The Effect of Over-Expression of ξ-Crystallin on Glutaminase mRNA Stability

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ABSTRACT

During metabolic acidosis, increased renal catabolism of glutamine generates ammonium and bicarbonate ions to partially restore normal acid-base balance. The remaining carbons derived from glutamine are then used to synthesize glucose. This adaptive response is sustained in part by a pH-responsive increase in glutaminase (GA) that results from selective stabilization of the GA mRNA. Previous studies have shown that the 3'-UTR of the GA mRNA contains a pH-response element that consists of a direct repeat of an eight-base AU sequence and that this element binds ζ-crystallin with high affinity and specificity. Increased binding of this protein during metabolic acidosis may initiate the pH-responsive stabilization of the GA mRNA.

A tetracycline-responsive expression system (tet-off) was developed to test the effect of over-expression of ζ-crystallin on the expression and the stability of the GA mRNA. Two constructs, pcDNA 3.1-βG-GA–Hygro and pTRE2-ζ-crystallin, were created. The pcDNA 3.1/Hygro vector is designed for high-level, constitutive expression in mammalian cell lines and contains the selectable marker, hygromycin. A chimeric βG-GA cDNA segment that encodes β-globin and the 3'-UTR of the GA mRNA was inserted into the pcDNA 3.1/Hygro vector. The construct, pTRE2-ζ-crystallin contains the tet-responsive element (TRE) that drives the expression of ζ-crystallin. The two plasmids were co-transfected into 8C cells that express high levels of the tTA protein that binds to and activates transcription from the TRE only in the absence of doxycycline (Dox). Clonal cell lines were selected with hygromycin. These cells were grown in the presence and absence of Dox and screened with ζ-crystallin specific antibodies to identify clonal lines that exhibit a large induction of ζ-crystallin when grown in the absence of Dox. RNA isolated from the selected line was quantified using Real-Time RT-PCR. The resulting data demonstrate that over-expression of ζ-crystallin does not increase GA mRNA levels.

INTRODUCTION

Metabolic acidosis occurs when the body has accumulated an excess of acid and lacks sufficient bicarbonate to effectively neutralize the effects of the acid. Metabolic acidosis can be a mild symptom associated with starvation, a high protein diet, or a gastrointestinal disorder such as vomiting or diarrhea. Alternatively, it can indicate a more serious problem such as the lack of insulin or a defect in function of the liver, lung, heart, or kidneys.

Acids are a natural by-product of various metabolic processes in the body including the breakdown of proteins and fats. In other conditions, the body does not produce enough bicarbonate, an acid neutralizer, to balance the acids being produced. This problem may result when the body uses fats for energy instead of carbohydrates. Conditions where metabolic acidosis may occur include chronic alcoholism, malnutrition, and diabetic ketoacidosis. The disorder may also be a direct result from another condition such as kidney failure, liver failure, or severe diarrhea. Lactic acid build up in the blood due to heart failure, shock, or cancer may also induce metabolic acidosis

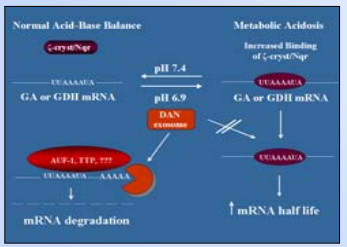

Figure 1. Mechanism by which the onset of metabolic acidosis leads to stabilization of the renal GA and GDH mRNAs. During normal acid-base balance, the weak binding of ζ-crystallin (ζ-cryst/Nqr) allows the 8-base AU-rich pH-RE to bind an alternative protein that recruits a 3'-deadenylase and the exosome that promote the rapid turnover of the two mRNAs. The enhanced binding of ζ-crystallin to the AU-element during acidosis may block this process and accomplish the selective stabilization of the mRNAs.

METHODS & RESULTS

Cloning

1. Construction of pcDNA3.1-βG-GA/Hygro

The **pcDNA3.1/Hygro** vector is designed for high-level, constitutive expression in a variety of mammalian cell lines. The vector contains a gene that imparts resistance to hygromycin. The **βG-GA** cDNA was ligated into the multiple cloning site using XbaI/HindIII (Figure 2A). Plasmids were then amplified and the insert was confirmed with NheI digestion (Figure 2B).

