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Altering the GTP binding site of the DNA/RNA-binding protein, Translin/TB-RBP, decreases RNA binding and may create a dominant negative phenotype

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ABSTRACT

The DNA/RNA-binding protein, Translin/Testis Brain RNA-binding protein (Translin/TB-RBP), contains a putative GTP binding site in its C-terminus which is highly conserved. To determine if guanine nucleotide binding to this site functionally alters nucleic acid binding, electrophoretic mobility shift assays were performed with RNA and DNA binding probes. GTP, but not GDP, reduces RNA binding by ~50% and the poorly hydrolyzed GTP analog, GTPγS, reduces binding by >90% in gel shift and immunoprecipitation assays. No similar reduction of DNA binding is seen. When the putative GTP binding site of TB-RBP, amino acid sequence VTAGD, is altered to VTNSD by site directed mutagenesis, GTP will no longer bind to TB-RBP$_{GTP}$ and TB-RBP$_{GTP}$ no longer binds to RNA, although DNA binding is not affected. Yeast two-hybrid assays reveal that like wild-type TB-RBP, TB-RBP$_{GTP}$ will interact with itself, with wild-type TB-RBP and with Translin associated factor X (Trax). Transfection of TB-RBP$_{GTP}$ into NIH 3T3 cells leads to a marked increase in cell death suggesting a dominant negative function for TB-RBP$_{GTP}$ in cells. These data suggest TB-RBP is an RNA-binding protein whose activity is allosterically controlled by nucleotide binding.

INTRODUCTION

RNA-binding proteins play numerous roles in the post-transcriptional regulation of gene expression including splicing and capping in the nucleus, transport of mRNAs and translation in the cytoplasm (1). Many of these processes are modulated by post-translational protein modifications, which alter binding specificities and the association–dissociation properties of RNA–protein complexes. Small molecules such as divalent metals and nucleotides also can modify protein binding to nucleic acids and influence processes such as mRNA transport (2–5).

The Testis Brain RNA-binding protein (TB-RBP) is the mouse ortholog of the human DNA-binding protein, Translin, a highly conserved 26 kD protein (6,7). As a DNA-binding protein, TB-RBP/Translin has been proposed to bind to single-stranded DNA sequences at translocation breakpoints in lymphocytes and at recombination hot-spots during meiosis in germ cells (8–13). As an RNA-binding protein, TB-RBP/Translin has been proposed to bind to conserved sequence elements, often in the 3′ untranslated regions of mRNAs. Many of these mRNAs are translationally regulated and some of the mRNAs expressed in post-meiotic male germ cells require transport through intercellular bridges to maintain haploid sufficiency (14–20).

TB-RBP/Translin functions as a multimer (8,21,22). The protein must form at least a dimer to bind DNA or RNA and larger complexes of tetramers and octamers have been detected by gel filtration chromatography and electron microscopy (8). In addition to forming homomultimeric complexes, TB-RBP/Translin interacts with a number of proteins including GADD 34 (23), a transitional endoplasmic reticulum ATPase (24), a cytoskeletal γ actin (24), and Translin associated factor X (Trax) (24). Trax is a 32 kD protein with extensive amino acid homology to TB-RBP (25–27). TB-RBP forms heterodimers or oligomers with Trax, which enhance TB-RBP binding to DNA, but strongly inhibit TB-RBP binding to RNA *in vitro* (28). TB-RBP also serves as a linker protein, binding specific mRNAs to microtubules, and TB-RBP co-immunoprecipitates a γ actin, suggesting a microtubule (14,19) and/or microfilament (24) based transport of mRNA for TB-RBP. Moreover, suppression of TB-RBP binding to transported mRNAs disrupts mRNA sorting in hypocampal neurons (18). In male germ cells, TB-RBP localizes in the nuclei of meiotic pachytene spermatocytes and in the cytoplasm in all subsequent germ cell stages, suggesting TB-RBP has distinct subcellular functions in the testis (17).

In vivo and *in vitro* studies with truncated and mutated forms of TB-RBP have revealed several essential domains needed for nucleic acid binding (21,22). A leucine zipper in the C-terminus

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is required for protein dimerization which is stabilized by a disulfide bond involving cysteine 225 (21,22). Dimerization or oligomerization of TB-RBP is required for either DNA or RNA binding, since monomers will not bind either nucleic acid (21,22). In addition, one basic domain in the N-terminus is essential for either DNA or RNA binding, while a second basic domain is also needed for RNA binding (22,28). A highly conserved leucine-rich nuclear export signal in the C-terminus is essential for the exit of TB-RBP from the nucleus (28).

In addition to the leucine zipper, nuclear export signal and two basic domains essential for TB-RBP function, sequence analysis suggests that TB-RBP contains a putative GTP binding domain. We demonstrate here that TB-RBP binds GTP and that GTP, but not GDP, suppresses RNA binding, but not DNA binding. Mutation of this site inhibits RNA binding and creates a dominant negative form of TB-RBP in transfected NIH 3T3 cells. To our knowledge, TB-RBP is the first example of a DNA/RNA-binding protein in a multicellular organism where GTP/GDP binding selectively affects its nucleic acid binding.

