pH-responsive stabilization of glutamate dehydrogenase mRNA in LLC-PK₁-F⁺ cells

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Schroeder, Jill M., Wenlin Liu, and Norman P. Curthoys. pH-responsive stabilization of glutamate dehydrogenase mRNA in LLC-PK₁-F⁺ cells. Am J Physiol Renal Physiol 285: F258–F265, 2003.—During chronic metabolic acidosis, the adaptive increase in rat renal ammoniagenesis is sustained, in part, by increased expression of mitochondrial glutaminase (GA) and glutamate dehydrogenase (GDH) enzymes. The increase in GA activity results from the pH-responsive stabilization of GA mRNA. The 3′-untranslated region (3′-UTR) of GA mRNA contains a direct repeat of an eight-base AU-rich element (ARE) that binds 3′-crystallin/NADPH:quinone reductase (3′-crystallin) with high affinity and functions as a pH-response element. RNA EMSAs established that 3′-crystallin also binds to the full-length 3′-UTR of GDH mRNA. This region contains four eight-base sequences that are 88% identical to one of the two GA AREs. Direct binding assays and competition studies indicate that the two individual eight-base AREs from GA mRNA and the four individual GDH sequences bind 3′-crystallin with different affinities. Insertion of the 3′-UTR of GDH cDNA into a β-globin expression vector (βG) produced a chimeric mRNA that was stabilized when LLC-PK₁-F⁺ cells were transferred to acidic medium. A pH-responsive stabilization was also observed using a βG construct that contained only the single GDH ARE and a destabilizing element from phosphoenolpyruvate carboxykinase mRNA. Therefore, during acidosis, the pH-responsive stabilization of GDH mRNA may be accomplished by the same mechanism that affects an increase in GA mRNA.

renal ammoniagenesis; metabolic acidosis; posttranscriptional regulation

RENSAL CATABOLISM OF GLUTAMINE is increased significantly following the onset of metabolic acidosis (27). The resulting increases in renal ammoniagenesis and gluconeogenesis contribute to the maintenance of acid-base balance (1). In rat kidney, this adaptation is sustained during chronic acidosis, at least in part, by an increased expression of mitochondrial glutaminase (GA) and glutamate dehydrogenase (GDH), cytosolic phosphoenolpyruvate carboxykinase (PEPCK), and various transport proteins (7). The resulting increases in the activities of these enzymes and transport systems contribute to the increased production and vectorial transport of ammonium and bicarbonate ions to facilitate the excretion of acids and partially compensate for the decrease in blood pH, respectively. The increased level of PEPCK results from an increased rate of transcription of the PEPCK gene (18). However, the increases in GA and GDH are caused by stabilization of their respective mRNAs (19, 20).

A protein in rat renal cortical cytosolic extracts was observed to bind with high affinity and specificity to the 3′-untranslated region (3′-UTR) of GA mRNA (23). This protein was subsequently identified as 3′-crystallin/NADPH:quinone reductase (3′-crystallin) (29). The protein-binding element within GA mRNA was mapped to a 29-base sequence that contained a direct repeat of two 8-base AU-rich elements (AREs) (23). The function of this sequence was established by characterizing the effect of medium pH on the half-lives (t₁/₂) of various chimeric β-globin (βG) mRNAs that were stably expressed in LLC-PK₁-F⁺ cells (21). The inclusion of a 76-nt GA mRNA segment containing the direct repeat of the two AREs was sufficient to produce a pH-responsive stabilization of a nonresponsive βG-PEPCK mRNA. Furthermore, when the two AREs within a βG-GA mRNA were mutated, the pH-responsive stabilization was abolished. The cumulative data indicate that the two AREs within GA mRNA function as a pH-response element (pHRE) and that enhanced binding of 3′-crystallin to this sequence during acidosis may mediate the stabilization of GA mRNA (7, 22).

