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Identification and Characterization of a Novel Protein That Regulates RNA–Protein Interaction

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Abstract In a previous study [Nachaliel et al., 1993], we identified an RNA-binding protein (RBP) in FTO-2B rat hepatoma cells whose activity was stimulated upon the dissociation of a protein factor. We report in this article that the RBP is a complex protein of about 400 kDa, composed of RNA-binding subunit(s) (RBS), and regulatory subunit(s) (RS). We purified the RS to near-homogeneity (M_r ~25,000) and determined the amino acid sequence of a peptide derived from RS. On the basis of this sequence information, the cDNA for RS was obtained. Recombinant RS protein expressed in *Escherichia coli* had the capacity to bind RBS and inhibit its RNA-binding activity. The cDNA contains the complete coding sequence because the recombinant protein has the same electrophoretic mobility as that of the native RS in SDS-polyacrylamide gels. Sequence comparison showed that RS is almost identical to DJ-1, a recently discovered protein with an oncogenic potential, and CAP1, a rat sperm protein. However, the protein does not contain any known motifs that can provide a clue as to its exact function. Indirect immunofluorescence analyses showed that in addition to the cytoplasm, where RS is associated with microtubular filaments, the polypeptide is localized to the cell nucleus. The possible role of RS is discussed. J. Cell. Biochem. 72:435–444, 1999.

Key words: RNA-binding protein; complex protein; microtubules

RNA-binding proteins play an important role in the post-transcriptional control of gene expression [Siomi and Dreyfuss, 1997]. The activity of this family of proteins is mediated through specific recognition of sequences and structural elements within the mRNA transcripts [McCarthy and Kollmus, 1995; Dreyfuss et al., 1996]. Characterization of these proteins resulted in the identification of several RNA-binding motifs, including the RNP motif, KH domains, and the RGG box [Burd and Dreyfuss, 1994]. It has been suggested [Herschlag, 1995] that at least some of these proteins may function as RNA chaperones, acting to facilitate proper folding of the RNA, thereby ensuring that the molecule is accessible for its biological function. In addition to the RNA-binding motifs, auxiliary domains that are involved in protein–protein interaction have been identified in several RNA-binding proteins [Biamonti and Riva, 1994]. For example, hnRNP protein A1 was shown to form both homo- and heterocomplexes with other hnRNP proteins through a specific glycine-rich domain located at the C-terminal region of hnRNP A1 [Cartegni et al., 1996]. Although the function of the protein–protein interaction in the control of RNA–protein interaction remains known, it is likely to have a regulatory role. The importance of these interactions is reflected in the recent discoveries of genetic disorders caused by aberrant expression of an RNA-binding protein in the fragile X syndrome [Siomi et al., 1996] and in myotonic dystrophy [Philips et al., 1998], or of a protein that interacts with an RNA-binding protein in spinal muscular atrophy [Liu and Dreyfuss, 1996].

We have previously identified a cyclic adenosine monophosphate (cAMP)-regulated RNA-binding protein (RBP) with an affinity for

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double-stranded sequences [Nachaliel et al., 1993]. The RBP did not show sequence preference and interacted with a variety of sequences with predicted stem-loop structures. Binding was lost when the RNA probe was heat-denatured but restored after being renatured by slow cooling. We also demonstrated that the RNA-binding activity was reduced in rat hepatoma FTO-2B cells incubated with cAMP, or in livers from starved rats, a metabolic adaptation known to have increased levels of cAMP [Claus and Pilkis, 1981]. Incubation of the protein extract with alkaline phosphatase stimulated RNA-binding activity, suggesting that phosphorylation may regulate the RNA-binding activity. Further experiments demonstrated that FTO-2B cell extract contains a protein factor that inhibits the RNA-binding activity, suggesting that protein–protein interaction regulates the RNA–protein interaction. In the present study, we report on the isolation of the protein factor (RS) that regulates the RNA-binding activity, the identification of a cDNA clone and that RS is a component of a high-molecular-mass complex protein that also contains an RNA-binding activity (RBS). RS is uniquely distributed in the cytosol and nucleus in different cells, and is associated with microtubular filaments. RS was found to be almost identical to DJ-1, an oncogene shown recently to have the capacity to transform mouse fibroblasts in cooperation with *ras* [Nagakubo et al., 1997] and to CAP1, a sperm protein that is important in fertilization [Wagenfeld et al., 1998], suggesting that RS may have a broader cellular role than anticipated.