MATERIALS AND METHODS

RNA and DNA mobility shift assays

Electrophoretic mobility shift assays were performed with an RNA probe, transcript c or a DNA probe, Bcl-CL1, as previously described (28). The RNA–protein complexes were digested with T1 RNase (1 U) and incubated with heparin (5 mg/ml). Transcript c was transcribed from a pGEM3Z plasmid using [α-^{32}P]CTP while Bcl-CL1 was radiolabeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Recombinant proteins were incubated with 40 000 c.p.m. of RNA or DNA probe for 10 min at room temperature in 20 µl of 20 mM HEPES pH 7.6, 3 mM $MgCl_2$, 40 mM KC1, 2 mM DTT and 5% glycerol. Nucleic acid–protein complexes were analyzed in 4% polyacrylamide gels in TBE buffer.

Isolation of TB-RBP–RNA complexes from testis extracts by immunoprecipitation

Immunoprecipitation of TB-RBP and its bound mRNAs was performed essentially as described previously (19). Testis extracts (10 mg) containing RNasin (100 U/ml) were incubated with protein A–agarose beads (100 µl), pre-immune serum (10 µl), and 20 mM Tris pH 7.6, 40 mM KCl and 1.5 mM MgCl (TKM; 4 ml) containing 0.1% Tween-20, RNasin (100 U/ml) and 0.1% Empigen BB (TKM-T) for 2 h at 4°C. After centrifugation at 2000 r.p.m. for 2 min (Eppendorf microfuge), TKM-T (500 µl), protein A–agarose (40 ul) and preimmune serum or an affinity purified antibody to TB-RBP (15 µg) were added to the supernatant. After incubation overnight at 4°C, the mixtures were centrifuged at 2000 r.p.m. for 2 min, and the pellets were washed four times with 5 ml TKM-T. The pellets were then suspended in 250 µl of Tri Reagent and RNA was purified. Following ethanol precipitation, the RNA was dissolved in 20 µl DEPC-treated water and assayed by RT–PCR.

Site-directed mutagenesis of the putative GTP binding domain of TB-RBP

Wild-type TB-RBP was subcloned into a pET28a vector for protein expression in *Escherichia coli*. To create TB-RBP_{GTP}, site directed mutagenesis was carried out using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. Using synthesized oligonucleotide primers (5′-GTCAACAGTGTCACTACT-AATAGCGACTACTCTCGGCCCCTTGAC-3′ and 5′-GTC-AAGGGGCCGAGAGTAGTCGCTATTAGTAGTGACACT-GTTGAC-3′), the putative GTP binding site, amino acid sequence VTAGD, was changed to VTNSD. DNA sequence analysis of the construct confirmed the altered sequence. Recombinant TB-RBP and TB-RBP_{GTP} were expressed in BL21 (DE3) cells and purified by HisBind Resin (Novagen, Madison, WI) following the manufacturer's protocol. The purities of the proteins were confirmed by SDS–PAGE. For transfection, wild-type TB-RBP and TB-RBP_{GTP} mutant cDNAs were subcloned in frame to the C-terminus of GFP in the pEGFP C2 vector.

Yeast two-hybrid assays

Complete cDNAs of TB-RBP, TB-RBP_{GTP} and Trax were subcloned into the *Eco*RI/*Sal*l sites of pBD-GAL4cam and pAD-GAL4 (Stratagene). Pairs of binding domain and activation domain plasmid constructs were co-transfected into yeast (strain YRG-2) and transformants were selected on SD medium lacking leucine and tryptophan. Protein–protein interactions were detected by the growth of yeast on SD medium lacking leucine, tryptophan and histidine, and by the X-Gal filter lift assay on SD medium lacking leucine and tryptophan. Strength of interactions was determined by the addition of 3-amino-1,2,4-triazole (Sigma, St Louis, MO) at concentrations from 5 to 100 mM. All transformation and filter lift assay procedures were performed following the manufacturer's instructions (Stratagene).

Cell culture, transfections and confocal fluorescence microscopy

NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and streptomycin. Cells were transiently transfected using FuGENE 6 reagent (Roche) according to the manufacturer's protocol, grown on two-well chamber slides and fixed, 48 h post-transfection, using 4% paraformaldehyde in PBS. After washing three times in PBS, the slides were mounted using Vectashield mounting medium and examined by fluorescent microscopy and by confocal microscopy with a Bio-Rad MRC-1024 confocal laser scanning microscope. ApopTag staining of transfected cells was carried out using the manufacturer's protocol (Intergen, NY).

RESULTS

GTP reduces TB-RBP binding to RNA but not to DNA

Sequence and mutational analysis of mouse TB-RBP (28) revealed several domains including a putative GTP binding site, VTAGD, which shares substantial homology to the DTAGQ sequence present in many G-proteins (Fig. 1A) (29). Comparing known TB-RBP/Translin sequences, this putative

