1) from mouse testes (A) and brain (B) were incubated with α KNDS (lane 2) or α TB-RBP (lane 3). The immunoprecipitated proteins were resolved on a 10% SDS−polyacrylamide gel. The TER ATPase denoted with an arrow was detected by Western blotting with the specific TER ATPase antibody (αVCP 821) (lanes 1−3). In addition to the TER ATPase, in brain extracts the antibody also recognizes a 30 kDa protein (denoted with an arrowhead).

brain extracts (Figure 4B, lane 3) when α TB-RBP was used, but not when α KNDS was the precipitating antibody (panels A and B of Figure 4, lanes 2).

The sequence of a proteolytic fragment from protein band II is identical to amino acids 89−106 of a mouse cytoskeletal γ actin (Figure 3) (*27*). Using a monoclonal antibody to actin for Western blotting, actin was found in the pellet of both testicular and brain extracts after immunoprecipitation with α TB-RBP (panels A and B of Figure 5, lanes 3), but not after incubation with the nonprecipitating antibody, α KNDS (panels A and B of Figure 5, lanes 2). A greater abundance of actin was routinely observed in the actin-rich brain extracts and brain immunoprecipitates than in testis extracts and testis immunoprecipitates (Figure 5, compare panel A to B).

The sequences of two proteolytic fragments from protein band III are 100% homologous to two regions [amino acids 41−49 and 63−69 of the human protein Trax (Figure 3) (*17*)]. Trax has been shown to bind to TB-RBP in vivo in the yeast two-hybrid assay and in in vitro binding assays with recombinant proteins (*17*).

Recombinant TB-RBP Binds to Recombinant TER ATPase. To establish whether TB-RBP and TER ATPase can interact in vitro, an assay was performed using a recombinant GST−TER ATPase fusion protein and recombinant TB-RBP. Through the specific binding of GST to glutathione−Sepharose 4B beads, the GST−TER ATPase can be easily

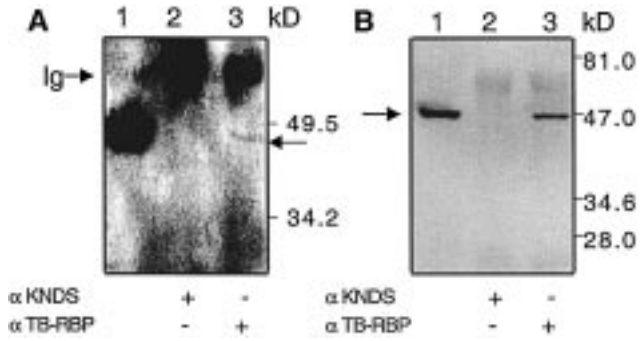

FIGURE 5: Cytoskeletal γ actin co-immunoprecipitates with TB-RBP. Cytoplasmic extracts of mouse testes (A, lane 1) and brain (B, lane 1) were immunoprecipitated with α KNDS (lane 2) or α TB-RBP (lane 3). The precipitates were electrophoresed on a 10% SDS−polyacrylamide gel. The γ actin, detected by Western blotting with a monoclonal antibody to actin (C4), is denoted with arrows.

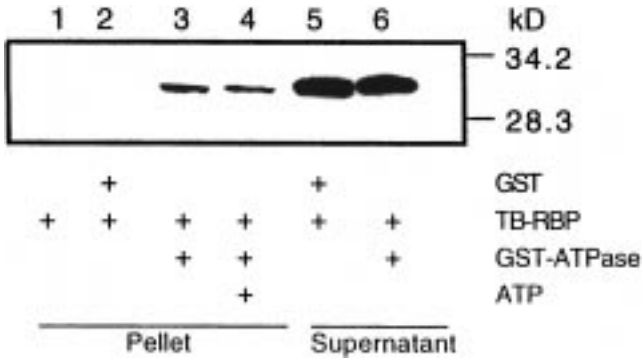

FIGURE 6: In vitro interactions between TB-RBP and GST−TER ATPase: lane 1, TB-RBP alone; lanes 2 and 5, TB-RBP incubated with GST; and lanes 3, 4, and 6, TB-RBP incubated with GST−TER ATPase. GST and GST−TER ATPase were pelleted with glutathione−Sepharose 4B beads. Aliquots of the pellets (lanes 1−4) and supernatants (lanes 5 and 6) were electrophoresed on 10% SDS−polyacrylamide gels. TB-RBP was detected by Western blotting using α KNDS. Lanes 5 and 6 contained aliquots from the supernatants of lanes 2 and 3, respectively. In lane 4, 1 mM ATP was added during the incubation.

