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Identification of ζ-Crystallin/NADPH:Quinone Reductase as a Renal Glutaminase mRNA pH Response Element-binding Protein*

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Increased renal ammoniagenesis and bicarbonate synthesis from glutamine during chronic metabolic acidosis facilitate the excretion of acids and partially restore normal acid-base balance. This adaptation is sustained, in part, by a cell-specific stabilization of the glutaminase mRNA that leads to an increased synthesis of the mitochondrial glutaminase. A direct repeat of an 8-base AU sequence within the 3′-nontranslated region of the glutaminase mRNA binds a unique protein with high affinity and specificity. Expression of various chimeric mRNAs in LLC-PK$_1$-FBPase$^+$ cells demonstrated that a single 8-base AU sequence is both necessary and sufficient to function as a pH response element (pH RE). A biotinylated oligoribonucleotide containing the direct repeat was used as an affinity ligand to purify the pH RE-binding protein from a cytosolic extract of rat renal cortex. The purified binding activity retained the same specific binding properties as observed with crude extracts and correlated with the elution of a 36-kDa protein. Microsequencing by mass spectroscopy and Western blot analysis were used to identify this protein as ζ-crystallin/NADPH:quinone reductase. The purified protein contained eight tryptic peptides that were identical to sequences found in mouse ζ-crystallin and three peptides that differed by only a single amino acid. The observed differences may represent substitutions found in the rat homolog. A second protein purified by this protocol was identified as T-cell-restricted intracellular antigen-related protein (TIAR). However, the purified TIAR neither bound nor affected the binding of ζ-crystallin/NADPH:quinone reductase to the pH RE. Furthermore, specific antibodies to ζ-crystallin, but not TIAR, blocked the formation of the complex between the pH RE and either the crude cytosolic extract or the purified protein. Thus, ζ-crystallin/NADPH:quinone reductase is a pH response element-binding protein.

In contrast to other tissues, where glutamine metabolism is largely constitutive, the renal catabolism of glutamine is acutely activated in response to the onset of metabolic acidosis (1, 2). During normal acid-base balance, very little of the plasma glutamine is extracted and catabolized within the kidney (3). However, during metabolic acidosis renal ammoniagenesis and gluconeogenesis are greatly increased, and the kidney becomes the primary site of glutamine catabolism (4). This process generates ammonium ions and bicarbonate ions that facilitate the excretion of acids and partially restore normal acid-base balance (2). During chronic acidosis, the increased glutamine catabolism in rat kidney is sustained, in part, by increased expression of multiple genes that encode the regulatory enzymes and transport proteins that participate in this adaptive response.

The mitochondrial glutaminase (GA)[1] catalyzes the initial reaction in the primary pathway of renal glutamine catabolism. Glutaminase activity is increased 7- to 20-fold within the renal proximal convoluted tubule during chronic acidosis (5, 6). This increase results from an increased rate of glutaminase synthesis (7) that correlates with a similar increase in the level of GA mRNA (8, 9). The two forms of GA mRNA that are expressed in rat kidney result from the use of alternative polyadenylation sites. The levels of both mRNAs are coordinately affected by changes in acid-base balance. However, the rate of transcription of the GA gene is not increased during either acute (9) or chronic acidosis (10). Instead, the increase in glutaminase activity results from the selective stabilization of the GA mRNA (11).

The stabilization of the GA mRNA was initially demonstrated by stable transfection of various β-globin (βG) constructs (12) into LLC-PK$_1$-FBPase$^+$ cells, a pH-responsive porcine proximal tubule-like cell line (13). Expression of pβG produced a high level of a very stable mRNA ($t_{1/2}$ > 30 h) that was not affected by transfer of the cells to acidic medium (pH 6.9, 10 mM HCO_3^-). In contrast, pβG-GA, which encodes an mRNA containing an additional 956 bases from the 3′-nontranslated region that is common to both GA mRNAs, was expressed at significantly lower levels. The decreased expression resulted from the more rapid turnover ($t_{1/2}$ = 4.6 h) of the βG-GA mRNA. Transfer of the latter cells to acidic medium resulted in a pronounced stabilization (6-fold) and a gradual induction of the βG-GA mRNA. These studies indicated that the 3′-nontranslated region of the GA mRNA contains a pH response element (pH RE).