Previous experiments suggest that the adaptive increase in rat renal GDH mRNA is also mediated through stabilization of its mRNA. For example, the increase in GDH mRNA following the acute onset of metabolic acidosis occurs in the same tubular segments (8, 33, 34) and with similar kinetics as observed for GA mRNA (20). For both mRNAs, there is an 8- to 10-h lag between the onset of acidosis and the initial increase in mRNA levels. In addition, when LLC-PK₁-F⁺ cells were transferred to acidic medium (pH 6.9) and treated with actinomycin D to inhibit transcription, an apparent threefold stabilization of endogenous GDH mRNA was observed (20). These data suggest that GDH mRNA may be stabilized by the same mechanism that is used to stabilize GA mRNA. The 3′-UTR of rat GDH

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Macromolecular Resources (Ft. Collins, CO). The sequences of the coding strands of the oligonucleotides are the following:

5’-GTACCTGTGACTCTTTAAATAATTACTGACT-3’
[GA(R2-IA)]

5’-GTACCTGTGACTCTTTAAATAATTACTGACT-3’
[GA(R2-IB)]

5’-GTACCTTTCCTTATAAGTTTTCT-3’ (GDH1)
5’-GTACCTTGAAACGCTAAATAGGAAATT-3’ (GDH1)
5’-GTACCAACATATCCTAAAATAGGTCTCT-3’ (GDH3)
5’-GTACCCAGATTATATTATAAGATAGT-3’ (GDH4)

The italicized letters designate the AREs. The resulting double-stranded DNAs encode the ARE sequences and form Asp718 and XbaI overhangs. The annealed oligonucleotides were inserted into pBSK that had been restricted with Asp718 and XbaI.

A DNA template containing a T7 promoter was obtained by digesting the pGDH1–4 plasmid with BssHII, which flanks the multi-cloning site of pBSK. Generating the other templates required additional digestion with XbaI and SacI. XbaI cleaves DNA immediately after the ARE sequence, and SacI cleaves the similarly sized non-template DNA into two pieces so that the template and promoter-less DNA fragments can be separated on an 8% polyacrylamide gel. The templates were extracted from the gel using the crush and soak method (26). The eluted DNA was precipitated with 2 vol of 100% ethanol, washed with 200 μl 70% ethanol, and resuspended in 11 μl diethylpyrocarbonate-treated water. In vitro transcription reactions using T7 RNA polymerase to synthesize 32P-labeled and unlabeled RNAs were performed as described previously (23). The concentrations of the 32P-labeled and unlabeled RNAs were determined by scintillation counting and by measuring the absorbance at 260 nm and using specific extinction coefficients calculated from the nucleotide composition, respectively.

A gel electrophoretic mobility shift assay. This assay was performed as reported previously (23). Briefly, 10–50 ng of purified ζ-crystallin were incubated for 10 min at room temperature in a 10-μl reaction containing 10 mM HEPES, pH 7.4, 25 mM potassium acetate, 2.5 mM magnesium acetate, 2 μg yeast tRNA, 0.5% Nonidet P-40, 5% glycerol, 1 mM dithiothreitol, and 10 U of RNasin. Approximately 20 fmol of labeled RNA were then added, and the sample was incubated at room temperature for 20 min. For the competition studies, a 30-, 100-, or 300-fold excess of an unlabeled RNA was added along with the labeled RNA. To compare the binding of the GDH1–4 and GA(R2-1) RNAs, the samples were also incubated for 10 min with 15 U of RNase T1 to reduce the length of the GDH1–4 RNA. The samples were subjected to electrophoresis for ~2 h at 170 V on a 5% polyacrylamide gel using a 90 mM Tris, 110 mM boric acid, 2 mM EDTA running buffer. Gels were then dried and exposed to a PhosphorImage screen.