recovered by low-speed centrifugation. If TB-RBP interacts with GST−TER ATPase, it will pellet with the fusion protein and be detected by Western blotting. TB-RBP was found in the pellet after incubation with the GST−TER ATPase (Figure 6, lanes 3 and 4), but not in the pellet after incubation with Sepharose beads alone (Figure 6, lane 1), or with GST alone (Figure 6, lane 2), or with bovine serum albumin (data not shown). The addition of 1 mM ATP did not increase or decrease the level of interactions between TB-RBP and TER ATPase under the binding assay conditions that were used (Figure 6, lane 4). These results confirm that TB-RBP can interact with the TER ATPase.

TB-RBP Partitions with the TER ATPase in a Membrane Fraction. Having established that recombinant TB-RBP interacts with the TER ATPase in in vitro assays (Figure 6), we sought to determine whether TB-RBP could be detected in the TER membrane fractions where the TER ATPase has been detected (*23*, *24*). An enriched membrane fraction was prepared from testes using discontinuous sucrose gradient centrifugation (*24*). Following SDS−polyacrylamide gel electrophoresis, TB-RBP and the TER ATPase were assayed by Western blotting using specific antibodies to each protein. TB-RBP and the TER ATPase were detected in the starting

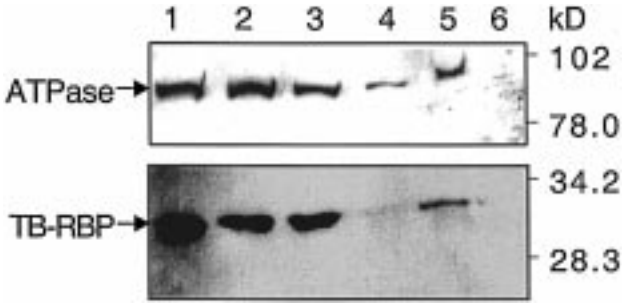

FIGURE 7: Western blot of the distribution of TER ATPase and TB-RBP in TER membrane fractions. (Top) The TER ATPase and (bottom) TB-RBP: lane 1, cytosolic extract from testes; lane 2, TER membrane fraction obtained by gradient centrifugation; lane 3, supernatant following gradient centrifugation; lane 4, TER membrane after second wash; lane 5, first-wash fraction; and lane 6, second-wash fraction.

testicular extract (Figure 7, lane 1), in the membrane fraction (Figure 7, lane 2), in postmembrane cytosolic fractions (Figure 7, lane 3), and in thoroughly washed membranes (Figure 7, lane 4). Continued washing of the membrane fraction releases both TB-RBP and the TER ATPase (Figure 7, lanes 5 and 6) from a membrane fraction. These data demonstrate that TB-RBP partitions with the TER ATPase in a membrane fraction.

EM Immunocytochemistry Reveals TB-RBP and the TER ATPase Colocalize in Male Germ Cells. To determine whether TB-RBP colocalizes with the TER ATPase in intact cells, mouse testicular sections were immunoreacted first with the α TB-RBP antibody followed by incubation with a secondary antibody conjugated to 10 nm gold particles, and then with the TER ATPase antibody followed by protein A conjugated to 15 nm gold particles. Examination at the electron microscope level focused on spermatocytes and spermatids where TB-RBP is known to be maximally expressed (*8*).

Immunogold labeling of TB-RBP (10 nm gold particles) was observed as single gold particles or clusters in the nuclei and cytoplasm of meiotic and postmeiotic male germ cells (Figures 8 and 9). Higher amounts of TB-RBP associated with the chromatin of pachytene spermatocytes or in the cytoplasm of round and early elongated spermatids were detected, as previously reported (*8*). In contrast, the TER ATPase was widely distributed as single particles in the nuclei and cytoplasm of meiotic and postmeiotic cells. The TER ATPase was often detected as single particles sometimes associated with mitochondria (Figure 8b).