MATERIALS AND METHODS

Purification of RS

Extracts of the rat hepatoma cell line FTO-2B (confluent cultures from 250 × 10-cm^2 plates) were prepared as previously described [Nachaliel et al., 1993]. All subsequent steps, unless otherwise indicated, were performed at 4°C. The lysate was subjected to differential precipitation with $(NH_4)_2SO_4$ into a fraction that contains the RBS (30–50% saturation of $(NH_4)_2SO_4$), and a fraction containing RS (>70% saturation of $(NH_4)_2SO_4$). The fraction containing RS was loaded at room temperature onto a 10 ml column of phenyl-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ), pre-equilibrated with 70% saturated solution of $(NH_4)_2SO_4$ and 1 mM DTT. The column was developed with 5-bed volumes successively of 50%, then 20% saturated solutions of $(NH_4)_2SO_4$. RS activity was eluted in the 20% $(NH_4)_2SO_4$ wash. After dialysis, the fraction was loaded onto a 5-ml column of hydroxylapatite (Calbiochem, San Diego, CA) pre-equilibrated with potassium-phosphate buffer (10 mM, pH 6.8) (KPB) containing 1 mM DTT, and the column was developed by stepwise incremental concentrations of KPB containing 1 mM DTT.

Digestion and Protein Sequencing

The proteins eluted from the hydroxylapatite were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA) and visualized using Ponceau S (0.2% w/v). Targeted polypeptides were excised for Edman degradation sequence analysis on an Applied Biosystems model 475a protein sequencing system using pulsed-liquid chemistry. To generate smaller peptides for internal sequencing, the membrane-bound polypeptide was cleaved with CNBr as described by Stone and Williams [1993]. The resulting peptides were resolved by SDS-PAGE using the Tris-Tricine buffer system [Schagger and von Jagow, 1987] and electroblotted onto a PVDF membrane (Immobilon-P, Millipore). The amino acid sequence obtained was compared to known sequences in the standard data base using BLAST.

Analysis of RNA–Protein Interaction

Determination of RNA–protein interaction was carried out by a gel shift mobility assay using a 30-nucleotide P-enolpyruvate carboxykinase-derived sequence as an RNA probe (Lp8), as previously described [Nachaliel et al., 1993]. The final volume of the binding reaction was 25 µl and an aliquot of 10 µl was loaded onto the gel.

Construction of Prokaryotic Expression Vector and Isolation of Recombinant Protein

The protein-coding sequence of RS was amplified by PCR (94°C 30 s; 56°C 30 s; 72°C 1 min, 30 cycles) using RS cDNA (GenBank Accession No. T35410; see under Results), and the oligomers 5′-CATATTACATAACCATGGCTTCC and 5′-GAGAATGGATCCCTAACGGCC as coding and complementary primers, respectively. The amplified product was digested with *Nco*I and *Bam*HI and inserted into the corresponding

sites in the T7 RNA polymerase-based bacterial expression vector pET-15b (Novagen, Madison, WI), to generate the construct pET-RS. Insertion into the *Nco*I site of pET-15b eliminated the vector sequence for His-Tag, resulting in a translation product identical to the wild-type RS. The constructed DNA was confirmed by sequencing using ABI PRISM Dye Terminator chemistry and an ABI 373 DNA Sequencer (PE Biosystems, Foster City, CA). The pET-RS construct was introduced into *Escherichia coli* BL21(λDE3), using standard transformation techniques, and expression of transfected gene was induced with IPTG (0.4 mM) for 4 h at 37°C. The recombinant RS was purified from bacterial extract by $(NH_4)_2SO_4$ precipitation (>70% saturation), followed by fractionation on phenyl-Sepharose and hydroxyapatite, as described for the native protein.

Coupling of RS to CNBr-Activated Sepharose

The purified recombinant RS was ligated to CNBr-activated Sepharose as recommended by the manufacturer (Amersham Pharmacia Biotech).

Production of Antibody Directed Against RS

Antibody to RS was prepared by initial subcutaneous injection of 500 µg of the purified recombinant RS in complete Freund's adjuvant into a rabbit, followed by one additional booster injection of 100 µg, 21 days later. Serum was collected before injection and 3 and 6 weeks later, and the IgG fraction was purified by absorption onto protein A-Sepharose CL-4B (Amersham Pharmacia Biotech).

SDS-PAGE and Western Blot Analysis

PAGE and electroblotting of proteins onto PVDF membrane (Immobilon-P; Millipore) were carried out under standard conditions. Blots were incubated with nonfat milk to block unreacted sites and then with the RS antibody (1:5,000 dilution of rabbit antiserum) for 1 h at room temperature. After washing and incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:15,000 dilution) (Sigma Chemical Co., St. Louis, MO), blots were developed using the SuperSignal enhanced chemiluminescence detection kit from Pierce (Rockford, IL).

Immunofluorescence Microscopy

Cells grown on glass coverslips for 24 h were fixed with methanol at −18°C for 10 min and blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at 22°C. The fixed cells were incubated with the rabbit polyclonal antiserum to RS (1:200 dilution) and mouse monoclonal antibody to β-tubulin (Sigma Chemicals, clone TUB 2.1, 1:100 dilution) in blocking solution. After washing, cells were exposed to fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Sigma) and lissamine rhodamine (LRSC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), each diluted 1:100 in blocking solution. The cells were visualized using confocal laser scanning microscopy as described [Berrios and Colflesh, 1995] .