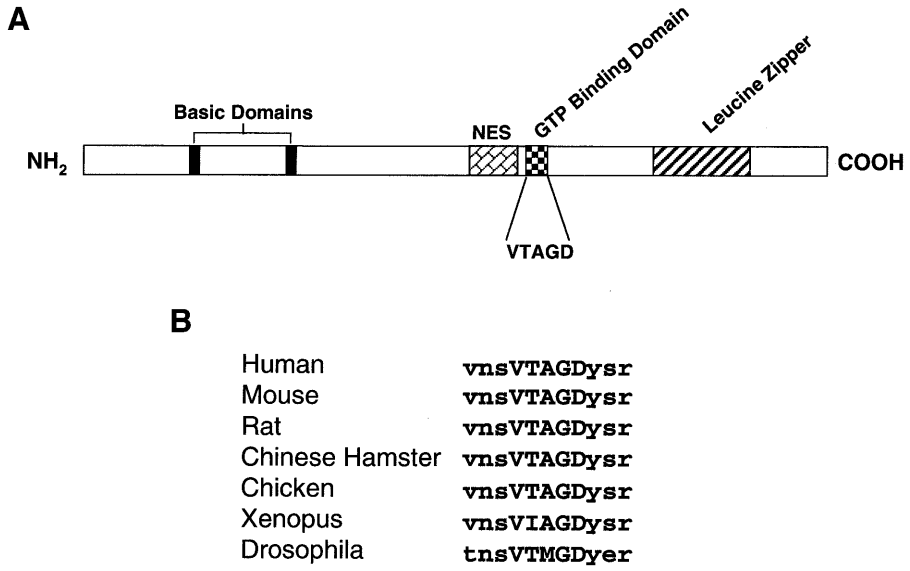

Figure 1. (**A**) Schematic representation of putative domains of TB-RBP. NES indicates nuclear export signal and VTAGD is the putative GTP binding site. (**B**) Sequence comparison of conserved GTP binding site of TB-RBP from diverse species. The sources of the sequences were GenBank accession nos: Human, Q15631; Mouse, AAF60295; Rat, NP068530; Chinese hamster, CAA66669; Chicken, P79769; *Xenopus*, AAF65620; and the *Drosophila* genome sequence.

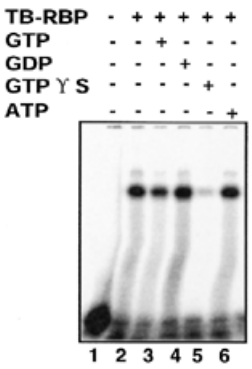

Figure 2. Effect of guanine nucleotides on TB-RBP binding to RNA. RNA gel shifts were performed as described in Materials and Methods. [^{32}P]-Labeled transcript c (40 000 c.p.m.) was added to each lane. Lane 1, transcript c alone; lane 2, plus recombinant TB-RBP (40 ng); lane 3, plus recombinant TB-RBP (40 ng) and 250 μM GTP; lane 4, plus recombinant TB-RBP (40 ng) and 250 μM GDP; lane 5, plus recombinant TB-RBP (40 ng) and 250 μM GTPγS; and lane 6, plus recombinant TB-RBP (40 ng) and 250 μM ATP.

GTP binding site is identical in species ranging from chicken to human and differs by one amino acid between *Drosophila* or *Xenopus* and human (GenBank accession nos, see Fig. 1B legend.

To determine if the GTP binding region could be a functional domain of TB-RBP, we investigated the effect of guanine nucleotides on the binding of TB-RBP to nucleic acids. In electrophoretic mobility shift assays using transcript c, an RNA known to bind TB-RBP (28), the presence of 250 μM GTP reduces recombinant TB-RBP binding to RNA to ~50% of control values (Fig. 2, lane 3). When GDP is substituted for GTP, no significant reduction from control binding levels is seen (Fig. 2, lane 4). Addition of 250 μM GTPγS, a poorly hydrolyzed analog of GTP, reduces the TB-RBP–transcript c interactions by ~10-fold (Fig. 2, lane 5). ATP or the poorly hydrolyzed analog of ATP, adenylyl imidophosphate, (data not shown) also does not decrease the RNA binding of TB-RBP (Fig. 2, lane 6). Similar decreases in RNA binding were obtained with endogeneous TB-RBP in mouse testicular extracts in the presence of GTPγS (see below).

To investigate the inhibitory effect of GTPγS on TB-RBP binding to RNA, two approaches were taken. First, a dose response curve increasing GTPγS from 50 μM to 1 mM was carried out (Fig. 3A). A concentration dependent decrease in RNA binding was observed as GTPγS levels increased (Fig. 3A, lanes 3–6). Secondly, utilizing the ability of our affinity purified antibody to TB-RBP to selectively precipitate TB-RBP bound endogenous mRNAs (19), we analyzed the effect of GTPγS on mRNA–TB-RBP co-precipitation from extracts. Following immunoprecipitation of TB-RBP, RT–PCR was used to assay two mRNAs that specifically bind TB-RBP and two control mRNAs that lack the TB-RBP binding sites from the precipitate (Fig. 3B). mRNAs encoding protamine 1 (Fig. 3B, lane 2) and protamine 2 (Fig. 3B, lane 6) were precipitated by antibody to TB-RBP, but not by preimmune serum (Fig. 3B, lanes 1 and 5). When GTPγS was added to the extracts, either no protamine 1 mRNA (Fig. 3B, lane 3) or a low level of protamine 2 mRNA were precipitated (Fig. 3B, lane 7). In contrast, control mRNAs encoding TB-RBP (Fig. 3B, lanes 9–11) and clusterin (Fig. 3B, lanes 13–15) were not precipitated under any conditions. RT–PCR assays of testicular RNA confirmed that the primers detected all four mRNAs (Fig. 3B, lanes 4, 8, 12 and 16). These two experimental approaches establish GTPγS has a profound effect on TB-RBP binding to RNA.