The majority of TB-RBP (detected as 10 nm gold particles) was found in association with the TER ATPase in both the nuclei and cytoplasm of male germ cells (Figures 8 and 9). TB-RBP was rarely detected alone. In contrast, the TER ATPase was abundant and was often detected alone (Figure 9e).

Interestingly, in the nuclei of spermatocytes and spermatids, TB-RBP was found associated with electrondense regions of the chromatin (Figure 8a,d) and in the cytoplasm associated with less electrondense structures (Figure 8b). Frequently, when TB-RBP was seen as particles or in clusters within or in the vicinity of intercellular bridges, the TER ATPase was also seen (Figure 9). No labeling was detected with normal rabbit serum (negative control) followed by goat anti-rabbit IgG and protein A conjugated to colloidal gold, indicating that the immunogold reactions with the α TB-RBP and the TER ATPase antibodies were specific (Figure 8c).

Confocal Microscopy Detects TB-RBP and Actin Colocalized in Mouse Germ Cells. Microfilaments have been proposed to help direct RNA localization, anchoring, and short-distance transport in cells (reviewed in refs *2* and *3*). Moreover, since actin is abundant in and near the intercellular bridges of male germ cells (*9*, *10*), it is not surprising that γ actin should coprecipitate with TB-RBP. To confirm the interactions between TB-RBP and actin in male germ cells, confocal microscopy was used. Actin was detected by indirect fluorescence with rhodamine with a monoclonal antibody, C4, which recognizes vertebrate actins (*28*). TB-RBP was detected by indirect fluorescence with FITC with the affinity-purified α TB-RBP. TB-RBP (green in Figure 10) is present primarily in the cytoplasm of germ cells, consistent with previous localization studies (*8*) (Figure 10A). In the cytoplasm of round spermatids, an area of intense TB-RBP localization is frequently seen (Figure 10). Actin is also detected throughout the cytoplasm of pachytene spermatocytes and round spermatids and in nuclei as punctate structures (Figure 10B). The detection of TB-RBP and actin is specific since the controls of rabbit anti-chicken Ig and anti-rabbit Ig conjugated with biotin only revealed background levels of fluorescence (data not shown). TB-RBP and actin colocalize in both nuclear and cytoplasmic regions of germ cells (Figure 10, yellow areas) with intense colocalization often seen in an unidentified region (structure?) in the cytoplasm of round spermatids (Figure 10C).

DISCUSSION

We identify here three proteins in mouse testes and brain which interact with TB-RBP. One protein, the TER ATPase, has been shown to be involved in transporting vesicles from membranes to Golgi; the second protein, a cytoskeletal γ actin, is a structural protein of microfilaments, and the third protein, Trax, is a member of the TB-RBP gene family. Trax associates with TB-RBP in both nuclear and cytoplasmic complexes from neural sources (*14*, *16*) and interacts with TB-RBP in yeast two-hybrid assays (*17*).

The transitional endoplasmic reticulum ATPase is an essential component of a complex of proteins involved in the ATP-dependent formation of transition vesicles from the transitional endoplasmic reticulum. It functions as a vesicle fusion protein in a homo-oligomeric ring-shaped particle. The TER ATPase is identical to the valosin-containing protein, an ATP binding homo-oligomeric protein related to *N*-ethylmaleimide-sensitive fusion proteins, proteins functioning in vesicle fusion of the endoplasmic reticulum, Golgi, endocytic vesicles, and secretory vesicles undergoing regulated fusion. Electron microscopy studies have suggested that TB-RBP or Translin also functions as a multimer forming in vitro a ring-shaped octamer (*12*). Binding studies in vitro and yeast two-hybrid assays have also demonstrated that TB-RBP must form at least dimers to be capable of binding DNA or RNA (*29*). Immunocytochemical analyses at the electron microscope level also detect multimeric forms of TB-RBP in male germ cells (*8*). The TER ATPase and TB-RBP interactions are likely to represent two components of a larger complex regulating mRNA transport and localization. In-