RESULTS

RS is a Component of a High-Molecular-Mass Complex Protein

We have previously shown [Nachaliel et al., 1993] that RNA-binding activity was markedly increased in the presence of high concentrations of KCl (>300 mM) (e.g., see Fig. 1, lanes 11 and 12) and proposed that the activation may have been mediated by dissociation of an inhibitory activity. Further experiments demonstrated that the RNA-binding and inhibitory

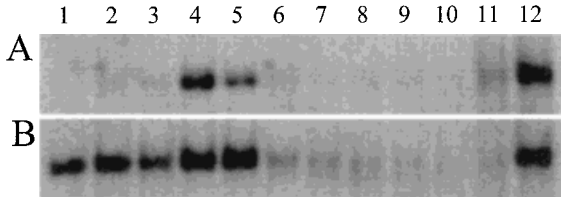

Fig. 1. RS is a component of a high-molecular-mass complex. RNA-binding activity was measured in fractions of FTO-2B cellular extract separated by gel filtration on Sephacryl S300. Cell lysis and chromatography were carried out using a low-ionic-strength buffer containing 40 mM KCl. **A:** RNA-binding activity in the Sephacryl S300 fractions measured in the presence of 40 mM KCl concentration (**lanes 1–10**). Activity of the whole cell extract applied to the column measured in the presence of 40 mM KCl (**lane 11**) or 500 mM KCl (**lane 12**). **B:** RNA-binding activity in the Sephacryl S300 fractions measured in the presence of 500 mM KCl concentration (**lanes 1–10**). **Lanes 11** and **12** are as in A. Complex formation in the RNA-binding assay was determined as described under Materials and Methods. Column calibration was carried out with ferritin (440,000 Da), catalase (230,000 Da), and alcohol dehydrogenase (150,000 Da). These proteins were eluted at fractions 1–2, 4–5, and 7, respectively.

activity were separable by chromatography on an anion exchanger and that reconstitution of the fractions containing the separated activities resulted in the arrest of RNA-binding activity. To examine whether the factor carrying the inhibitory activity (RS) is a component of the RBP complex, extracts from FTO-2B cells were fractionated by gel filtration on Sephacryl S300. To maintain the association of RS with RBS, cell lysis and gel chromatography were carried out using a low-ionic-strength buffer containing 40 mM KCl [Nachaliel et al., 1993]. The eluted fractions were analyzed for RNA-binding activity by performing the assay in the presence of low (40 mM) or high (500 mM) concentrations of KCl. As shown in Figure 1A, RNA-binding activity was detected only in fractions 4–5 when assayed in the presence of low concentration of KCl. However, when assayed in the presence of high concentrations of KCl, an additional peak of activity was detected in fractions 1–2 (Fig. 1B), suggesting the presence of an inhibitory activity co-migrating with the RBS in a complex of higher molecular mass. Fractions 4–5 contain an uninhibited form of RBS, since fractionation of the protein extract in the presence of 0.5 M KCl, a condition that favors RBP dissociation, resulted in a single peak of the RNA-binding activity in these fractions (not shown). Calibration of the gel filtration column with several proteins of known molecular mass, indicated that the active RBS (fractions 4–5) migrated as a 250-kDa protein, and the inhibited RBP complex in fractions 1–2 is >400 kDa. Because RBS was previously found to have a molecular mass of 110–120 kDa under denaturing conditions [Nachaliel et al., 1993], we suspect that RBP contains 2 subunits of RBS.

Purification of RS and Identification of a cDNA Clone

RS was purified from the rat hepatoma cells, FTO-2B, as described under Materials and Methods. Fractionation of the final active fraction (eluted with 200 mM KPB from hydroxylapatite) by SDS-PAGE showed that this fraction contained two major proteins with molecular masses of ~35 and 25 kDa (Fig. 2). Partial sequencing of the 35- kDa polypeptide identified this protein as the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a 37-kDa tetrameric protein, which has been shown to have several other additional activities unrelated to its role in glycolysis [Meyer-Siegler et al., 1991; Singh and Green, 1993; Nagy and Rigby, 1995]. Because several commercial preparations of GAPDH failed to inhibit complex formation, and several monoclonal antibodies to GAPDH/uracil DNA glyco-