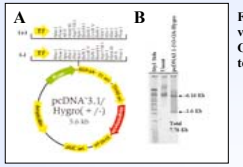

Figure 2. pcDNA3.1/Hygro vector and pcDNA 3.1-βG-GA/Hygro digested with Nhe I to show correct orientation.

2. Construction of pTRE2- ζ -crystallin

The **pTRE2** vector contains the tet-responsive element (TRE) that is regulated by the tTA and rTA regulatory proteins in the Tet-off and Tet-on systems, respectively (Figure 3A). The vector was digested with NheI and EcoRV. The **ζ-crystallin** gene was digested from the MLR 107 plasmid, purified, and inserted into the multiple cloning site of pTRE2 using XbaI and DraI. Plasmids were then amplified and the insert was confirmed by BamHI digestion (Figure 3B).

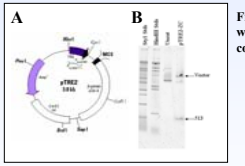

Figure 3. pTRE2- ζ -crystallin was digested with BamHI to confirm the correct orientation.

Cell Selection

8C (LLC-PK$_1$-FBPase$^+$) Cells

The 8C cell line is derived from the well characterized porcine kidney cell line LLC-PK$_1$-FBPase$^+$. These cells exhibit properties characteristic of renal proximal tubular cells including a pH-responsive increase in glutamine metabolism. 8C cells also express high levels of the tetracycline-responsive transcriptional activator (tTA) protein that binds to and activates transcription from the tetracycline response element (TRE) in the absence of doxycycline (Dox).

Calcium Phosphate Transfections

All recombinant DNA constructs were introduced into the 8C cells using a calcium phosphate transfection method. For the luciferase assay, pTRE2-Luc was transiently transfected into the 8C cells. The constructs, pcDNA3.1-βG-GA-Hygro and pTRE2-ζ-crystallin, were co-transfected into the cells and selected with hygromycin.

Dual-Luciferase Reporter™ Assay System

In the Dual-Luciferase Reporter™ Assay, the activities of firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*) luciferases are measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LARII) to generate a "glow-type" luminescent signal. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is initiated simultaneously by adding Stop & Glo® reagent to the same tube. In the dual assay system, each reporter produces a linear response with sub attomole sensitivities and no endogenous activity in the experimental host cells.

Selection of Stable Cell Lines

All cells were treated with 0.8 mg/ml of hygromycin. The cells that incorporated the plasmid DNA were resistant to the toxic effects of this drug, whereas cells that did not incorporate the plasmid underwent apoptosis after 7 days of selection. Surviving cell colonies were selectively removed with cloning rings. 24 clones were chosen, placed into 12 well plates and grown with 0.2 mg/ml G418, 0.8 mg/ml hygromycin and 50 ng/ml doxycycline.

Screening Clonal Lines for ξ-crystallin

A Western blot was performed to determine which clonal lines contained the ζ-crystallin construct and were responsive to Dox. Cells were harvested 5 days after being treated +/- Dox and screened with a ζ-crystallin specific antibody.

Screening Clonal Lines for the Presence of β-globin (βG)

A Northern blot was performed to determine that βG was present in the chosen clonal line.

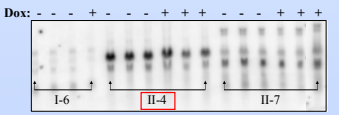

Figure 7. Northern blot of RNA isolated from clonal lines that were grown in absence and presence of Dox and probed with a βG cDNA.

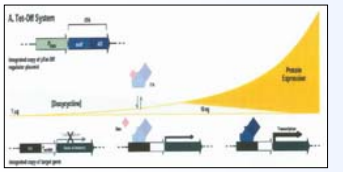

Figure 4. The tet-off system in the 8C cells contains the tTA protein that binds to the tet-responsive element (TRE) only in the absence of Dox. In high concentrations, Dox will bind to the tTA protein, inhibit it from binding to the TRE, and transcription ceases. In the absence of Dox, tTA will bind to the TRE and transcription occurs.

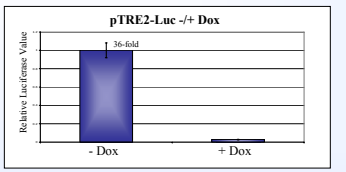

Figure 5. Luciferase assay results of pTRE2-Luc transfection +/- Dox. A 36-fold increase is seen with – Dox.