Experiments using additional chimeric βG constructs indicated that a 340-base segment of the GA mRNA, termed R-2, retained most of the functional characteristics of the 3′-nontranslated region (14). Mapping studies, using RNA electrophoretic mobility shift assays, demonstrated that the specific binding of a unique protein mapped to the 29-nucleotide R-2I RNA that contained a direct repeat of an 8-base AU sequence. Site-directed mutation of the direct repeat of the 8-base AU sequence completely abolished the pH-responsive stabilization of the βG-GA mRNA (15). A βG reporter construct that contained the 3′-nontranslated region of the phospho*enol*pyruvate carboxykinase mRNA, pβG-phosphoenolpyruvate carboxykinase, was designed to further test the function of the AU

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[1] The abbreviations used are: GA, glutaminase; βG, β-globin; pH RE, pH response element; MS, mass spectrometry; TIAR, T-cell-restricted intracellular antigen-related protein; PAGE, polyacrylamide gel electrophoresis.

element. When expressed in LLC-PK$_1$-FBPase$^+$ cells, the half-life of the βG-phosphoenolpyruvate carboxykinase mRNA was only slightly stabilized (1.3-fold) by growth in acidic medium. However, insertion of short segments of GA cDNA containing either the direct repeat or a single 8-base AU sequence was sufficient to impart a 5-fold pH-responsive stabilization to the chimeric mRNA. Thus, either the direct repeat or a single copy of the 8-base AU sequence is both necessary and sufficient to function as a pH RE. The apparent binding to the pH RE is increased 3-fold in cytosolic extracts prepared from LLC-PK$_1$-FBPase$^+$ cells that were grown in acidic medium (16). Extracts prepared from the renal cortex of rats that were made acutely acidotic also exhibit a similar increase in binding to the direct repeat of the pH RE. The time course for the increase in binding correlates with the temporal increase in GA mRNA. Thus, the protein that binds to the pH RE may mediate the pH-responsive stabilization of the GA mRNA.

In the current study, an affinity ligand containing the pH RE was used to purify a 36-kDa protein from a cytosolic extract of rat renal cortex. This protein binds to the R-2I RNA with the same specificity as the pH RE-binding protein that is contained in a crude extract. Through MS/MS microsequencing and Western blot analysis, the purified protein was identified as ζ-crystallin/NADPH:quinone reductase. Furthermore, antiserum specific for ζ-crystallin blocks the formation of the complex formed between the R2-I RNA and either the purified protein or the crude cytosolic extract. Thus, ζ-crystallin/NADPH:quinone reductase is a pH RE-binding protein.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats (140–160 g) were acquired from Charles River Laboratories. [α-^{32}P]UTP (specific activity, 800 Ci/mmol), horseradish peroxidase-conjugated secondary antibody, and ECL-Plus kits were obtained from Amersham Pharmacia Biotech. Restriction enzymes, RNase T1, T7 polymerase, and yeast tRNA were obtained from Roche Molecular Biochemicals and New England Biolabs. Chemicals for acrylamide gels, Micro Bio-spin chromatography columns, and protein standards were purchased from Bio-Rad. Immobilon NC membrane and Microcon columns were obtained from Millipore. RNasin was obtained from Promega. GelBond PAG films were purchased from Intermountain Scientific. DNA preparation kits were from Promega or Qiagen. Slide-a-lyzer cassettes were obtained from Pierce. Other chemicals were acquired from Sigma. Anti-sera against ζ-crystallin/NADPH:quinone reductase (17) were kindly provided by Dr. J. Samuel Zigler, Jr. (National Eye Institute). A monoclonal antibody against the T-cell-restricted intracellular antigen-related protein, TIAR (18), was kindly provided by Dr. Nancy Kedersha (Harvard Medical School).

Rat Kidney Cytosolic Extract—Rats were made acidotic by stomach loading with 20 mmol of NH_4Cl/kg of body mass and then providing 0.28 mM NH_4Cl as the sole source of drinking water. After 18–24 h, the rats were anesthetized with 1 mg of pentobarbital/kg of body mass and opened with a midline incision. The kidneys were perfused *in situ* with Krebs-Henseleit solution, then removed, decapsulated, sliced longitudinally, and placed in ice-cold Krebs-Henseleit solution. The cortex was dissected free of papilla and medulla and then cut into small pieces. The cortical tissue was placed in an equal volume of 40 mM Hepes buffer, pH 7.4, containing 100 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 10 μM leupeptin, 10 μM antipain, and 5 μg/ml phenylmethylsulfonyl fluoride and disrupted with a Dounce homogenizer. The homogenate was centrifuged for 10 min at 1,000 × *g* to pellet intact cells and nuclei. The supernatant was centrifuged at 10,000 × *g* for 10 min to pellet the mitochondria and then for 90 min at 100,000 × *g* to pellet membrane-bound organelles and polyribosomes. The final supernatant was divided into 100-μl aliquots and stored frozen at −70 °C. The protein concentration of the cytosolic extract was determined by a Bradford assay (19) using bovine serum albumin as the standard.