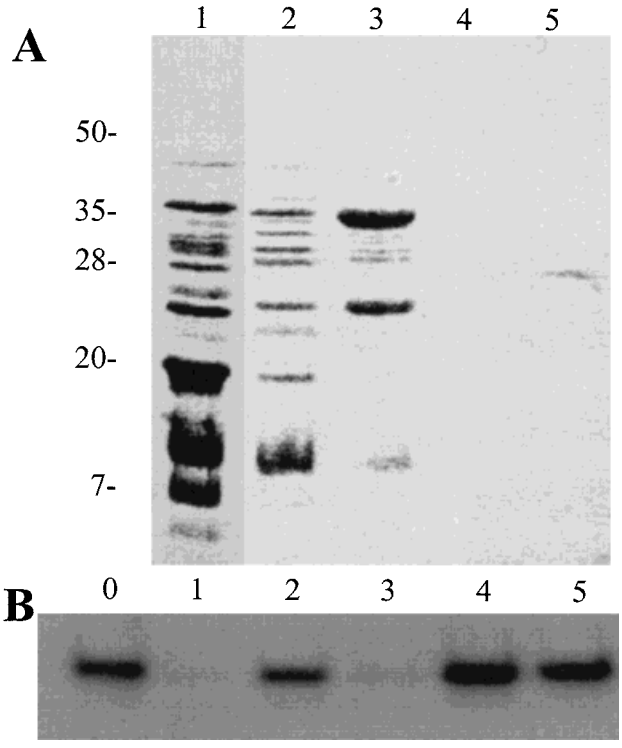

Fig. 2. Purification of regulatory subunit (RS) by chromatography on hydroxylapatite. RS was fractionated on hydroxylapatite as described under Materials and Methods. **A:** SDS-polyacrylamide gel analysis of proteins in an aliquot of the extract applied to the column (**lane 1**), and those eluted with 100, 200, 300, and 500 mM of KPB, (**lanes 2–5**), respectively. The position of the molecular-mass markers (kDa) on the gel is indicated. **B:** Analysis of RNA-binding activity of partially purified RBS (0.8 mg/ml) alone (**lane 0**), or in the presence of the fraction loaded on the column (**lane 1**), or hydroxylapatite fractions eluted with 100, 200, 300, and 500 mM of KPB (**lanes 2–5**), respectively. Prior to the assay, each fraction was desalted on Sephadex G-25 pre-equilibrated with 100 mM KPB, and an aliquot of 10 µl was used in the assay. The RNA-binding assay was carried out as described under Materials and Methods.

sylase [Arenaz and Sirover, 1983] were incapable of neutralizing RS activity (data not shown), we concluded that GAPDH is not likely to carry RS activity.

Our initial attempts to directly sequence the 25-kDa protein were unsuccessful, suggesting that it may be blocked at its N-terminus. Cleavage of the polypeptide with CNBr yielded two major peptides with molecular masses of about 10.5 and 5 kDa. Sequence analysis of the 10.5-kDa peptide provided the amino acid sequence RRAGIKVTVAGLAGKDPVQ, which was found to be homologous with several partially sequenced cDNAs deposited in the standard database. One of these clones (GenBank Accession No. T35410), originally isolated from a human colon cDNA library by the Institute for Genetic Research (TIGR), was purchased and sequenced in its entirety. This sequence has been deposited in the GenBank with the accession number AF021819. The cDNA is 904 base pairs (bp) long, which is consistent with the size of the mRNA, as subsequently determined by Northern blot analysis (not shown). The cDNA contains an open reading frame encoding a polypeptide of 189 amino acids, and a calculated molecular mass of 19,847 Da, which is close to the estimated mass of RS isolated from the hepatoma cells (see below). While the sequence of RS does not contain any known motif that can provide a clue as to its function, RS was found to be almost identical to the recently identified DJ-1 (GenBank Accession No. D61380), a potential product of an oncogene [Nagakubo et al., 1997] and CAP1 (Accession No. AJ007291), a sperm protein that is important in fertilization [Wagenfeld et al., 1998]. RS and DJ-1 share the same sequences in their translated and 3′-untranslated regions, but RS-encoding cDNA contains an extended 5′-untranslated region (not shown).

Isolated cDNA Codes for RS

Using polymerase chain rection (PCR), we subcloned a fragment from RS cDNA containing the entire predicted coding sequence into the bacterial expression vector pET-15b and introduced it into *E. coli* BL21(λDE3). Induction by IPTG of the expression of the transfected gene showed a protein with the expected molecular mass of ~25 kDa (not shown); this protein was purified from the bacterial extracts as described under Materials and Methods. Figure 3 shows the results of the last purification