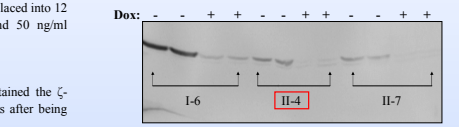

Figure 6. Western blot of clonal lines grown +/- Dox for 5 days and screened with a ζ-crystallin specific antibody. Clonal line II-4 exhibits a 7.82-fold increase in ζ-crystallin expression when grown in the absence of Dox.

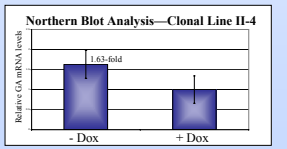

Figure 8. Northern blot analysis of clonal line II-4 reveals a 1.63-fold induction in GA mRNA levels when cells are grown without Dox.

Glutaminase mRNA Analysis

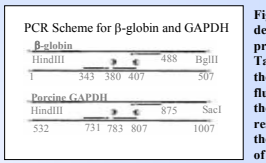

Figure 9. Two template specific primers define the endpoints of the amplicon and provide an initial level of specificity. The Taqman probe is also complementary to the target sequence. Therefore, a fluorescence signal is obtained only when the correct segment is amplified. The resulting signal is directly proportional to the number of molecules present at the end of the cycle.

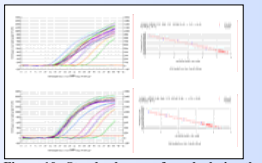

Figure 10. Standard curves for calculating the concentration of βG-GA and GAPDH mRNAs.

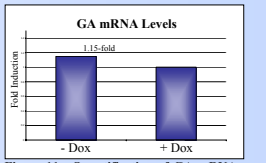

Figure 11. Quantification of GA mRNAs (from clonal line II-4) with Real-Time RT-PCR. The cells grown without Dox showed a 1.15-fold induction in GA mRNA levels.

SUMMARY

Increased renal catabolism of glutamine generates ammonium and bicarbonate ions to partially restore the normal acid-base balance during metabolic acidosis. This response is sustained by a pH-responsive increase in glutaminase (GA) that results from GA mRNA stabilization. The 3'-UTR of GA mRNA contains a pH-response element consisting of a direct repeat of an eight-base AU sequence that binds ζ-crystallin with high affinity. The increased ζ-crystallin binding seen during metabolic acidosis may be responsible for the pH-responsive stabilization of the GA mRNA.

Two constructs, pcDNA3.1-βG-GA/Hygro and pTRE2-ξ-crystallin, were created. The construct, pcDNA3.1-βG-GA/Hygro, contains a pcDNA3.1/Hygro vector and a βG-GA cDNA insert. The vector is designed for high-level, constitutive expression in mammalian cell lines and contains hygromycin, a selectable marker. The insert encodes β-globin (βG) and the 3'-UTR of the GA mRNA. An additional construct, pTRE2- ζ-crystallin, was developed which contains the tet-responsive element (TRE) that drives ζ-crystallin expression.

To determine if the 8C cells are responsive to the presence or absence of doxycycline (Dox), the cells were transfected with pTRE2-Luciferase and grown +/- Dox. In the absence of Dox, the tTA protein binds TRE and initiates transcription. However, in the presence of Dox, transcription will not occur. A luciferase assay was performed, and a 36-fold increase was seen in luciferase in the cells grown - Dox.

Both constructs, pcDNA3.1-βG-GA-Hygro and pTRE2-ζ-crystallin, were co-transfected into 8C cells using a calcium phosphate transfection method. Clonal cell lines were selected with 0.8 mg/ml hygromycin. Surviving colonies were removed with cloning rings and were selected with G418, Dox, and hygromycin.

A Western blot was performed on the clonal lines to determine which line showed over-expression of ξ-crystallin without Dox. Clonal line II-4 showed a 7.82-fold increase in ξ-crystallin in the absence of Dox. A Northern blot determined the presence of βG-GA mRNA in clonal line II-4. To accurately quantify the level of βG -GA mRNA, Real-Time RT-PCR was performed.

With Real-Time RT-PCR analysis, only a 1.15-fold induction in GA mRNA levels was seen in the cells grown without Dox (over-expression of ζ-crystallin). It appears that either ζ-crystallin is present in excess and its effect on GA mRNA stability is not affected by over-expression or it does not participate in the stabilization of the GA mRNA.

ACKNOWLEDGEMENTS

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