βG expression vectors. Various βG constructs were synthesized to contain either the 3’-UTR of the GDH cDNA or an individual ARE. A 930-nt segment containing the 3’-UTR of GDH cDNA was PCR-amplified from pGDH1–4 using primers that added SpeI and XbaI sites to the 5‘- and 3‘-ends, respectively. The PCR product was cloned into the SrfI site of pCR-Script-SK (+), and the SpeI/XbaI fragment was excised from the plasmid and inserted into the XbaI site within the multi-cloning site of pBG (17). The pBG-GA(R2-1) and pBG-GA vectors were constructed by annealing complimentary oligonucleotides (Macromolecular Resources, Ft. Collins, CO) that encode the GA(R2-1) or the GDH4 sequence and form SpeI and XbaI overhangs and inserting them into the XbaI site of pBG. The coding sequences of the GA(R2-1) and GDH4 oligonucleotides are 5’-CTAGTTCCTTAAAAATATTTAATTAC-3’.
ATTCTAAAT-3’ and 5’-CTAGTAGACATTATTATAGAAAATGG-3’, respectively. The italicized letters in the oligonucleotide sequences designate the AREs. The pβG-GDH4-PEPCK vector was constructed by K. Propst. Complementary oligonucleotides (Macromolecular Resources) that encode the GDH4 sequence and form XbaI and Nhel overhangs were annealed and inserted into the XbaI and Nhel sites of pGEM4Z-PEPCK (24). The sequence of the coding strand of the GDH4 oligonucleotide is 5’-CTAGTAGACATTATTATAGAAAATGG-3’, where the bold italicized letters designate an EcoRV site that was used to detect the presence of the inserted sequence, and the italicized lightface letters designate the ARE. The pGEM4Z-GDH4-PEPCK vector was digested with XbaI and SpeI to obtain the GDH4-PEPCK sequence that was inserted into XbaI/SpeI-digested pβG to produce pβG-GDH4-PEPCK.

**Cell culture.** LLC-PK1-F+ cells were obtained from Gerhard Gstauthaler and cultured as described previously (16). Cells were grown in a 50:50 mixture of DMEM and Ham’s F-12 containing 5 mM glucose and 10% fetal bovine serum at 37°C in a 5% CO2-95% air atmosphere. Normal medium (pH 7.4) contains 25 mM sodium bicarbonate, whereas acidic medium (pH 6.9) contains 10 mM sodium bicarbonate supplemented with 15 mM sodium chloride to maintain an equivalent osmolarity and sodium ion concentration. LLC-PK1-F+ cell lines that stably express the chimeric mRNAs were produced by transfection of 3-day postsplit cells with calcium phosphate-precipitated DNA (3) and selection with medium containing 0.8 mg/ml G418. The medium was changed every 2 days. After 14–21 days, three 10-cm plates containing multiple colonies were combined. Following the next split, the cells were grown in normal medium containing 0.2 mg/ml G418.

**Analysis of T1.** The various transfected LLC-PK1-F+ cell lines were generally split 1:10 and grown for 7–10 days in pH 7.4 medium containing 0.2 mg/ml G418. They were then maintained in pH 7.4 medium without G418 for 24 h and subsequently treated for 12 h with normal or acidic medium. At time 0, 65 μM 5–6 dichloro-1-β-ribofuranosylbenzimidazole (DRB), a specific inhibitor of RNA polymerase II transcription (11), dissolved in 95% ethanol was added to each plate. An equivalent concentration of 95% ethanol was added to control plates. The final concentration of ethanol never exceeded 0.5%. At 0, 3, 6, and 9 h post-DRB treatment, total cellular RNA was isolated using the method of Chomczynski and Sacchi (6). The RNA concentration was determined by measuring the absorbance of the RNA at 260 nm.

**Northern blot analysis.** A 507-bp fragment of rabbit βG cDNA was excised by restricting pRSV-βG (15) with HindIII and BglII. A 2.0-kb fragment of the 18S ribosomal RNA cDNA from Acanthamoeba castellanii was excised by restricting pAR2 with HindIII and EcoRI (9). The fragments were separated on 1% agarose gels, excised, and purified using a GENECLEAN kit. A synthesis of oligolabeled cDNA probes and Northern blot analysis was performed as described previously (17). The blots were exposed to a PhosphorImager screen. The percentage of radioactivity contained in the RNA/protein complex is indicated as % shifted. The reported data are representative of 3 separate experiments.