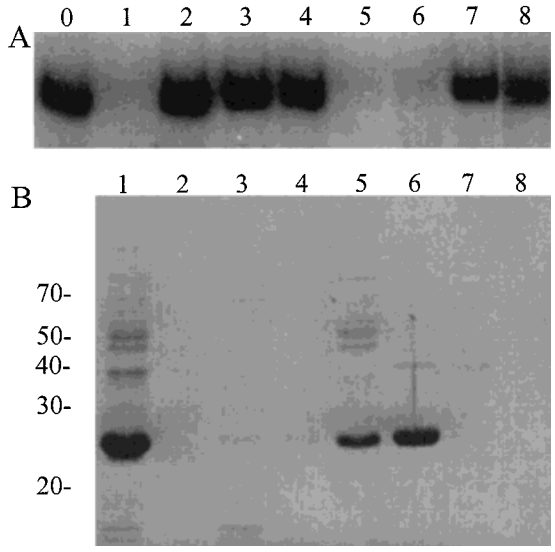

Fig. 3. Recombinant regulatory subunit (RS) inhibits RNA-binding activity. Chromatography of recombinant RS on hydroxylapatite was carried out as described under Materials and Methods. **A:** RNA-binding activity of partially purified RBS from FTO-2B cells (0.8 mg protein/ml) alone (**lane 0**), and in the presence of the fraction applied to the column (**lane 1**), in the flowthrough fraction (**lane 2**), and in the fractions eluted by 1 M KCl then 10, 100, 200, 300, and 500 mM of KPB (**lanes 3**–**8**), respectively. Samples were desalted as described in the legend to Fig. 2. **B:** Coomassie blue-stained SDS-polyacrylamide gel of proteins in the sample applied to the column (**lane 1**), in the flowthrough fraction (**lane 2**), and in the fractions eluted by 1 M KCl, then 10, 100, 200, 300, and 500 mM of KPB (**lanes 3**–**8**), respectively. The position of molecular-mass standards (kDa) is indicated.

step on hydroxylapatite in which inhibition of RNA-binding activity (Fig. 3A, lanes 1, 5 and 6) correlates with the presence of RS in the various fractions (Fig. 3B, lanes 1, 5 and 6).

The results presented in Figure 3B suggest that the size of the recombinant RS is somewhat larger than that deduced from the cDNA (19.9 kDa). To resolve this inconsistency, we examined the electrophoretic mobility of the purified recombinant protein and that of RS from FTO-2B cells using Western blot and an antibody raised against the recombinant RS. As shown in Figure 4, both proteins have the same mobility on SDS-polyacrylamide gel, consistent with an apparent molecular mass of 24 kDa. Similar results were observed when an independent source of molecular size standards was employed. While we cannot rule out the possibility that both proteins are modified post-translationally, we suspect that RS exhibits anomalous mobility on SDS-polyacrylamide gels.

To further corroborate the identity of the bacterially expressed RS, the recombinant pro-

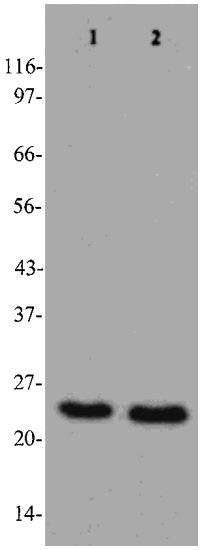

Fig. 4. Recombinant regulatory subunit (RS) has the same molecular size as the eukaryotic polypeptide. Western blot analysis of purified recombinant RS (20 ng) (**lane 1**), and FTO-2B cell extract (50 μg) (**lane 2**) employing the rabbit antiserum to RS. A similar analysis using preimmune serum did not detect any protein. Gel electrophoresis and Western blot analysis were carried out as detailed under Materials and Methods. The position of molecular-mass standards (kDa) is indicated.

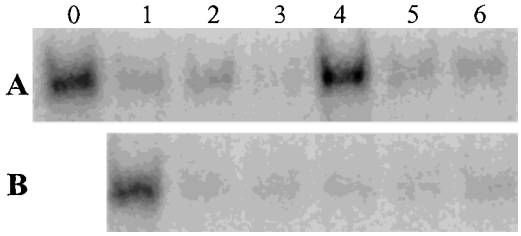

Fig. 5. Enrichment of RBS by regulatory subunit (RS)-affinity chromatography. Recombinant RS was immobilized to CNBr-activated Sepharose-4B as described under Materials and Methods. A partially purified preparation of RBS (a 30–50% $(NH_4)_2SO_4$ fraction of FTO-2B extract containing 4 mg of protein) was applied to a 1.5-ml column of either RS-bound Sepharose 4B **(A)** or CNBr-activated Sepharose 4B blocked with 0.1 M Tris-HCl, pH 8 **(B)**. The columns were pre-equilibrated with binding buffer containing 40 mM KCl. RNA-binding activity was measured in an aliquot (10 μl) of the extract applied to the column (**A**, **lane 0**), or in the flowthrough fractions (**A**,**B**, **lane 1**) and in fractions eluted from the resin with buffer containing 100, 200, 300, 400, and 500 mM of KCl (**A**,**B**, **lanes 2**–**6**), respectively. Final KCl concentration in the RNA-binding assay was adjusted to 200 mM.

tein was immobilized to CNBr-activated Sepharose 4B, as described under Materials and Methods. A partially purified preparation of RBS was applied to either RS-linked (Fig. 5A) or RS-free resin (Fig. 5B) in the presence of a low-ionic-strength buffer. The resins were washed with increasing concentrations of KCl, and the eluates were tested for the presence of RNA-binding activity. As demonstrated in Figure 5, while RBS was recovered in the flowthrough fraction of the RS-free column, RBS was retained by the RS-bound Sepharose-4B and eluted at 0.3 M KCl, showing that RS has the capacity to be involved in direct protein–protein interaction. Considering the cumulative data, we concluded that the recombinant RS is identical to the native RS, and that the putative protein originally selected for sequencing is indeed the regulatory component of the RNA-binding protein.