In Vitro Transcription of RNA—The R-2I plasmid (14) was digested with *Bss*HII and *Xba*I, and the DNA fragment containing the T7 promoter and the R-2I template was purified by electrophoresis on an 8% polyacrylamide gel. The R-2I RNA was transcribed in a 10-μl reaction mixture containing the following components: 100 ng of template DNA; 20 μCi of [α-^{32}P]UTP; 0.5 mM ATP, CTP, and GTP; 50 μM unlabeled UTP; 20 units of RNAsin; and 10 mM dithiothreitol. After the mixture was incubated at 37 °C for 1 h, 1.0 unit of RNase-free DNase was added, and the reaction mixture was incubated at 37 °C for 15 min. The RNA was then purified by chromatography on a Micro Bio-spin column, and its concentration was determined by scintillation counting.

Syntheses of unlabeled RNAs were performed using a 100-μl reaction volume lacking [α-^{32}P]UTP but containing a 0.5 mM concentration of each ribonucleotide. The appropriate templates were prepared as described previously (14, 15). The concentrations of the unlabeled RNA samples were determined by measuring their absorbance at 260 nm and using specific extinction coefficients calculated from the nucleotide composition of the individual transcripts.

RNA Electrophoretic Mobility Shift Assays—Either 3 μg of a rat renal cortical extract or 10–50 ng of purified protein were preincubated for 10 min at room temperature in a 10-μl reaction mixture containing 10 mM Hepes, pH 7.4, 25 mM potassium acetate, 2.5 mM magnesium acetate, 0.5 μg of yeast tRNA, 0.5% Igepal CA 630, 5% glycerol, 1 mM dithiothreitol, and 10 units of RNAsin. Where indicated, specific antibodies were added, and the mixture was incubated for 20 min at room temperature. Subsequently, 20 fmol of labeled R2-I RNA was added. For competition experiments, specific or nonspecific competitors (100-fold excess) were also added. The samples were incubated for 20 min at room temperature and then subjected to electrophoresis for 90 min in a 5% polyacrylamide gel at 170 V using a 90 mM Tris, 110 mM boric acid, 2 mM EDTA running buffer. The gels were dried and imaged using a PhosphorImager screen.

Synthesis of Affinity Ligand—The following oligonucleotide was synthesized by Macromolecular Resources (Ft. Collins, CO): 5′-TTAGUGUGACUCUUUAAAUAUUAAAAUAAUUACUACUAACUGUUCA-**TT**dA**TT**-3′. The 5′-end contained two deoxythymidines, and the 3′-end contained six deoxyribonucleotides including two biotinylated thymidines (Glen Research, Sterling, VA; indicated in *bold*). The remainder of the oligonucleotide was composed of a sequence of ribonucleotides derived from the GA mRNA. The underlined nucleotides are the direct repeat of the 8-base AU sequence that constitutes the pH response element of the GA mRNA (15). A comparison of the affinity ligand and the R-2I and R-2H RNAs is shown in Fig. 1.

Affinity Purification of the pH RE-binding Protein—A 1-ml sample of a rat renal cortical cytosolic extract (~20 mg of total protein) was dialyzed overnight against 1× binding buffer containing 10 mM Hepes, pH 7.4, 25 mM potassium acetate, 2.5 mM magnesium acetate, and 1 mM dithiothreitol. The following additions were made to the dialyzed extract: 40 μl of 10% Igepal CA 630, 1 mg of tRNA, 20 μmol of dithiothreitol, and 20 μl of RNAsin (40 units/μl). The sample was then incubated at 4 °C for 10 min. The biotinylated RNA ligand was centrifuged through a Micro Bio-spin P6 column. Approximately 2 nmol of the purified RNA were added, and the mixture was incubated at 4 °C for an additional 20 min. A fast protein liquid chromatography column was packed with 0.5 ml of avidin agarose (10-fold excess with respect to the biotinylated RNA) and washed extensively with 1× binding buffer. The total sample was loaded at a flow rate of 0.1 ml/min, and the column was then washed at 0.05 ml/min with 110 ml of binding buffer in which the concentrations of the potassium acetate and magnesium acetate were gradually increased 4-fold. The binding activity was then eluted with 5 ml of buffer in which the potassium acetate and magnesium acetate concentrations were linearly increased from 4× to 20×. The collected fractions (0.5 ml) were dialyzed *versus* 1× binding buffer and assayed by RNA gel shift analysis. To assess purity, samples from the collected fractions containing 10 ng of protein were separated on a 10% polyacrylamide gel containing 1% SDS and stained with 0.1% silver nitrate.