**RESULTS**

An RNA/protein complex was formed when GDH1–4 RNA, containing the entire 3′-UTR of GDH mRNA (bases 1696–2874), was incubated with purified ζ-crys-
tallin (Fig. 1). The shifted complex had the same mobility as the complex that is formed when ζ-crystallin is bound to GA(R2-I) RNA. However, relative to the GA(R2-I)/ζ-crystallin complex, very little of the total radioactivity is contained in the GDH1–4/ζ-crystallin complex. The observed difference could be due, in part, to the relative sizes of the two RNAs. To resolve the unbound GDH1–4 RNA from the shifted complex, it was necessary to treat the samples with RNase T1. This treatment had no effect on the complex formed with GA(R2-I) RNA. Thus most, if not all, of the nucleotides in bound GA(R2-I) RNA are protected from digestion. However, only a few of the ~1,200 nucleotides within GDH1–4 RNA are retained in the complex. As a result, most of the label in GDH1–4 RNA is digested and migrates as short oligonucleotides, whereas nearly all of the bound GA(R2-I) RNA appears in the shifted complex. Despite this difference, the observed complex indicates that the 3′-UTR of GDH mRNA contains one or more elements that bind ζ-crystallin.

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<tr>
<th>GA(R2-I)</th>
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<td>ζ-crys</td>
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Fig. 1. Binding of ζ-crystallin to the R2-I RNA segment of glutaminase [GA(R2-I)] and glutamate dehydrogenase oligonucleotide (GDH1–4) RNAs. GA(R2-I) RNA is a 29-base segment from the 3′-untranslated region (UTR) of GA mRNA that contains the direct repeat of the two 8-base AU-rich elements (AREs). GDH1–4 RNA (1.2 kb) contains the entire 3′-UTR of the GDH mRNA. Either 2 (lanes 3, 4, and 6) or 4 μl (lane 2) of purified ζ-crystallin (ζ-crys) were added to the samples. Samples in lanes 2 and 4–7 were treated with RNase T1 to digest the unbound RNA. The RNA/protein complexes were resolved on a nondenaturing polyacrylamide gel. The gel was then dried and imaged with a PhosphorImager screen. The arrow indicates the position of ζ-crystallin/RNA complex. The percentage of total radioactivity contained in the RNA/protein complex is indicated as % shifted. The reported data are representative of 3 separate experiments.
To determine whether \( \zeta \)-crystallin can bind to individual eight-base ARE sequences, it was necessary to synthesize short RNAs of similar lengths that contain a single ARE. As a control, RNAs containing the individual AREs from GA mRNA were synthesized. GA(R2-IA) RNA contained the UUUAAAUA element (bases 2596–2603), and GA(R2-IB) RNA contained the UUAAAUUA element (bases 2604–2611) in the context of the surrounding sequence of the 3′-UTR of GA mRNA (28). Purified \( \zeta \)-crystallin binds to the two RNAs to form complexes that have mobilities identical to those observed with GA(R2-I) RNA (Fig. 2). Thus it is unlikely that multiple copies of \( \zeta \)-crystallin bind to GA(R2-I) RNA even though it contains two potential binding elements.