Specificity of the Interaction of RS with RBS

The selectivity of the interaction of RS with RBS was tested in the experiment illustrated in Figure 6. In this experiment, we examined the capacity of RS to modulate the activities of RBS and a second RNA-binding protein with an affinity to the mRNA for the rat cationic amino acid transporter-1 (*cat-1*) [Aulak et al., 1996], identified recently in the nuclear extract of FTO-2B cells (Y. Hod and M. Hatzoglou, to be published). RBS and *cat-1* RBP are likely to be different proteins because the mobility of the RNA–protein complexes on native polyacrylamide gels is significantly different (Fig. 6), and they are enriched in different cellular fractions (postpolysomal Vs nuclear), neither of which exhibits the other RNA-binding activity (not

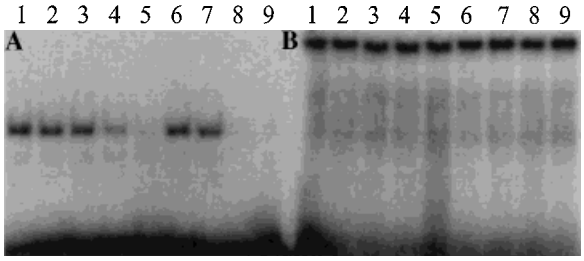

Fig. 6. Selective interaction of regulatory subunit (RS) with other proteins. RNA-binding activity in cellular fractions from FTO-2B cells enriched with RBS **(A)** and *cat-1* RBP **(B)**. RBS was determined using a 30–50% $(NH_4)_2SO_4$ cut of postpolysomal fraction (0.2 mg/ml) and Lp8 as an RNA probe [Nachaliel et al., 1993]. *cat-1* RBP was determined using a 0.5 M KCl wash of isolated nuclei (0.1 mg/ml) and ~500 nt RNA probe from the 3′-region of *cat-1* mRNA. Both activities **(A,B)** were measured in the presence of no other additions (**lanes 1**) or in the presence of 0.5, 5, 50, and 500 μg/ml (**lanes 2**–**5**) of a fraction from FTO-2B cells enriched with RS (70–90% $(NH_4)_2SO_4$ cut of postpolysomal fraction), or 0.01, 0.1, 1, and 10 μg/ml (**lanes 6**–**9**) of a purified recombinant RS (the same fraction shown in Fig. 3, lane 6).

shown). As seen, while the activity of RBS was inhibited by either a cellular fraction from FTO-2B cells enriched with RS, or a highly purified fraction of recombinant RS (Fig. 6A), none of these fractions was effective in modulating *cat-1* RBP activity (Fig. 6B), suggesting that the interaction of RS with other proteins is selective.

Intracellular Localization of RS

Indirect immunofluorescence labeling employing confocal laser microscopy was used to examine the intracellular distribution of RS (Fig. 7). In these experiments, the cells were reacted with the IgG fractions of rabbit anti-RS, as well as with mouse anti-β-tubulin, to enhance the visualization of cellular boundaries. The two proteins were distinguished by using the appropriate conjugated secondary antibodies that labeled RS and β-tubulin with green and red (Fig. 7, lanes 1 and 2), respectively. Immunolabeling of RS in FTO-2B cells shows that the polypeptide is concentrated mostly in the extranuclear region (Fig. 7A,B), and this pattern was found irrespective of cell density. Also, replacing the immune serum with preimmune serum resulted in loss of staining (not shown), suggesting that immunolabeling is highly specific. Examination of the intracellular distribution of RS in several other cells including rat liver, NIH 3T3 mouse fibroblasts, and the porcine kidney epithelial cell, LLC-PK_1, showed that, in contrast to FTO-2B cells, RS is highly concentrated in the cell nuclei. Figure 7C shows a representative immunolabeling results with LLC-PK_1 cells. Further analysis by confocal microscopy using isolated nuclei corroborated these results and showed that RS