Western Blots—The proteins contained in 150-μl samples of the individual column fractions were concentrated by chloroform/methanol precipitation (20) and washed with methanol. The protein pellets were then resuspended in Laemmli sample buffer. The samples were separated by SDS-PAGE using a 10% separating gel, transferred to a nitrocellulose membrane, and incubated with either a 1:500 dilution of ζ-crystallin/NADPH:quinone reductase antiserum or a 1:2000 dilution of TIAR monoclonal antibody. The membrane was then incubated with a 1:1500 dilution of horseradish peroxidase-conjugated secondary antibody. Images were developed with the ECL-Plus kit and visualized on a Storm system (Molecular Dynamics).

Preparation of Sample for Microsequencing—The electrophoresis unit and all glassware were incubated overnight with 2% cleansing concentrate (Bio-Rad), followed by extensive washing with doubly deionized H_2O. The purified protein was concentrated, subjected to SDS-PAGE, and stained with freshly prepared Coomassie Blue. The

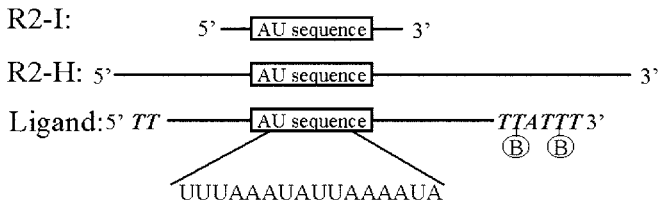

FIG. 1. **Comparison of the RNAs used for gel shift assays and as a ligand for affinity purification.** All three RNAs contain the pH RE from the GA mRNA that is a direct repeat of two 8-nucleotide AU sequences. In total, the R2-I and R2-H RNAs contained 29 and 72 nucleotides, respectively. The affinity ligand contained 44 ribonucleotides and the indicated 5′- and 3′-deoxyribonucleotides (in *italics*) including two biotinylated thymidines (labeled *B*).

36-kDa protein band was excised, sealed in an Eppendorf tube, and sent to the Harvard Microchemistry Facility (William S. Lane) for analysis. The protein was subjected to in-gel reduction, carboxyamidomethylation, and tryptic digestion. Multiple peptide sequences were determined by microcapillary reverse-phase chromatography coupled to a Finnigan LCQ quadrupole ion trap mass spectrometer. Interpretation of the resulting MS/MS spectra were facilitated by software developed in the Harvard Microchemistry Facility and by data base correlation with the algorithm SEQUEST (21).

RESULTS

Affinity Purification of pH RE-binding Protein—A biotinylated RNA (Fig. 1) was designed as a ligand for the affinity purification of the pH RE-binding protein. When used as a probe in an RNA gel shift assay, the affinity ligand bound to the pH RE-binding protein with an affinity equivalent to that observed with the R-2I RNA (data not shown). Therefore, a cytosolic extract from rat kidney cortex was incubated with a sufficient excess of the affinity ligand to bind all of the pH RE-binding protein. The resulting complex was then applied to a fast protein liquid chromatography column that had been packed with avidin-agarose and equilibrated with the binding buffer. The column was washed extensively and then eluted with binding buffer in which the concentrations of potassium acetate and magnesium acetate were increased proportionately. The eluant through the wash with 4× binding buffer (100 mM potassium acetate and 10 mM magnesium acetate) contained very little R-2I RNA binding activity. Thus, 0.5-ml fractions were routinely collected only as the steep gradient of 4× to 20× binding buffer was applied to the column. An aliquot of each fraction was analyzed by SDS-PAGE and silver staining. As shown in Fig. 2*A*, two proteins were recovered in these fractions. The elution of a 43-kDa protein peaked in fraction 4, and a 36-kDa protein peaked in fraction 7. When larger volumes of the peak fractions were concentrated by chloroform/methanol precipitation and subjected to SDS-PAGE, additional protein bands were observed. However, the additional proteins constituted less than 5% of the total protein (data not shown).