Electrophoretic mobility shift assays using recombinant TB-RBP and Bcl-CL1, a single-stranded DNA oligonucleotide, as probe (28) were carried out to determine what effects guanine nucleotides have on TB-RBP binding to a known

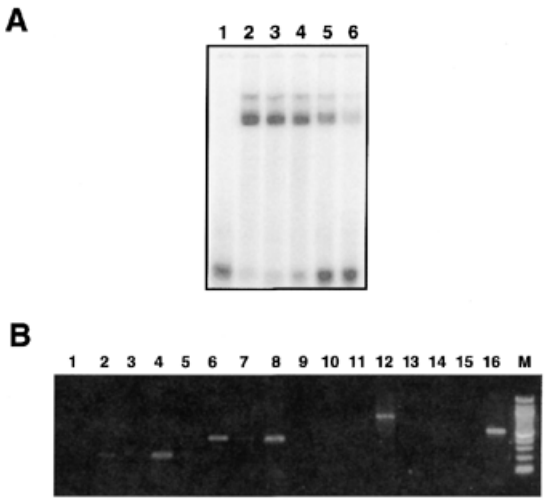

Figure 3. Effect of GTPγS on TB-RBP binding to RNA. (**A**) RNA gel shifts were performed as described in Figure 2. Lane 1, transcript c alone; lane 2, recombinant TB-RBP (20 ng); lane 3, plus 50 µM GTPγS; lane 4, plus 100 µM GTPγS; lane 5, plus 250 µM GTPγS; and lane 6, plus 1 mM GTPγS. (**B**) Immunoprecipitations of TB-RBP followed by RT–PCR assays for mRNAs were performed as described in Materials and Methods (19). Lanes 1–4, RT–PCR assays for protamine 1; lanes 5–8, protamine 2; lanes 9–12, TB-RBP; and lanes 13–16, clusterin. Lanes 1, 5, 9 and 13, RT–PCR assay of the pellet from the pre-immune serum control; lanes 2, 6, 10 and 14, RT–PCR assays of the pellet from precipitation with affinity purified antibody to TB-RBP; lanes 3, 7, 11 and 15, RT–PCR assays of the pellet from precipitation with affinity purified antibody to TB-RBP in the presence of 500 µM GTPγS; and lanes 4, 8, 12 and 16, control RT–PCR assays of aliquots of total testicular purified RNAs. M, marker DNA fragments. RNA was isolated from the pellets and suspended in 20 µl of water and equal aliquots (1 µl) of each were assayed.

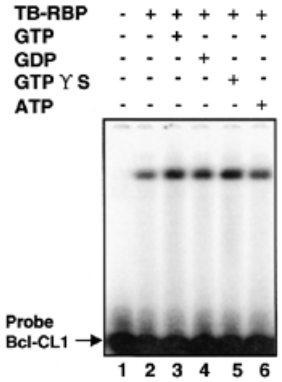

Figure 4. Effect of guanine nucleotides on TB-RBP binding to DNA. DNA gel shifts were performed as described in Materials and Methods. [^{32}P]-labeled Bc1-CL1 (40 000 c.p.m.) was added to each lane. Lane 1, Bc1-CL1 alone; lane 2, plus recombinant TB-RBP (40 ng); lane 3, plus recombinant TB-RBP (40 ng) and 250 µM GTP; lane 4, plus recombinant TB-RBP (40 ng) and 250 µM GDP; lane 5, plus recombinant TB-RBP (40 ng) and 250 µM GTPγS; and lane 6, plus recombinant TB-RBP (40 ng) and 250 µM ATP.

DNA target. In contrast to the decreases in RNA binding seen with GTP and GTPγS, neither 250 µM GTP (Fig. 4, lane 3) or GTPγS (Fig. 4, lane 5) significantly decrease TB-RBP binding to DNA. In fact, we detect a modest, but reproducible, increase

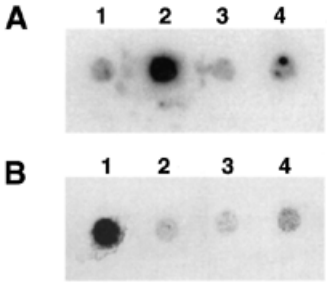

Figure 5. Recombinant TB-RBP binds GTP. (**A**) Protein samples were dotted onto a supported nitrocellulose membrane (Bio-Rad, CA) and the membrane was incubated with [α^{32}P]GTP in TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6) containing 5% non-fat dry milk. The membrane was washed three times for 5 min each at room temperature with TBST and exposed for autoradiography. Lane 1, Control BSA (25 µg); lane 2, recombinant TB-RBP (1 µg); lane 3, recombinant Trax (1 µg); and lane 4, recombinant TB-RBP$_{GTP}$ (2 µg). (**B**) Protein staining of the above membrane with 0.1% Ponceau S in 5% acetic acid.

for the protein–DNA complex in the presence of GTP or GTPγS (Fig. 4, lanes 3 and 5), but not with GDP (Fig. 4, lane 4). As seen for RNA, ATP (Fig. 4, lane 6) or adenyl imidophosphate (data not shown) do not significantly influence the binding of TB-RBP to single-stranded DNA. When mouse testicular extracts are substituted for the recombinant TB-RBP, a similar small increase in DNA binding is seen in the presence of GTP. Thus, under identical assay conditions, we detect, in the presence of GTPγS, a ~10-fold decrease in TB-RBP binding to RNA and a small enhancement of DNA binding.