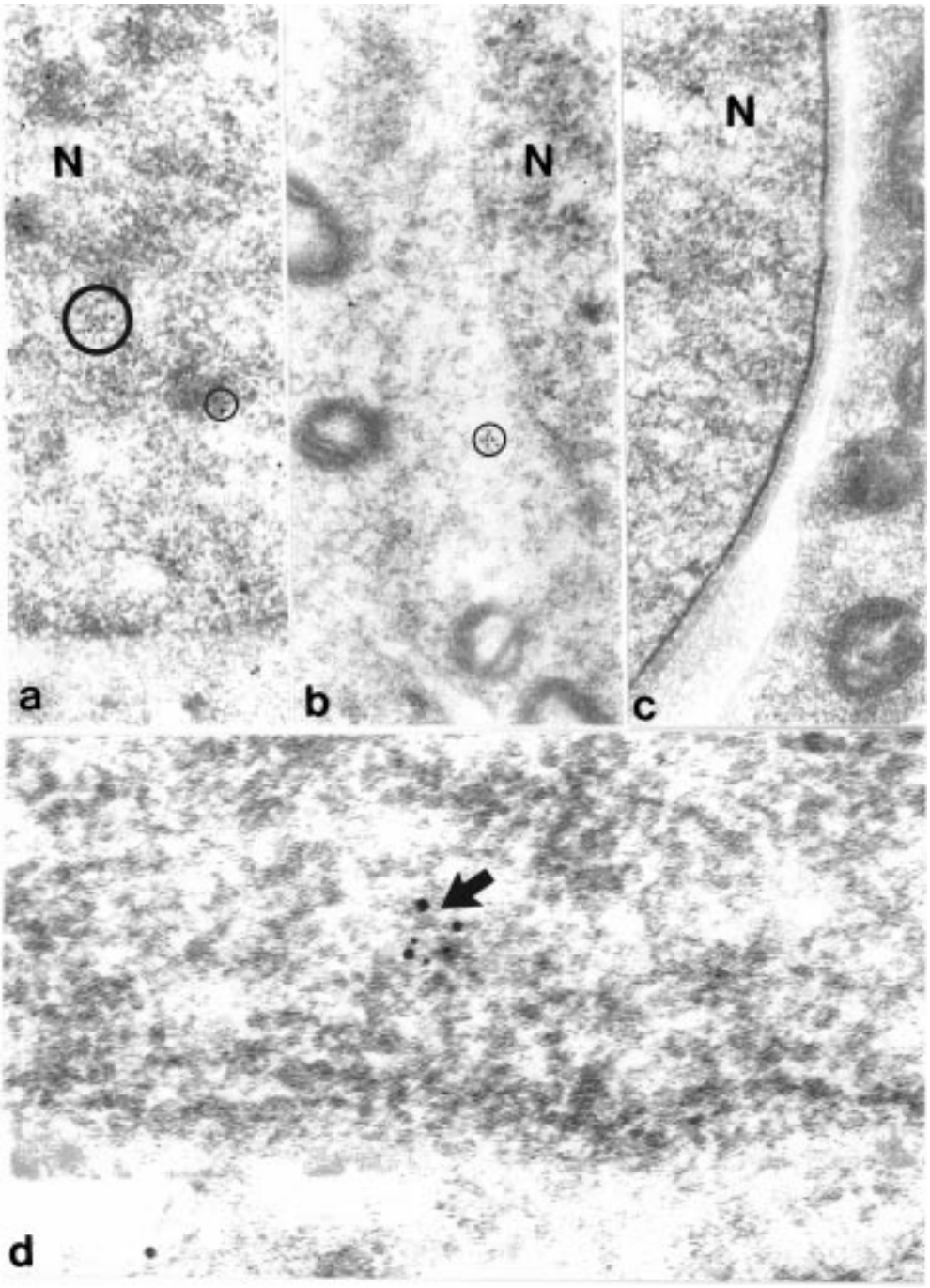

FIGURE 8: Thin sections of mouse seminiferous tubules analyzed for the presence of TB-RBP and the TER ATPase by EM immunocytochemistry with the α TB-RBP antibody (10 nm gold particles) or the TER ATPase antibody (15 nm gold particles). (a) Clusters (encircled) of 10 nm gold particles associated with 15 nm colloidal gold particles are seen over a spermatocyte nucleus (magnification of 38000×). (b) Clusters of 10 nm gold particles (encircled) overlying the cytoplasm in a spermatid (magnification of 38000×). (c) Control normal rabbit serum postincubated with goat anti-rabbit IgG and protein A conjugated to colloidal gold did not detect any protein (magnification of 38000×). (d) Higher-power photograph of a cluster of 10 and 15 nm gold particles (arrow) overlying the nucleus of a spermatocyte (magnification of 50000×). N is the nucleus.