To measure the R-2I RNA binding activity, the fractions were dialyzed overnight against 1× binding buffer and then analyzed in an RNA electrophoretic mobility shift assay. As shown in Fig. 2*B*, the amount of R2-I-protein complex formed closely correlates with the elution profile for the 36-kDa protein. The ratio of binding activity to the amount of 36-kDa protein quantified by silver staining was nearly constant across the elution profile (Fig. 2*C*), suggesting that this protein is the pH RE-binding protein. To further support this conclusion, the affinity purification was repeated using a shallower gradient of the 4× to 20× binding buffer to separate the proteins into 28 fractions. Analysis by SDS-PAGE and silver staining (data not shown) indicated that fraction 13 contained only the 43-kDa protein, whereas fraction 20 contained only the 36-kDa protein. After dialysis, the two fractions were tested for R2-I RNA binding activity (Fig. 3). Again, only the fraction containing the

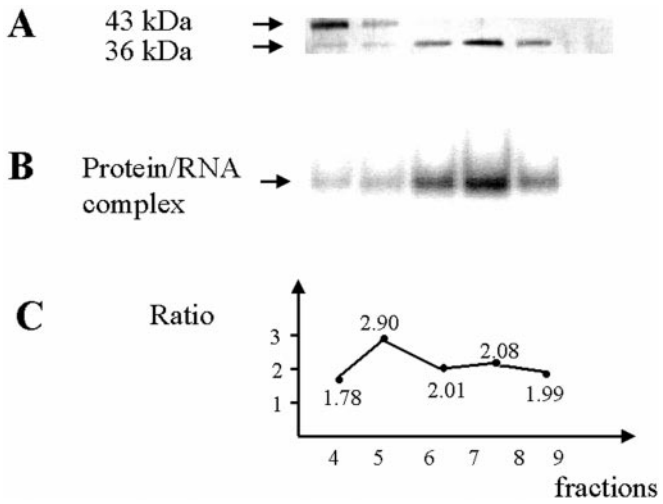

FIG. 2. **Analyses of samples that were collected from the avidin-agarose column and then dialyzed overnight against 1× binding buffer.** *A*, aliquots of *fractions 4–9* were separated by 10% SDS-PAGE and silver-stained. *B*, aliquots of the same fractions were analyzed for binding activity using an RNA gel shift assay containing 25 fmol of ^{32}P-labeled R2-I RNA. *C*, specific binding activity was estimated as the ratio of the digitized intensities of the protein-RNA complex observed in *B* to those of the 36-kDa protein from *A*.

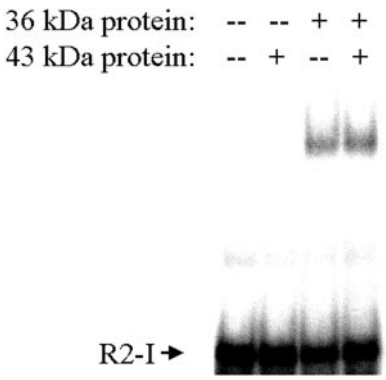

FIG. 3. **Comparison of the binding activities observed with fractions that contained either the 36- or 43-kDa proteins.** Samples containing ~10 ng of protein from each fraction were incubated with 25 fmol of ^{32}P-labeled R2-I RNA and then separated on a native polyacrylamide gel.

36-kDa protein formed an RNA-protein complex. The fraction containing the 43-kDa protein neither bound the R-2I RNA nor affected the level or mobility of the complex formed with the 36-kDa protein. Thus, the purified 43-kDa protein does not form a specific complex with the R-2I RNA.