TB-RBP binds to GTP

In order to verify that the effect of guanine nucleotides on TB-RBP is due to a direct interaction between the protein and guanine nucleotide, we studied the binding of GTP to TB-RBP and to a mutated form of TB-RBP, TB-RBP$_{GTP}$, in which the putative binding site has been altered. In TB-RBP$_{GTP}$ the putative GTP binding site is disrupted by site directed mutagenesis of A161N and G162S. Proteins were dotted onto nitrocellulose membranes and incubated with [^{32}P]GTP. Control TB-RBP binds significant levels of [^{32}P]GTP (Fig. 5A, lane 2), whereas GTP binding was greatly reduced when the putative binding site was changed to VTNSD in TB-RBP$_{GTP}$ (Fig. 5A, lane 4). As controls, 25 µg of a non-GTP binding protein, bovine serum albumin (Fig. 5A, lane 1), and 1 µg of Trax, a TB-RBP binding protein which does not bind to nucleic acids nor has a GTP binding domain, were used (Fig. 5A, lane 3).Only background binding of GTP to bovine serum albumin or Trax was observed (Fig. 5A, lanes 1 and 3). Similar binding to TB-RBP, but not to TB-RBP$_{GTP}$, was obtained when GTP[γ^{35}S] was substituted for [^{32}P]GTP, or when the nucleotide and TB-RBP binding assays were performed in solution and then spotted onto membranes (data not shown). Ponceau S staining of the proteins on the membrane after the binding assays confirmed that the proteins remained on the membrane, indicating the difference in bound radiolabeled GTP on the filter reflects true differences in GTP binding to each protein (Fig. 5B).

The GTP binding site of TB-RBP is required for TB-RBP binding to RNA but not to DNA

To confirm that GTP was causing the reduction in TB-RBP binding to RNA, we compared nucleic acid binding of TB-RBP and the TB-RBP_{GTP} mutant protein in RNA and DNA gel shifts. Using transcript c as probe in electrophoretic mobility shift assays, control TB-RBP exhibited identical RNA binding as described in Figure 1 (Fig. 6A, lanes 2–4) and GppNHp, another poorly hydrolyzed analog of GTP, decreased TB-RBP–RNA binding by ~10-fold (Fig. 6A, lane 4), similar to the decrease observed with GTPγS (Fig. 2). Following removal of the GTP binding site, TB-RBP_{GTP} does not bind significantly to transcript c RNA (Fig. 6A, lane 5). No binding of TB-RBP_{GTP} to RNA is seen in the presence of GDP or GppNHP (Fig. 6A, lanes 6 and 7). These data indicate that in addition to the two essential basic domains and leucine zipper of TB-RBP (21,22,28), the intact structure of the GTP binding site is necessary for TB-RBP–RNA interactions.

To establish whether the altered GTP binding site in TB-RBP affects all nucleic acid binding to TB-RBP, DNA gel shifts were performed with TB-RBP and TB-RBP_{GTP}. Recombinant TB-RBP_{GTP} protein bound to the DNA probe (Fig. 6B, lane 5) at a comparable level to that seen with the wild-type protein (Fig. 6B, lane 2). The presence of GDP (Fig. 6B, lanes 3 and 6) or GppNHp (Fig. 6B, lanes 4 and 7) had no significant effect on the binding of control TB-RBP or TB-RBP_{GTP} to DNA, demonstrating that GTP binding to TB-RBP greatly influences RNA but not DNA binding to TB-RBP. The need for TB-RBP or TB-RBP_{GTP} to dimerize to bind DNA (21,22) argues that the selective loss of RNA binding in the mutant protein is not due to inability to dimerize.

TB-RBP_{GTP} homodimerizes and interacts with TB-RBP

Although multimerization is essential for TB-RBP to bind to RNA or DNA (8,21,22), altering the GTP binding site of TB-RBP could indirectly change nucleic acid binding by affecting protein–protein interactions essential for TB-RBP interactions. To directly determine whether TB-RBP_{GTP} can form dimers in cells, we investigated the *in vivo* interaction of TB-RBP_{GTP} with itself, with wild-type TB-RBP and with Trax (a TB-RBP binding protein) in a yeast two-hybrid assay. Using a filter lift assay for β-galactosidase activity, we determined that TB-RBP_{GTP} homodimerizes (Fig. 7E), heterodimerizes with wild-type TB-RBP (Fig. 7D) and also interacts with Trax (Fig. 7B). These interactions are identical to previous reports showing that wild-type TB-RBP homodimerizes (Fig. 7A) and heterodimerizes with Trax (Fig. 7B) (28). Trax does not interact with itself (Fig. 7C).

To provide an estimate of strength of protein–protein interactions, we have used 3-amino 1,2,4 Triazole as a competitive inhibitor of the *HIS 3* gene product (30). The TB-RBP homodimers, TB-RBP_{GTP} homodimers and the TB-RBP–TB-RBP_{GTP} heterodimers appear to bind with similar strength, since growth of each of the co-transformants is suppressed by 75 mM, but not by 60 mM 3-amino-1,2,4-triazole. No substantial difference in TB-RBP or TB-RBP_{GTP} binding to Trax is detected by this criterion (data not shown).