terestingly, two-hybrid screens with the *Drosophila* bag of marbles gene (bam) have shown it interacts with a homologue of the vertebrate TER ATPase (*30*). Bam is a component of the fusome, a special organelle composed of membrane-associated structural proteins and vesicles, believed to regulate germ cell differentiation (*31*). The transport of TB-RBP-bound mRNAs may be mediated through the ER membrane in association with the TER ATPase, a process analogous to the processes controlling Vg1 mRNA localization where a subdomain of the endoplasmic reticulum facilitates mRNA localization during oogenesis (*20*, *21*). Vg1 mRNA localizes to the vegetal cortex in a microtubule-dependent manner, mediated by a 69 kDa microtubule-associated protein that binds to a localization element in the 3′ UTR of Vg1 mRNA (*32*). The 3′ untranslated region also binds a 75 kDa protein, Vera or VG1 RBP, which is associated with the endoplasmic reticulum through TRAPα, an integral protein of the endoplasmic reticulum membrane (*20*, *21*). These interactions allow Vg1 mRNA to attach to the ER, and the ER to be transported on microtubules (*20*,

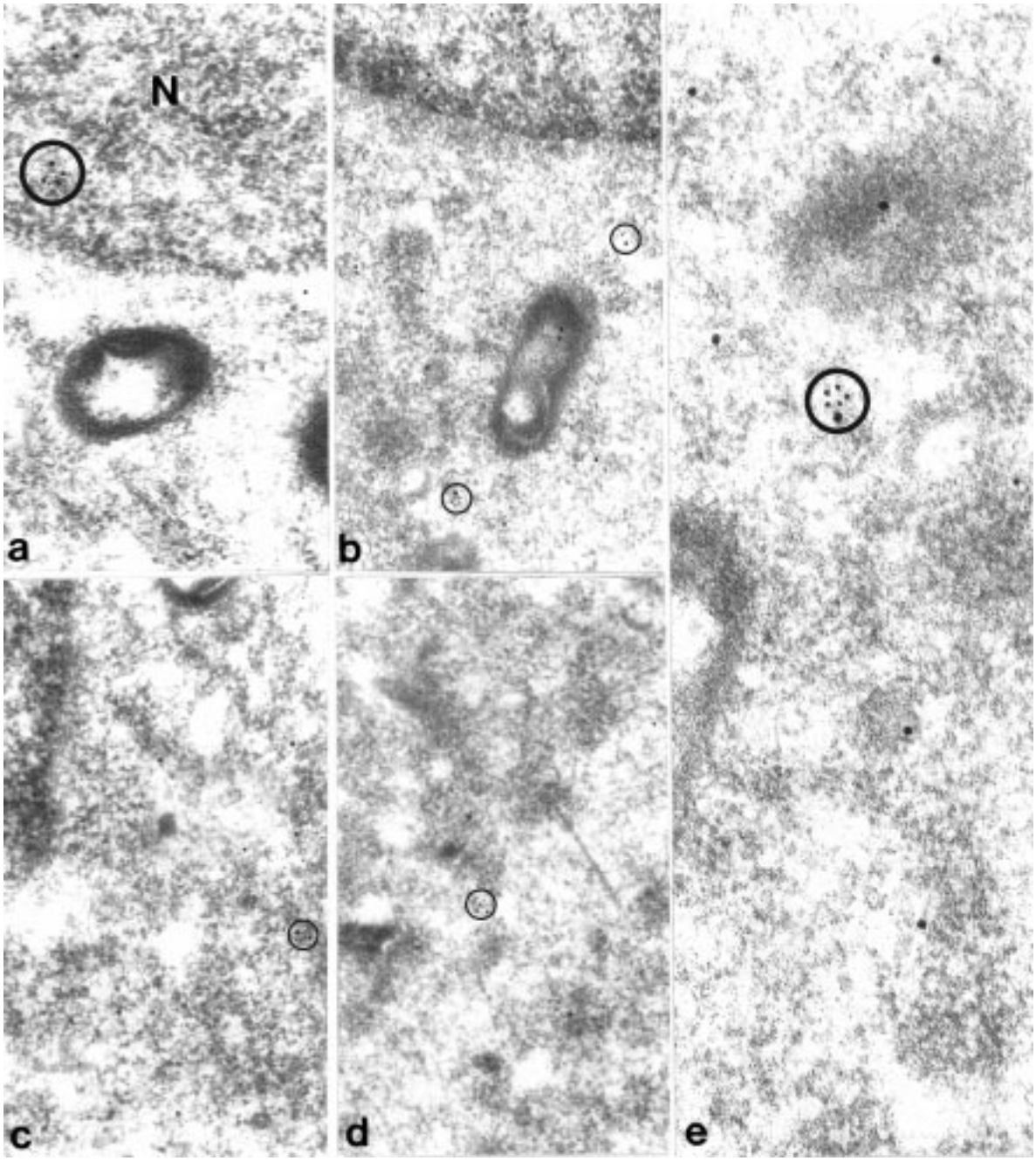

FIGURE 9: Thin sections of mouse seminiferous tubules analyzed for the presence of TB-RBP and the TER ATPase by EM immunocytochemistry. (a) Clusters of 10 nm gold particles associated with 15 nm gold particles (encircled) are seen in the nucleus of a spermatocyte (magnification of 38000×), (b) in the cytoplasm of a round spermatid (magnification of 38000×), and (c and d) in early elongated spermatids (magnification of 38000×). In panel b, two 15 nm (ATPase) particles are seen