Experiments were also performed to determine whether \( \zeta \)-crystallin binds to the individual eight-base AREs from the 3′-UTR of GDH mRNA. The sequences and locations of the AREs within the 3′-UTR of GDH are UUUAAGUA (GDH1; bases 1973–1980); CUAAAAUA (GDH2; bases 2313–2320); UUCAAUA (GDH3; bases 2537–2544); and UUUAUUA (GDH4; bases 2749–2756) (10). Purified \( \zeta \)-crystallin binds to each of the RNAs containing a single ARE from the GDH 3′-UTR (Fig. 2) but to a lesser extent than observed for GA(R2-I) RNA or the individual GA AREs. Of the four GDH AREs, GDH2 and GDH4 RNAs demonstrate the highest apparent affinity for \( \zeta \)-crystallin. Thus the individual GDH AREs can function as binding elements for \( \zeta \)-crystallin. An additional, more slowly migrating band was reproducibly observed only with GDH4 RNA.

Then composition or the significance of this band is unknown.

Competition studies were performed to assess the relative affinity of \( \zeta \)-crystallin binding to individual GA and GDH AREs. Purified \( \zeta \)-crystallin was bound to \( ^{32}P \)-labeled GA(R2-I) RNA, and increasing amounts of unlabeled RNAs were added as competitors. Competition studies demonstrated that a 100-fold excess of unlabeled GA(R2-IA) RNA or GA(R2-IB) RNA was required to produce a level of competition similar to that observed with a 30-fold excess of GA(R2-I) RNA (Fig. 3). Thus the two individual elements exhibit similar affinities for \( \zeta \)-crystallin. Furthermore, the relative affinity for the two individual elements is approximately one-third of that for the RNA that contains the direct repeat of the individual binding sites.

RNAs containing individual GDH AREs were also tested as competitors of labeled GA(R2-I) RNA (Fig. 4). GDH2 and GDH4 RNAs were more effective competitors than GDH3 and GDH1 RNAs. A 300-fold excess of GDH2 or GDH4 RNA competes slightly less effectively than a 100-fold excess of GA(R2-I) RNA. In contrast, GDH1 and GDH3 RNAs were weaker competitors. Thus the competition pattern observed with individual GDH elements confirms the results of the direct binding studies. The lower bands observed when GA(R2-IA) and GA(R2-IB) RNAs (Fig. 2) and GDH2 RNA (Fig. 3) were added as competitors represent undissociated dimers of \( ^{32}P \)-labeled GA(R2-I) RNA.

Functional studies were performed to determine whether the 3′-UTR of GDH mRNA is sufficient to produce a pH-responsive stabilization of a reporter
mRNA. This was accomplished by measuring the half-lives of various chimeric βG mRNAs expressed in LLC-PK₁-F⁺ cells treated with either normal (pH 7.4, 25 mM HCO₃⁻) or acidic (pH 6.9, 10 mM HCO₃⁻) medium. The pβG plasmid contains a promoter derived from the Rous sarcoma virus long terminal repeat, a transcription start site, the coding region from rabbit βG genomic DNA containing three exons and two introns, a multicloning site, and a 3'-UTR of bovine growth hormone (bGH) cDNA (17).

As a control, pβG was stably expressed in the split of LLC-PK₁-F⁺ cells used in the present experiments. Neither the level nor the t1/2 of βG mRNA was affected by growing the cells in either normal or acidic medium (data not shown). As a second control, the LLC-PK₁-F⁺ cells were also stably transfected with the pβG-GA plasmid that contains 955-bases from the 3'-UTR of GA mRNA (17). The pβG-GA vector produced an unstable mRNA that had a 1/2 of t1/2 (12 h) when the cells were transferred to acidic medium (Fig. 6). When the same cells were transferred to acidic medium, the chimeric RNA was degraded with a t1/2 of >30 h. This indicates that the sequence contained within the GA(R2-I) region of GA 3'-UTR is

tive stabilization to GDH mRNA in response to treatment with an acidic medium.