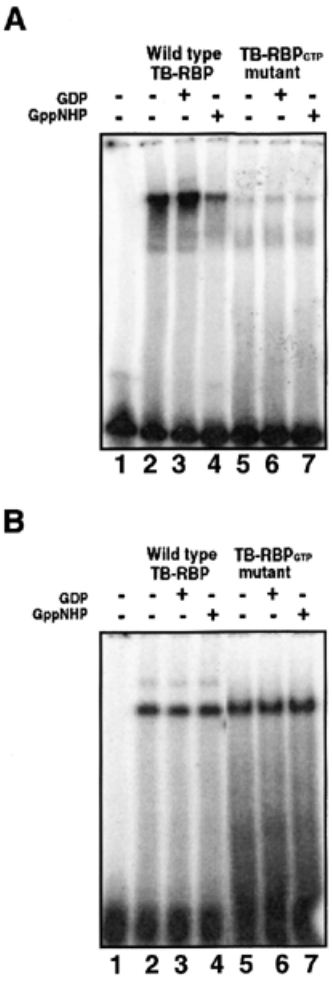

Figure 6. (**A**) Effect of guanine nucleotides on RNA and DNA binding to TB-RBP GTP. RNA gel shifts were performed as described in Materials and Methods. [^{32}P]-Labeled transcript c (40 000 c.p.m.) was added to each lane. Lane 1, transcript c alone; lane 2, plus recombinant TB-RBP (40 ng); lane 3, plus recombinant TB-RBP (40 ng) and 250 μM GDP; lane 4, plus recombinant TB-RBP (40 ng) and 250 μM GppNHp; lane 5, plus recombinant TB-RBP_{GTP} (40 ng); lane 6, plus recombinant TB-RBP_{GTP} (40 ng) and 250 μM GDP; and lane 7, plus recombinant TB-RBP_{GTP} (40 ng) and 250 μM GppNHp. (**B**) DNA gel shifts were performed as described in Materials and Methods. [^{32}P]-Labeled Bcl-CL1 (40 000 c.p.m.) was added to each lane. Lane 1, Bcl-CL1 alone; lane 2, plus recombinant TB-RBP (40 ng); lane 3, plus recombinant TB-RBP (40 ng) and 250 μM GDP; lane 4, plus recombinant TB-RBP (40 ng) and 250 μM GppNHp; lane 5, plus recombinant TB-RBP_{GTP} (40 ng); lane 6, plus recombinant TB-RBP_{GTP} (40 ng) and 250 μM GDP; and lane 7, plus recombinant TB-RBP_{GTP} (40 ng) and 250 μM GppNHp.

TB-RBP_{GTP} expression in NIH 3T3 fibroblasts causes cell death

Since we know that TB-RBP_{GTP} can dimerize with TB-RBP or with Trax in eukaryotic cells (Fig. 7) and TB-RBP_{GTP} has altered nucleic acid binding properties (Fig. 6), we set out to determine if this change in TB-RBP binding to nucleic acids could have a physiological effect in cells. To do this, we transiently transfected NIH 3T3 fibroblasts with either a wild-type TB-RBP cDNA (Fig. 8A and D) or TB-RBP_{GTP} cDNA (Fig. 8B, C and E), each cloned in frame to the C-terminus of GFP.

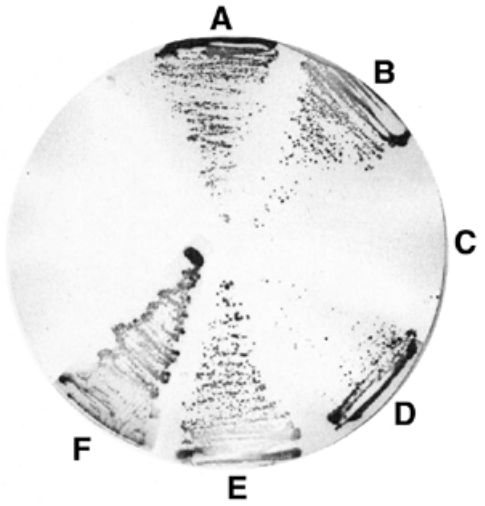

Figure 7. TB-RBP and TB-RBP_{GTP} can interact *in vivo*. The open reading frames of TB-RBP, TB-RBP_{GTP} and Trax were cloned in frame into both the pBDGAL4cam and pADGAL4 plasmids and interactions were examined in a yeast two-hybrid assay. Filter lift assay for β-galactosidase activity produced by co-transformants streaked on SD plates lacking leucine and tryptophan. All combinations interact except for BD Trax + AD Trax. (**A**) BD TB-RBP + AD TB-RBP; (**B**) BD TB-RBP + AD Trax; (**C**) BD Trax + AD Trax; (**D**) BD TB-RBP + AD TB-RBP_{GTP}; (**E**) BD TB-RBP_{GTP} + AD TB-RBP_{GTP}; (**F**) BD TB-RBP_{GTP} + AD Trax. No self-activation was observed with any of the AD or BD constructs assayed alone.

Control constructs containing the TB-RBP cDNA alone or the pEGFP C2 vector alone were used as transfection controls. Cells expressing GFP fused to wild-type TB-RBP appeared normal (Fig. 8A), whereas transfection with identical constructs expressing the TB-RBP_{GTP} protein instead of wild-type TB-RBP showed substantial increases in cell death (Fig. 8B). Cell death was seen in cells expressing TB- RBP_{GTP} even at lower levels than the wild-type TB-RBP transfections (Fig. 8, compare A to B) as well as at higher levels (Fig. 8C). Punctate particles rarely seen in control transfections are often seen in the cytoplasm following TB-RBP_{GTP} transfection (Fig. 8, compare A to B).