associated with a mitochondrion. Panel e is a higher-power picture of panel c (magnification of 50000×). The 15 nm gold particles, detecting the TER ATPase, are evenly scattered throughout the cytoplasm. A cluster of 10 nm gold particles over less electrondense structures is seen associated with one 15 nm gold particle (encircled).

21). TB-RBP may interact with the TER ATPase and the ER in a similar manner.

Our detection of the Translin-like protein, Trax, in immunoprecipitates from brain, testis, PC 12 cells, and other tissues confirms that the immunoprecipation procedure we are using is recognizing specific protein−protein interactions, since Trax has been shown to interact with TB-RBP in vivo and in vitro (*17*). Trax has been identified in a GS1 strand-specific DNA-binding complex enriched in brain (*14*), and a protein of about the size of Trax has also been reported in a putative transport particle for BC1 RNA in brain cytosolic extracts (*16*). To date, the function(s) of Trax remains unknown, although the bipartite nuclear targeting sequences in Trax suggest it may aid in the movement of associated proteins into the nucleus.

Many essential proteins are encoded by genes on the X chromosome (reviewed in ref *33*), and a growing number of functional proteins have been mapped to the Y chromosome (*34*). Among the proteins encoded on sex chromosomes with specialized roles in male germ cells are the Y-linked DNA-binding protein, Sry, the RNA-binding proteins, AZF and RBM, and the X-linked anchoring protein, AKAP 82 (*35*−*37*). As a result of the two meiotic divisions in the germ cells of the testis, the haploid spermatids contain either an