To test the function of shorter segments, the 29-base R2-I fragment of GA cDNA encoding the direct repeat of the two eight-base AREs of GA mRNA was cloned into pβG. This construct lacks the 3'-UTR of PEPCK mRNA that was included in previous studies (21). Therefore, it was used to determine whether the GA sequence can function as both a destabilizing element and a pHRE. When LLC-PK₁-F⁺ cells were stably transfected with pβG-GA(R2-I) and grown in normal medium, βG-GA(R2-I) mRNA decayed with a t1/2 of 12 h (Fig. 6). When the same cells were transferred to acidic medium, the chimeric RNA was degraded with a t1/2 of >30 h. This indicates that the sequence contained within the GA(R2-I) region of GA 3'-UTR is

Fig. 4. Competition analysis of the relative binding of ζ-crystallin to GDH1, GDH2, GDH3, and GDH4 RNAs. 32P-labeled GA(R2-I) RNA was incubated with purified ζ-crystallin (lanes 2–10). A 100- or 300-fold excess of an unlabeled RNA competitor was added as indicated. The samples were resolved on a nondenaturing gel. The gel was then dried and imaged with a PhosphorImager screen. The arrow indicates the position of ζ-crystallin/RNA complex. The percentage of total radioactivity contained in the RNA/protein complex is indicated as % shifted. The reported data are representative of 3 separate experiments.

Fig. 5. Half-life analysis of β-globin (βG)-GDH mRNA. A: schematic of βG-GDH mRNA. B: Northern blot analysis of βG-GDH mRNA isolated from LLC-PK₁-F⁺ cells stably transfected with pβG-GDGH. Cells were either maintained in normal (pH 7.4) medium or transferred to acidic (pH 6.9) medium for 12 h and then treated with 65 μM 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB) for 0, 3, 6, and 9 h. C: relative levels of βG-GDH mRNA were determined by PhosphorImager analysis. The level of βG-GDH mRNA was divided by the corresponding level of 18S rRNA to correct for errors in sample loading. The log of normalized data was then plotted vs. the time of treatment with DRB. The reported data are the mean of duplicate experiments. The error bars represent the range of the 2 data points for the 9-h time points.
sufficient to function as an instability element and a pHRE.

The direct binding and competition analyses indicated that the fourth ARE was one of two sites within GDH mRNA that binds H9256-crystallin with the greatest affinity. Therefore, the GDH4 sequence was cloned into pH9252G to test whether a single GDH element is sufficient to function as a pHRE. The t1/2 of pH9252G-GDH4 mRNA in LLC-PK1-F cells maintained in either normal or acidic medium was 30 h (data not shown). Thus the 35-base GDH4 sequence does not significantly destabilize pH9252G mRNA. Given the inherent stability of this construct, it could not be used to determine whether the GDH4 sequence can function as a pH-responsive stabilizing element.

To assess whether the GDH4 sequence can function as a pHRE, the 3′-UTR of PEPCK cDNA was cloned downstream of the GDH4 element to produce the pH9252G-GDH4-PEPCK plasmid. Insertion of the PEPCK sequence destabilizes the resulting pH9252G-GDH4-PEPCK mRNA (Fig. 7). The t1/2 of this mRNA in LLC-PK1-F cells grown in pH 7.4 medium was 16 h. However, when the cells were treated with pH 6.9 medium, the t1/2 was increased to >30 h. Therefore, the GDH4 sequence can function as a pHRE but not as a destabilizing element.

**DISCUSSION**

The control of mRNA degradation plays an important role in the posttranscriptional regulation of gene expression. Some mRNAs turnover rapidly with a t1/2 of <30 min, whereas others are very stable and have a t1/2 of >30 h (25). Furthermore, the t1/2 of an mRNA can be modulated during growth and differentiation and in response to various hormonal changes and stresses (25, 31). The resulting change in mRNA t1/2 produces a corresponding change in mRNA abundance. The rela-

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**Fig. 6.** Half-life analysis of βG-GA(R2-I) mRNA.  
A: schematic of βG-GA(R2-I) mRNA.  
B: Northern blot analysis of βG-GA(R2-I) mRNA isolated from LLC-PK1-F cells stably transfected with βG-GA(R2-I). Cells were either maintained in normal (pH 7.4) medium or transferred to acidic (pH 6.9) medium for 12 h and then treated with 65 μM DRB for 0, 3, 6, and 9 h.  
C: relative levels of βG-GA(R2-I) mRNA were determined by PhosphorImager analysis. The level of βG-GA(R2-I) mRNA was divided by the corresponding level of 18S rRNA to correct for errors in sample loading. The log of normalized data was then plotted vs. the time of treatment with DRB. The reported data are the mean of duplicate experiments. The error bars represent the range of the 2 data points for the 9-h time points.