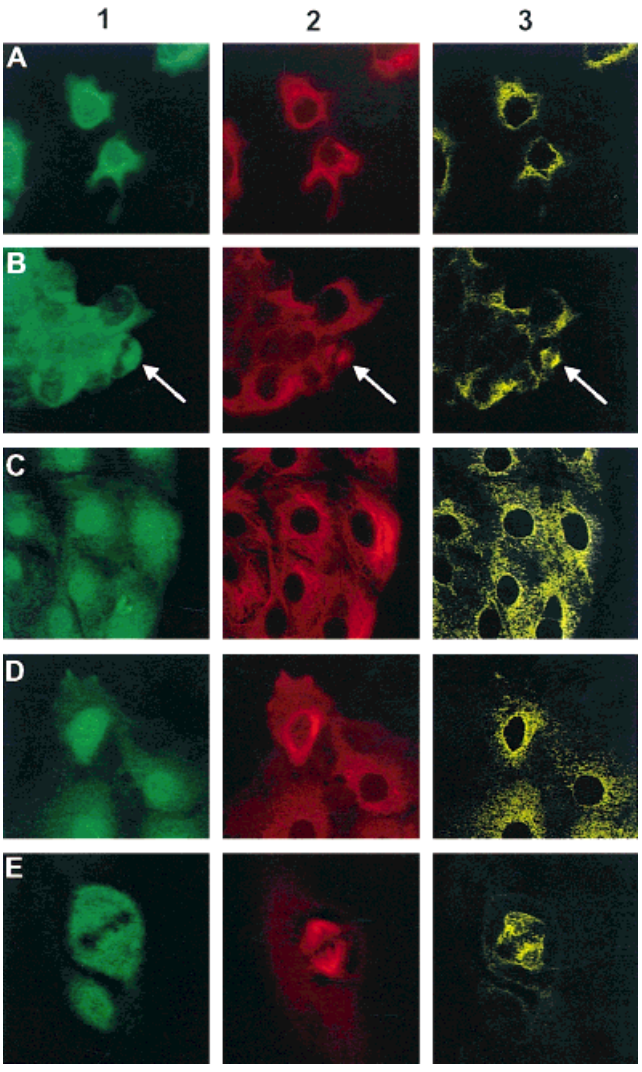

Fig. 7. Subcellular distribution of RS. Confocal microscopy analysis of RS in FTO-2B (rows A,B) and LLC-PK_1 (rows C–E) cells. LLC-PK_1 cells were exposed to colcemid (10 μM) for 3 h (row D) or vehicle itself (0.1% DMSO) (row C). Fixed cells immunolabeled with rabbit antibody to RS (**lane 1**) and mouse antibody to β-tubulin (**lane 2**) were exposed to fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and lissamine rhodamine (LRSC)-conjugated goat anti-mouse IgG. The green (representing RS) and red (representing β-tubulin) fluorescent signals in lanes 1 and 2, respectively, were overlaid by computer assistant (**lane 3**). Arrow, row B, points to a mitotic cell. See text and Materials and Methods for other details.

is localized within the nuclear interior in both FTO-2B and LLC-PK_1 cells (not shown). We concluded from these experiments that RS is commonly distributed in both the cytosol and nuclear compartments of many cells, but that its relative abundance differs in different cells. FTO-2B may represent a special class of cells where RS is more abundant in the cytoplasm than in the nucleus. Assuming that the cytosolic and nuclear forms of RS are the product of the same gene, the unique cellular distribution of RS in FTO-2B cells may be due to either overexpression of the cytosolic form and/or inhibition of its nuclear translocation.

Figure 7 also demonstrates that RS is associated with the microtubules. As seen in lane 3, superimposition of the fluorescent stainings of RS and β-tubulin resulted in yellow staining, indicating that the two proteins are co-localized both in FTO-2B and LLC-PK_1 cells (Fig. 7, rows A and C). This was further supported by the findings that the distribution of RS in the cytosol followed that of the microtubules in cells treated with colcemid (10 μM), a drug known to disrupt microtubules organization (Fig. 7D), and that during mitosis RS is found associated with the spindle (Fig. 7B,E), a microtubular-based organelle. In contrast to the typical filamentous structure of the microtubules, RS pattern is punctuated, suggesting that it is concentrated in unique centers along the microtubular filaments. While the significance of the intracellular distribution of RS is yet to be elucidated, the presence of RS in the cytosol and nuclei, and in association with microtubules, may suggest that RS is involved in more than a single cellular function.

DISCUSSION

The present study describes the initial characterization of the regulatory subunit (RS) of an RNA-binding complex. We purified the protein, identified a clone encoding RS, and sequenced the entire cDNA. Antibody prepared against the recombinant protein recognized the predicted size protein in a variety of mammalian cells, and the predicted amino acid sequence encoded by the cDNA matched that of a peptide derived from the native RS, confirming the authenticity of the clone. The present study also identifies RS as a component of a high-molecular-mass complex (RBP) that contains an RNA-binding activity (RBS), and shows that the purified recombinant RS has the capacity to bind and inhibit RBS activity, suggesting that RS is directly involved in protein–protein interaction.