Specificity of the Purified pH RE-binding Protein—Competition experiments were performed to determine the specificity of the binding interaction between the purified pH RE-binding protein and the R2-I RNA (Fig. 4). For a control, the same binding experiment was performed using a cytosolic extract of rat renal cortex. The complexes produced with both protein samples exhibited identical electrophoretic mobilities. A 100-fold excess of various unlabeled RNAs was added to compare specificity. The mut1, mut2, and mut3 competitors (14) are R2-I RNAs in which the first, second, and both of the AU-rich regions, respectively, were mutated. The $(AUUU)_5A$ RNA contains five tandem repeats of an AUUUA sequence. This sequence binds a variety of AUUUA-binding proteins (22). The pBS RNA was transcribed from the multicloning site of pBlueScript SK(+) and was included as a nonspecific competitor. Finally, the R2-H RNA is a 76-nucleotide sequence that includes all of R2-I plus flanking sequences from the GA mRNA

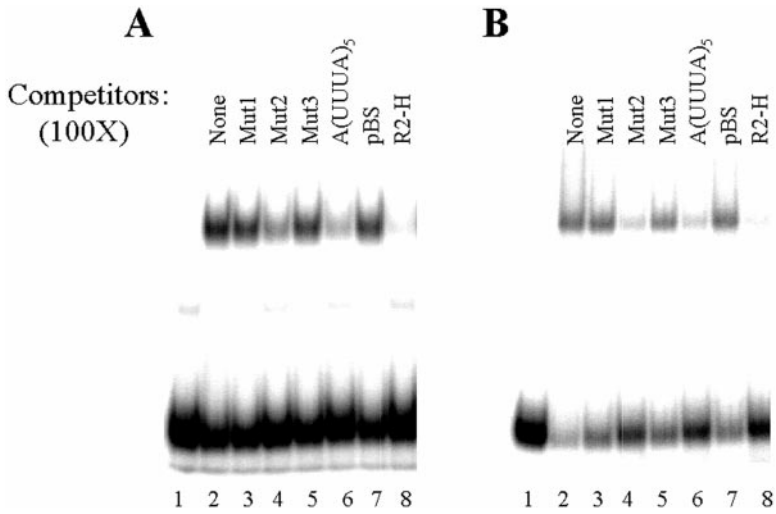

FIG. 4. **Comparison of the binding specificity of the pH RE binding activity of a crude extract (*A*) and the purified protein (*B*).** In both experiments, 25 fmol of ^{32}P-labeled R2-I RNA was incubated either in the absence (*lane 1*) or presence (*lanes 2–8*) of either 3 μg of a cytosolic extract of rat kidney cortex or 10 ng of purified pH RE-binding protein. Samples in *lanes 3–8* also contained a 100-fold excess of the indicated competitor. *pBS*, pBlueScript.

(Fig. 1*A*). Formation of the protein-R2-I complex with either the purified protein or the crude extract was almost completely inhibited by the R2-H RNA. In addition, both complexes were slightly competed by the $(AUUU)_5A$ and mut2 RNAs, but not any of the other RNAs. Thus, the purified pH RE-binding protein exhibits a specificity identical to that observed with the crude extract.

MS/MS Microsequencing—The purified and concentrated pH RE-binding protein was subjected to SDS-PAGE and stained with Coomassie Blue. The 36-kDa protein band was cut from the gel, digested with trypsin, and sequenced by mass spectroscopy. A total of 13 peptides were identified. The peptides were classified into two groups. Class I peptides include eight sequences that are identical to tryptic peptides derived from the 36-kDa mouse ζ-crystallin/NADPH:quinone reductase protein. Three other Class I peptides differ from tryptic peptides of mouse ζ-crystallin by a single amino acid substitution and may represent sequences from the rat homolog. The two Class II peptides correspond to tryptic peptides contained in the 43-kDa mouse RNA-binding protein, TIAR.

The identities of the two proteins contained in the purified preparation of the pH RE-binding protein were confirmed by Western blot analysis (Fig. 5). Antisera produced against the full-length ζ-crystallin/NADPH:quinone reductase purified from a guinea pig lens (17) reacted with only the single 36-kDa protein when tested *versus* a crude cytosolic extract of rat renal cortex (data not shown). When used *versus* a purified sample containing both the 43- and 36-kDa proteins (Fig. 5*A*), the anti-ζ-crystallin antibody again bound only to the 36-kDa protein (Fig. 5*B*). Similarly, a monoclonal antibody (18), which is specific for TIAR, bound to only the 43-kDa protein. Thus, a slight contamination of the isolated 36-kDa protein band with the larger protein accounts for the two Class II peptides that were identified by microsequencing.