**Fig. 7.** Half-life analysis of βG-GDH4-phosphoenolpyruvate carboxykinase (PEPCK) mRNA.  
A: schematic of the βG-GDH4-PEPCK mRNA.  
B: Northern blot analysis of βG-GDH4-PEPCK mRNA isolated from LLC-PK1-F cells stably transfected with βG-GDH4-PEPCK. Cells were either maintained in normal (pH 7.4) medium or transferred to acidic (pH 6.9) medium for 12 h and then treated with 65 μM DRB for 0, 3, 6, and 9 h.  
C: relative levels of βG-GDH4-PEPCK mRNA were determined by PhosphorImager analysis. The level of βG-GDH4-PEPCK mRNA was divided by the corresponding level of 18S rRNA to correct for errors in sample loading. The log of normalized data was then plotted vs. the time of treatment with DRB. The reported data are the mean of duplicate experiments. The error bars represent the range of the 2 data points for the 9-h time points.
tive stability of an mRNA is determined by specific sequences or structures within the mRNA that function as cis-acting elements. The regulation of mRNA turnover is mediated by specific trans-acting proteins that bind the individual elements with high affinity.

The 5′- and 3′-ends of most eukaryotic mRNAs are protected from exonucleolytic degradation by the binding of eIF4E and poly(A) binding proteins (PABP) to the 7-methyl-guanosine cap and the poly(A) tail, respectively. The two ends are then linked together by eIF4G that acts as a bridging protein by binding to eIF4E and PABP (13, 14). Deadenylation is the initial step in the degradation of most mRNAs (4, 31). In yeast, deadenylation is usually followed by decapping and 5′→3′ exonucleolytic degradation. However, in mammals the prominent pathway involves initial deadenylation followed by 3′→5′ degradation. Various AREs, including the canonical AUUUA sequence (4), function as instability elements by binding proteins, such as TTP (2), that recruit the 3′→5′ poly(A)-specific deadenylase DAN/PARN (13) and a complex of 3′→5′ exoribonucleases that has been termed the exosome (30). The deadenylation and subsequent degradation of an mRNA can be averted by the alternative recruitment of a stabilizing ARE-binding protein such as HuR (5).

On the basis of previous experiments, it was hypothesized that the pHRE of GA mRNA also functions as the recognition site for a sequence-specific endonuclease (7). During normal acid-base balance, the weak interaction of ζ-crystallin with the pHRE may allow for recruitment of the endonuclease that initiates the rapid degradation of GA mRNA. The onset of acidosis leads to an enhanced interaction of ζ-crystallin with the pHRE (22) that may be mediated by a kinase that is upstream of the p38 stress-activated protein kinase (12). The enhanced binding of ζ-crystallin may stabilize GA mRNA by blocking the recruitment of the sequence-specific endonuclease. However, more recent pulse-chase experiments indicate that the degradation of the chimeric βG-GA mRNA in LLC-PK1-F+ cells is preceded by deadenylation and occurs without apparent endonucleolytic cleavage (Schroeder JM and Curthoys NP, unpublished observations). Thus the pHRE may function as a site that alternatively binds the stabilizing protein, ζ-crystallin, or an ARE-binding protein that recruits a deadenylase and the exosome.