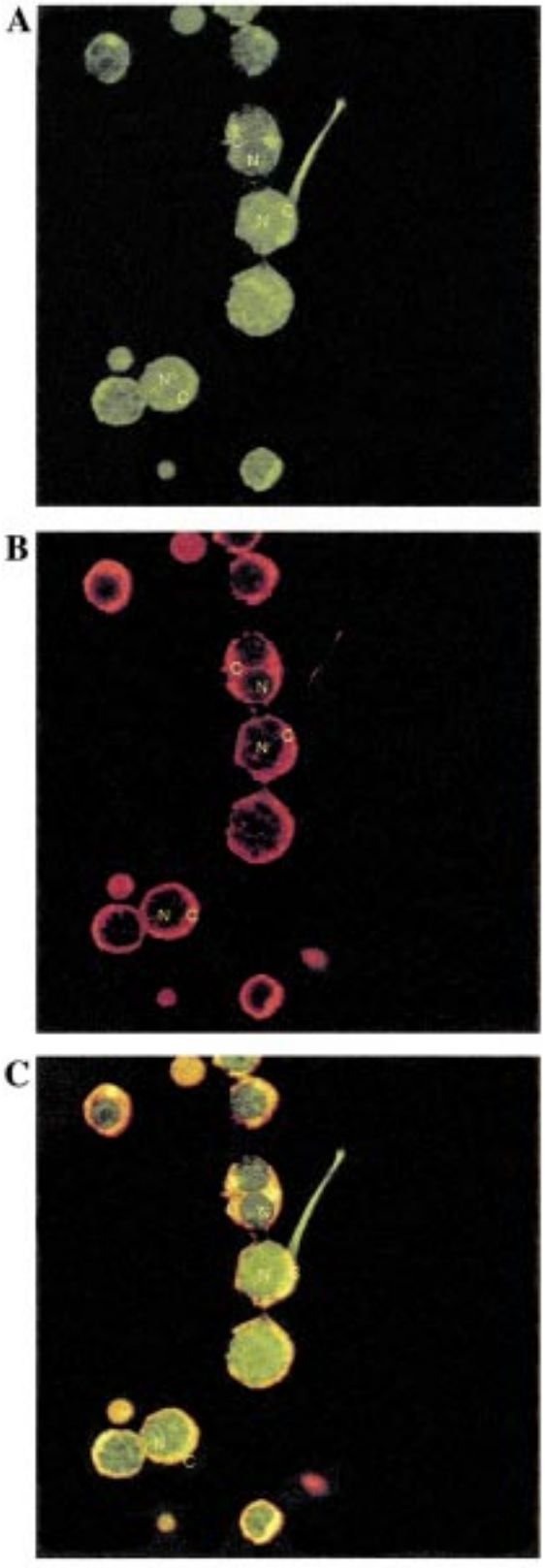

FIGURE 10: Confocal microscopy of the colocalization of TB-RBP and actin. Mouse male germ cells were fixed with 4% paraformaldehyde. (A) TB-RBP (green) was labeled with antibody α TB-RBP and anti-rabbit IG antibody conjugated with FITC. (B) Actin (red) was labeled with a monoclonal antibody to actin (C4) and detected with biotinylated anti-mouse IG antibody and streptavidin conjugated with rhodamine. (C) Colocalization of TB-RBP and actin (yellow). N is the nucleus and C the cytoplasm.

X or Y chromosome. However, proteins such as AKAP 82 are essential components of all spermatozoa. This requires transport of either the proteins or the mRNAs between the X- and Y-bearing spermatids through intercellular bridges (*38*). To date, transport of the mRNA encoding protamine 1 has been demonstrated to occur through the intercellular bridges of male germ cells (*39*, *40*). The mRNAs of both protamine 1 and 2 bind TB-RBP through conserved sequences in their 3′ untranslated regions (*5*, *41*), and TB-RBP specifically binds to similar sequences in the mRNAs encoding the X-linked AKAP 82 (data not shown). Moreover, AKAP 82 mRNA and protamine 1 and 2 mRNA can be selectively precipitated from testes extracts by α TB-RBP (data not shown). In light of the binding of TB-RBP to protamine 1 and 2 and AKAP 82 mRNAs and the abundance of microfilaments in and near the intercellular bridges of male germ cells (*9*, *10*), interactions between TB-RBP and actin are reasonable. We propose that TB-RBP binds to microfilaments, facilitating mRNA transport through intercellular bridges. Such interactions would implicate TB-RBP in a pathway of mRNA localization with cytoskeletal requirements different from its role in neuronal microtubule mRNA transport (*19*).

Multiple cytoskeletal binding associations have been reported for a number of RNA-binding proteins, including Vera/VG1 RBP (*20*, *21*), which attaches VG1 RNA to microtubules while its chicken homologue targets β actin mRNAs to the leading edge of motile fibroblasts by microfilament associations (*22*). We propose that TB-RBP also links specific mRNAs by microfilament or microtubule association to different cytoskeletal structures in different cell types and/or for different physiological functions. The detailed mechanism(s) whereby TB-RBP, the TER ATPase, Trax, and microfilaments or microtubules transport mRNAs in male germ cells and the brain remains to be determined.

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