To quantitate the apparent increase in death of NIH 3T3 cells following transfection with TB-RBP_{GTP}, fluorescent activated cell sorting was performed. Quantitation of dead green fluorescent NIH 3T3 cells 24 h after transfection revealed ~2-fold (from 12 to 22%) increase in dead cells following their transfection with TB-RBP_{GTP}–EGFP compared to transfection with TB-RBP–EGFP. This increase in cell death in the TB-RBP_{GTP} expressing cells compared to wild-type TB-RBP expressing cells was detected by Apoptag staining (Fig. 8, compare D to E). We conclude that TB-RBP_{GTP} has a dominant negative function in transfected cells, causing cell death.

DISCUSSION

We demonstrate here that the DNA- and RNA-binding protein, TB-RBP, contains a functional GTP binding site similar to domains found in small G proteins (31). Although RNA binding is greatly reduced by GTP, or any of its poorly hydrolyzable analogs, the DNA binding of TB-RBP is not decreased. The marked reduction of TB-RBP binding to transcript c in the presence of GTPγS is seen with both recombinant TB-RBP and with endogenous TB-RBP in testicular extracts. We have shown earlier that specific mRNAs can be immunoprecipitated with affinity purified antibody to recombinant TB-RBP (19). Addition of GTPγS to testicular extracts greatly reduces the amount of the specific TB-RBP–protamine 1 and 2 mRNA complexes that are immunoprecipitated (Fig. 3B). The influence of the GTP binding site on RNA binding is further established by the drastic reduction in RNA binding seen when two amino acids in the putative GTP binding site of TB-RBP are altered by site directed mutagenesis (Fig. 6). Since TB-RBP/Translin must be either a homodimer or oligomer to bind DNA (8,21) or RNA (21), the selective loss in RNA binding is not due to inability of TB-RBP_{GTP} to form homodimers, because TB-RBP_{GTP} interacts with itself in yeast two-hybrid assays (Fig. 7) and the altered TB-RBP gives DNA gel shifts identical to wild-type TB-RBP (Fig. 5). These data mirror those reported for the bacterial protein, Era-G, a widely conserved protein involved in ribosomal maturation. Era-G also forms dimers, binds RNA *in vitro* and contains a GTP binding site (32). As we see here for TB-RBP, mutations in the GTP binding site of Era-G also perturb RNA binding (31). These data suggest that TB-RBP and Era-G represent eukaryotic and prokaryotic proteins that modulate their RNA binding through their GTP/GDP binding sites, most likely as a result of change in native protein structure caused by nucleotide binding.

The amino acid sequence of the GTP binding site of TB-RBP is similar to that found in many members of the Ras family (29). From sequence analysis and crystallographic studies, it has been shown that a large number of proteins that bind ATP or GTP and are involved in intracellular transport share similar conserved sequence motifs (29). GTPase assays of recombinant TB-RBP have only detected basal levels of enzyme activity, suggesting the need for an activator of guanine nucleotide exchange and a GTPase activating protein (data not shown). Yeast two-hybrid assays and immunoprecipitation studies have been used to identify TB-RBP interacting proteins including Trax (24), the Ter ATPase (24) and a putative homolog of the yeast sec 7 protein. Trax does not bind DNA or RNA as a monomer or homomultimer, but forms heterodimers and/or oligomers with TB-RBP. The heterodimer does not bind to RNA, although it binds to DNA well (28). The Ter ATPase is a ubiquitously expressed protein involved in vesicle transport which binds to TB-RBP both *in vitro* and in yeast two-hybrid assays (24). In *Drosophila*, TER 94, an ortholog of the vertebrate Ter ATPase, has been proposed to facilitate the growth of cisternae from the endoplasmic reticulum by vesicle fusion (33). The Ter ATPase is present in the *Drosophila* fusome, a germ cell-specific organelle composed of membrane proteins and vesicles involved in germ cell differentiation (33). The identity and functions of the sec 7-like protein await further studies.

GTP binding may play a functional role in TB-RBP mRNA transport along microtubules. Orbit, a *Drosophila* gene product, is a microtubule associated protein that has GTP-binding motifs and binds directly to microtubules in a GTP-dependent manner (2). Attachment of mRNA to insect ovarian microtubules is greatly reduced by the application of GTP, ATP or AMP–PNP, but not ADP (3), indicative of kinesin motor protein-mediated interactions (4). This pattern of