The revised hypothesis is also supported by the analysis of the turnover of the chimeric βG-GA(R2-1) mRNA that contains the direct repeat of the eight-base AREs of GA mRNA (Fig. 6). Insertion of this segment was sufficient to destabilize βG mRNA in LLC-PK1-F+ cells maintained in normal medium (pH 7.4) and produce a pH-responsive stabilization when the cells were transferred to acidic medium (pH 6.9). Furthermore, the magnitude of both effects was similar to that observed in the control experiment that utilized βG-GA mRNA. Therefore, the 29-base R2-I segment functions as both a destabilizing element and a pHRE. This may occur through the alternate recruitment of a destabilizing ARE-binding protein and ζ-crystallin.

The binding of ζ-crystallin to the individual AREs of the GA 3′-UTR was previously studied using a crude rat renal cytosolic extract and GA(R2-I) RNAs in which the eight-base AREs were mutated to include five guanine and cytosine residues (23). The RNAs containing a single mutated ARE exhibited a large reduction in apparent binding affinity for ζ-crystallin compared with wild-type GA(R2-I) RNA. When both of the AREs were mutated, binding was abolished. In contrast, both the direct binding studies (Fig. 2) and the competition analysis (Fig. 3) performed with GA(R2-1A) and GA(R2-1B) RNAs, which contain a single ARE in the context of the surrounding sequence from the 3′-UTR of GA mRNA, indicate that a single eight-base element binds ζ-crystallin with only slightly less affinity than the complete R2-I sequence. Thus the decreased binding affinity observed with the single-site mutations may have been caused by the increase in the GC content of the mRNAs and not the missing ARE.

Previous studies suggested that the increase in renal GDH mRNA during acidosis is also mediated by a cell-specific stabilization (20). The 3′-UTR of GDH mRNA contains four well-spaced eight-base segments that are 88% identical to one of the AREs in the GA mRNA (9). The reported experiments establish that the 3′-UTR of GDH mRNA binds ζ-crystallin and contains both an instability element and a pHRE. Furthermore, the direct binding studies and the competition analysis indicate that all four putative AREs bind ζ-crystallin with varying affinities, all of which were less than that observed for the individual elements from GA mRNA. The GDH2 and GDH4 sequences showed a greater binding affinity than the GDH1 and GDH3 elements. The putative element with GDH4 RNA contains only A and U residues, whereas the GDH2 element contains a single C residue at the 5′-end of the eight-base sequence. In contrast, the GDH3 and GDH1 elements contain C and G substitutions at the number 3 and 6 positions, respectively. Therefore, substitutions that interrupt the stretch of AU nucleotides may have a greater negative impact on the binding of ζ-crystallin.

The greater affinity of ζ-crystallin for the direct repeat of the AREs within GA mRNA compared with the individual AREs within GDH mRNA is consistent with the observed changes in the levels of the two enzymes that occur during acidosis. The GA activity is increased 8- to 20-fold within the renal proximal convoluted tubule during chronic acidosis (8, 34), whereas the GDH activity within the same cells is increased only threefold (33). Therefore, the preferential binding of a limiting amount of ζ-crystallin could contribute to the greater fold-stabilization of GA mRNA.

Functional studies using βG-PEPCK mRNA indicated that the GDH4 ARE can function as a pHRE. However, either the multiple AREs or an alternative element within the 3′-UTR is needed to impart a significant destabilization to GDH mRNA. Identification of the destabilizing element will require the synthesis and characterization of additional constructs that individually assess the function of the three additional
AREs or that mutate the individual sites within the pGDH1–4 plasmid.

The combined binding and functional studies establish that individual AREs within GA and GDH mRNAs bind ζ-crystallin with different affinities and that a single ARE from GDH mRNA is sufficient to mediate a pH-responsive stabilization. A recent analysis using cDNA microarrays indicated that the onset of metabolic acidosis leads to increased expression of a large number of genes within the renal cortex (32). Thus it will be interesting to determine how many of these genes encode an eight-base sequence in their 3' UTR that is similar to pHRE sequences of GA or GDH mRNAs.

DISCLOSURES

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REFERENCES


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