A search of the database showed that RS is a novel protein because its primary sequence is unique among eukaryotic species, and it does not have any striking features that could yield a clue as to its actual activity. However, RS has a high degree of sequence similarity to that of the *E. coli* protein *ThiJ*. Both proteins are composed of almost identical numbers of amino acids (189 Vs. 191), of which 76 (42%) are identical, and an additional 48 amino acids (25%) are conservative substitutions. *ThiJ* (EC 2.7.1.49) is a kinase, catalyzes the phosphorylation of hydroxymethylpyrimidine (HMP), a precursor of thiamine pyrophosphate synthesis [Mizote et al., 1996]. Thiamine is a water-soluble B-complex vitamin (B1) playing an essential role in carbohydrate metabolism and neural function. We do not yet know what is the significance of the similarity between RS and *ThiJ*, since thiamine is an essential dietary vitamin in humans, ruling out the possibility that RS is involved in thiamine synthesis.

RS is widely expressed in many human tissues [Nagakubo et al., 1997], and several closely related sequences have been deposited in the databanks. These include sequences from human fetal brain and heart, endothelial cells, colon, and lung carcinoma, and from mouse kidney, diaphragm, and testis. The nucleotide sequence of a mouse cDNA is 85% identical to that of the human RS. In the present study, a peptide isolated from the rat RS was found identical to that from the human homologue. These results suggest that the RS gene is ubiquitously expressed, and that its encoded protein may have an important housekeeping role.

The immunostaining analysis of RS in several different cells clearly suggests that RS is bound to microtubular filaments of the cytoskeleton. Among many cellular functions, the cytoskeleton also plays an important role in mRNA metabolism, as a large fraction of poly(A)-containing mRNA is anchored to either microtubules [Ainger et al., 1993] or actin filaments [Taneja et al., 1992]. In addition, many components essential for polypeptide synthesis have been demonstrated to be complexed with the cytoskeleton. Among those are ribosomes [Toh et al., 1980; Hesketh et al., 1991], 5′-mRNA-binding protein [Zumbe et al., 1982] , and both initiation and elongation factors [Condeelis,

1995], strongly suggesting that mRNA translation may occur in association with the cytoskeleton [Hesketh, 1996].

A number of cytoskeleton-associated RNA-binding proteins that are involved in mRNA sorting have been identified [Bassell and Singer, 1997]. Among those are the *Xenopus* Vg1 RBP and the *Drosophilla* staufen. Vg1 RBP, which facilitates vegetal localization of Vg1 RNA in oocytes [Elisha et al., 1995] and other mRNAs [Schwartz et al., 1992; Litman et al., 1996], exerts its activity by direct association with specific sequence at the 3′-UTR of these mRNAs. Staufen is a double-stranded RNA binding protein [St Johnston et al., 1992], which is required for *bicoid* and *oskar* mRNAs localization [St. Johnston et al., 1991]. An RNA-binding protein involved in sorting β-actin mRNA to the leading edge of fibroblasts has been recently identified [Ross et al., 1997]. This RNA-binding protein is a component of a complex protein where one or more of the other subunits are likely to be involved in the regulation of the RNA-binding activity and β-actin mRNA sorting.

The association of RS with the microtubules, and its being a component of a complex protein with an RNA-binding activity may suggest that one potential role of RS is to link RBS to the cytoskeleton. Because RBS may be involved in the control of mRNA degradation [Nachaliel et al., 1993], an attractive hypothesis is that the cytoskeleton is a site of mRNA breakdown, as this compartment is also enriched with mRNAs and involved in its translation (discussed above). In this model, the role of RS is to sort enzyme(s) involved in mRNA degradation to the cytoskeleton either to bring them in close proximity to the cytoskeletal-bound mRNAs, or as a mean to control the activity of these degradative enzymes. The demonstration that the interaction between RS and RBS is regulated by phosphorylation [Nachaliel et al., 1993] is clearly consistent with the hypothesis that RS may play a regulatory role.

The present study also demonstrates that RS may be localized in the nuclei of rat liver cells, as well as, in several other unrelated cell lines (Fig. 7). Because nuclei do not contain RBS (to be published), it is likely that nuclear RS may be involved in other activities unrelated to RBS. For instance, DJ-1, a homologue of RS, has been reported to have the potential to transform cells in cooperation with *ras*, and that its nuclear translocation is cell-cycle dependent [Nagakubo et al., 1997]. The unique intracellular distribution of RS and its association with an RNA-binding activity [Nachaliel et al., 1993 and the present study], and its potential role in the control of cell proliferation [Nagakubo et al., 1997] and fertilization [Wagenfeld et al., 1998] suggest that RS is a multifunctional protein that may be involved in several cellular functions. Experiments to elucidate the multifaceted roles of RS are in progress.

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