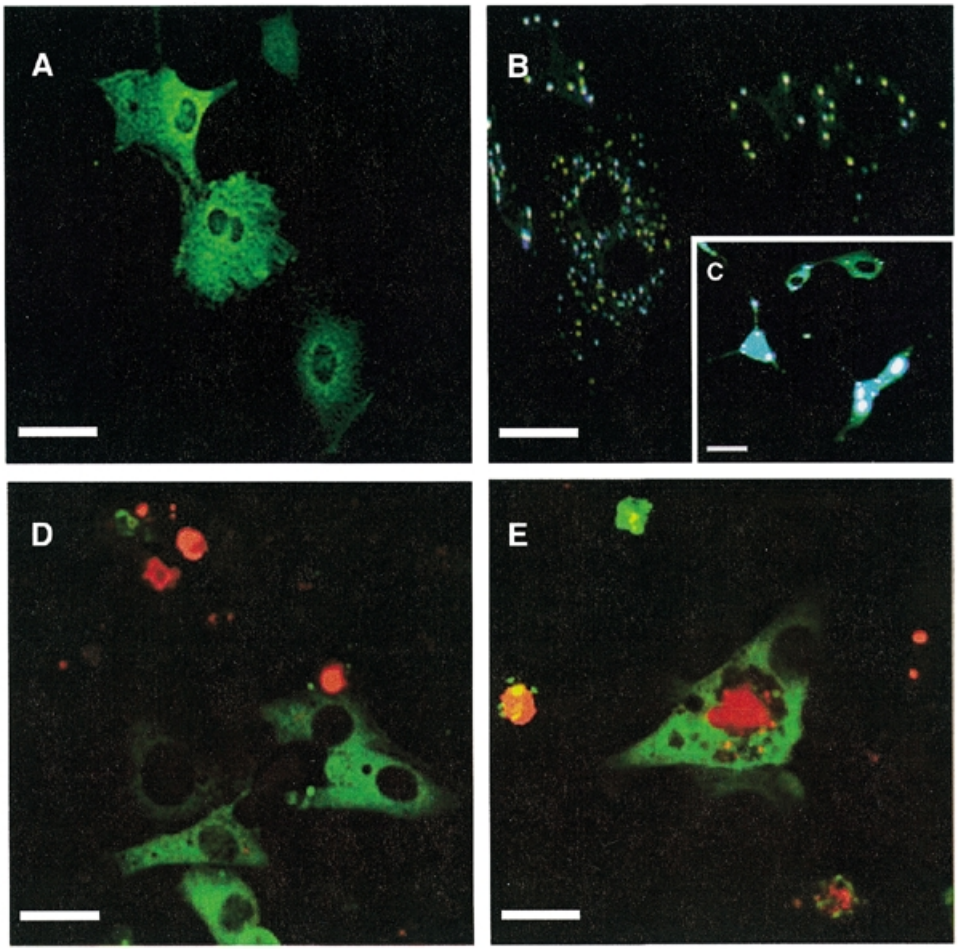

Figure 8. TB-RBP$_{GTP}$ is lethal to NIH 3T3 fibroblasts. (**A** and **D**) NIH 3T3 mouse fibroblasts were transiently transfected as described in Materials and Methods with an N-terminal fusion of EGFP attached to control wild-type TB-RBP (pEGFP–TB-RBP). (**B**, **C** and **E**) An N-terminal fusion of EGFP attached to a TB-RBP cDNA (pEGFP–TB-RBP$_{GTP}$) lacking the putative GTP binding domain (mutations were A161N and G162S) was transiently transfected into NIH 3T3 fibroblasts. The cells in (C) represent a higher level of expression of transfected protein than the cells in (A) or (B). In (D) and (E) cells are stained for DNA fragmentation using an ApopTag kit whose signal is represented by red fluorescence (Rhodamine B) in the nuclei. Cells were examined 48 h post-transfection. Bars represent 15 µm.

nucleotide sensitivity is also seen for a Staufen-like RNA-binding protein that attaches to, and is also transported along, *Notonecta* ovarian microtubules (5). In *Drosophila* oogenesis, the plus end-directed microtubule motor kinesin I is required for the posterior localization of oskar mRNA and associated Staufen protein (34). Endogeneous and recombinant TB-RBP have been demonstrated to bind to reconstituted microtubules from brain and testis extracts (15,19). Recently, we have co-immunoprecipitated TB-RBP and a member of the kinesin family with antibody to TB-RBP, suggesting there is a motor protein component of a TB-RBP protein complex in testis (data not shown).

The death of NIH 3T3 cells following transfection with TB-RBP$_{GTP}$ is quite remarkable. Previous studies transfecting wild-type TB-RBP into NIH 3T3 cells have detected a normal distribution of TB-RBP in both nuclei and cytoplasm and normal cell viability (28). In contrast, transfection with TB-RBP$_{GTP}$ leads to numerous cytoplasmic punctate structures and to cellular death at modest levels of expression. Co-staining TB-RBP$_{GTP}$ transfected cells with LysoTracker Red DND 99 dye has established that the punctate structures are not lysosomes, but particles containing TB-RBP$_{GTP}$. Since we do not know the precise functions of endogeneous TB-RBP in NIH 3T3 cells, we can only speculate why clusters of TB-RBP$_{GTP}$ in the cytoplasm may lead to cell death. Based upon the binding of TB-RBP$_{GTP}$ to itself, to wild-type TB-RBP and to Trax (Fig. 7), we propose that TB-RBP$_{GTP}$ could be titrating interacting proteins, thereby preventing them from participating in their normal cellular functions. This appears to be specific to TB-RBP lacking the GTP binding site, since a form of TB-RBP lacking the nuclear export signal, TB-RBP$_{NES}$, remains in the nucleus, but does not cause cell death in similarly transfected NIH 3T3 cells (28). We propose that TB-RBP$_{GTP}$ in NIH 3T3 cells sequesters TB-RBP interacting proteins such as the Ter ATPase, Trax, GADD34 or yet to be identified proteins preventing normal assembly of endogeneous TB-RBP into functional particles. This dominant negative effect is similar to the mutants of anthrax toxin that co-assemble with wild-type protein and block its ability to translocate enzymic moieties across membranes, thereby inhibiting toxin action (35). Similarly, aggregations of TB-RBP$_{GTP}$ into large granules in transfected NIH 3T3 fibroblasts may indicate

failure in the transport of particles containing the mutated TB-RBP protein. A detailed analysis of the components of TB-RBP_{GTP} particles is likely to shed light on how this mutation alters RNA-binding functions of TB-RBP.

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