Immunoblocking assays were performed to confirm that ζ-crystallin/NADPH:quinone reductase is a pH RE-binding protein (Fig. 6*A*). Preincubation of a cytosolic extract of rat renal cortex with increasing amounts of anti-ζ-crystallin antiserum completely blocked the formation of the specific R2-I RNA-protein complex. The same inhibition pattern was observed when the gel shifts were performed with the purified pH RE-binding protein (data not shown). The observed inhibition was specific, because anti-glutaminase antiserum (23) had no effect on the complex formation (Fig. 6*A*). The affinity ligand used to purify the pH RE-binding protein was slightly longer than the R2-I RNA probe. Thus, labeled R-2H RNA, which contains all of the ribonucleotides present in the RNA affinity ligand, was

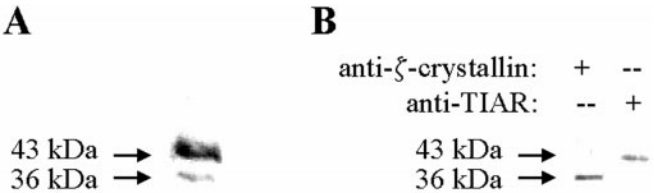

FIG. 5. **Western blot analysis of the two peptides contained in the purified preparation of the pH RE-binding protein.** *A*, the fractions from an avidin-agarose column that contained pH RE binding activity were pooled, and an aliquot was separated by 10% SDS-PAGE and silver-stained. *B*, an identical sample was used for Western blot analysis with either antiserum against ζ-crystallin/NADPH:quinone reductase or a monoclonal antibody *versus* TIAR.

synthesized and used as a probe for gel shift analysis (Fig. 6*B*). Because of the larger size of this probe, the resulting complex was digested with RNase T1 before electrophoresis. Again, the resulting complex is completely blocked by preincubation with anti-ζ-crystallin antiserum but is not affected by pretreatment with antibody specific for TIAR. Thus, ζ-crystallin/NADPH:quinone reductase binds with high affinity and specificity to the pH RE.

DISCUSSION

RNA-binding proteins play an important role in the turnover of mRNAs (24). For example, mRNAs that encode various cytokines (25), transcription factors (26), and other immediate-early gene products (27) generally turn over with half-lives of less than 1 h. Specific AU-rich elements within the coding sequence or the 3′-nontranslated region of the mRNAs function as instability elements (22). They recruit proteins that enhance 3′-deadenylation and the loss of poly(A)-binding proteins, leading to the rapid exonucleolytic degradation of the mRNA. Alternatively, the turnover of an mRNA may be initiated by a site-specific endonucleolytic cleavage that generates sites for rapid exonucleolytic degradation (28, 29). Selective stabilization of the latter class of mRNAs is mediated by sequence-specific binding of unique proteins that inhibit the endonucleolytic cleavage.

In the current study, an affinity ligand was used to purify from rat kidney cortex the protein that binds to the pH RE of the GA mRNA. The purified protein formed a complex with the R-2I RNA that retained the same electrophoretic mobility and specificity as observed with the crude cytosolic extract. In both cases, the observed binding was strongly competed by an oligonucleotide (R-2H) that contained both AU elements. When characterized as independent binding sites, the initial 8-nucle-

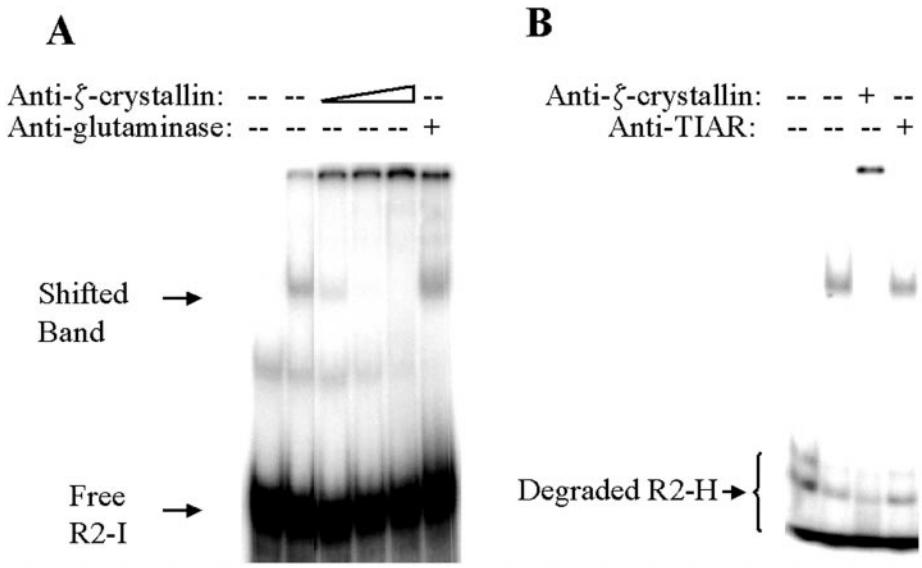

FIG. 6. **Immunoblocking analysis of the pH RE-binding protein.** *A*, samples containing either zero (*lane 1*) or 3 μg (*lanes 2–6*) of a crude extract were preincubated with increasing amounts (0.25, 0.5, or 1 μg) of ζ-crystallin antiserum or 1 μg of glutaminase antiserum. Then 25 fmol of ^{32}P-labeled R2-I RNA was added, and the samples were separated on a native polyacrylamide gel. *B*, samples containing either zero (*lane 1*) or 5 ng of purified protein (*lanes 2–4*) were preincubated with either 1 μg of ζ-crystallin antiserum or 4 μg of TIAR monoclonal antibody. Then 10 fmol of ^{32}P-labeled R2-H RNA was added, and the samples were digested with RNase T1 and separated on a native polyacrylamide gel.

otide element of the direct repeat was shown to have greater affinity for the pH RE-binding protein.[2] These data are consistent with the observation that the mut2 oligonucleotide, which retains the initial 8-base element, functions as a weak competitor. The $(AUUU)_5A$ oligonucleotide was previously shown to be a weak competitor (15). None of the other tested oligonucleotides was able to compete the specific binding observed with either the crude extract or the purified pH RE-binding protein.

The preparation of purified pH RE-binding protein contained two proteins that were identified by MS/MS sequencing and Western blot analysis. One of the proteins was the TIAR, a well known RNA-binding protein that contains three RNA recognition motifs (30). TIAR may participate in Fas-mediated apoptotic cell death (18, 31). It also binds to the AU-rich element that mediates the translational regulation of tumor necrosis factor α mRNA (32). An *in vitro* analysis of its binding preference revealed that TIAR also has an affinity for RNAs that contain short stretches of uridylate residues (30). This broad specificity may account for the purification of TIAR by the AU-rich pH RE affinity ligand. However, the purified TIAR failed to bind to either the R-2I or the longer R-2H RNA. In addition, specific antibodies to TIAR did not block formation of specific complexes that are formed by incubating either RNA with a cytosolic extract of rat kidney cortex. Thus, it is unlikely that TIAR functions as a pH RE-binding protein.

The second protein identified in the purified preparation of the pH RE-binding protein was ζ-crystallin/NADPH:quinone reductase. ζ-crystallin constitutes 10% of the total protein present in the lens of hystricomorph rodents (33) and camelids (34). In these species, the ζ-crystallin gene contains an alternative promoter that accounts for its lens-specific overexpression (17, 35, 36). Similar to other lens crystallins with a limited phylogenetic distribution, ζ-crystallin also has a catalytic activity and is expressed at enzymatic levels in various tissues of different species (17, 37, 38). ζ-crystallin possesses a novel NADPH-dependent quinone oxidoreductase activity that reduces various quinones through the sequential transfer of single electrons (33).

ζ-Crystallin/NADPH:quinone reductase was not previously known to function as an RNA-binding protein. However, the ability of bovine ζ-crystallin to bind to different forms of DNAs had been quantified through an ELISA assay (39). It preferentially binds to double-stranded Z-DNA and to single-stranded DNA but has a 5–7-fold lower affinity for double-stranded B-DNA. The sequence specificity of the observed interactions was not examined. However, the binding of ζ-crystallin to single-stranded DNA was effectively competed by NADPH. Thus, the NADPH binding site of ζ-crystallin may constitute a portion of its DNA binding site. The observation that antiserum *versus* mouse ζ-crystallin reacts specifically with the 36-kDa protein contained in the purified pH RE-binding protein and blocks formation of the specific complex formed with the pH RE indicates that the ζ-crystallin/NADPH:quinone reductase also has a high affinity binding site for the AU-rich pH RE.

The onset of metabolic acidosis may activate a signal transduction pathway that results in increased expression and/or covalent modification of the pH RE-binding protein. Identification of ζ-crystallin/NADPH:quinone reductase as a pH RE-binding protein will facilitate the characterization of the process by which this binding activity is enhanced during acidosis and the mechanism by which this interaction leads to the cell-specific stabilization of the GA mRNA.

[2] J. Schroeder and N. P. Curthoys, unpublished data.

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