Biochemical and Biophysical Research Communications 426 (2012) 539–543

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

CUG binding protein 1 binds to a specific region within the human albumin 3′ untranslated region

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ARTICLE INFO

Article history:
Received 7 August 2012
Available online 7 September 2012

Keywords:
RNA-protein binding
UV cross-linking
RNA-affinity
Proteomics
CELF1

ABSTRACT

3′ Untranslated regions (3′UTRs) of messenger RNAs have important roles in post-transcriptional regulation of gene expression and this is partly achieved through binding of specific proteins to sequences or structures within these regions. Previously, replacement of a native luciferase 3′UTR with the human albumin 3′UTR has been found to lead to a 10-fold increase in luciferase reporter activity. In this work we investigated protein binding to the human albumin 3′UTR. Electrophoretic mobility shift and UV cross-linking assays indicate that a ~50 kDa protein from Chinese Hamster Ovary (CHO) cells binds to the albumin 3′UTR, and affinity experiments followed by proteomics identified this protein as CUG binding protein 1 (CUG-BP1, also known as CELF1). Deletion analysis of the albumin 3′UTR showed that nucleotides 1–50 and nucleotides 101–150 are not required for binding but that removal of nucleotides 51–100 caused a loss in binding. The results suggest that CUG-BP1 binds to nucleotides 51–100 of the human albumin 3′UTR. In human cells CUG-BP1 binding may thus play a role in regulation of albumin expression and, additionally, it may have a function in post-transcriptional control in CHO cells.

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1. Introduction

In mammals the 3′ untranslated regions (3′UTRs) of messenger RNAs (mRNAs) are longer, have greater tertiary structure and have a wider functionality than the 5′UTRs [1,2]. It is now known that 3′UTRs are important in the regulation of gene expression through effects on mRNA stability, localisation and translation [3–6]. Usually a motif within a discrete part of the 3′UTR, not the whole region, is a sufficient signal to bring about such a regulatory effect and this motif can either be a sequence or a specific RNA structure (e.g. [3]). 3′UTR signals achieve their biological effects through binding to *trans*-acting factors or miRNAs [3–6].

Recently, in proof of principle experiments to test whether it was possible through rational selection and exchange of 3′UTR sequences in a reporter luciferase vector system to significantly enhance recombinant protein titre, we found that the human albumin 3′UTR increased reporter activity 10-fold [7]. Since nothing is known about the binding of proteins to the albumin 3′UTR, the aim of the present work was to firstly investigate whether proteins in fact do bind and secondly, if so, to determine their nature. Using electrophoretic mobility shift assays, UV cross-linking and RNA pull-down experiments we found the albumin 3′UTR to bind CUG binding protein 1 (CUG-BP1), a known RNA-binding protein that is a member of the CELF/Bruno-like family of RNA-binding proteins [8].

2. Materials and methods

2.1. Cell culture

The CHO AA8 tet-off cell line (Clontech) was used throughout. Cells were cultured in Dulbecco's modified Eagle's medium complete growth medium (DMEM + GlutaMAX; Gibco) supplemented with 10% foetal calf serum (FCS; Gibco), 1% penicillin/streptomycin (10,000 U penicillin sodium, 10,000 μg/ml streptomycin sulphate in 0.85% saline; Gibco), and gentamycin (300 μg/ml; Gibco). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. In vitro transcription, electrophoretic mobility shift assays and UV cross-linking

RNA transcripts were synthesised with the MEGAshortscript™ Kit (Ambion) by performing *in vitro* transcription and subsequently quantified by spectrophotometry. Templates for *in vitro* transcription were generated by PCR using primers (listed in Table 1) corresponding either to the 5′ and 3′ ends of the full-length human

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http://dx.doi.org/10.1016/j.bbrc.2012.08.123

Table 1
Primers used for *in vitro* transcription. Regions underlined in italics indicate the minimal T7 promoter.

Oligo	Sequences (5′–3′)
Full3UTR.for	*TAATACGACTCACTATAGG*CATCTACATTTAAAAGC
Del(1–50).for	*TAATACGACTCACTATAGG*ATGAAGATCAAAAGC
Del(1–100).for	*TAATACGACTCACTATAGG*CAACACCCTGTCTAAAAAAC
Del(1–150).for	*TAATACGACTCACTATAGG*CTCTGTGCTTCAATTAATA
Del(1–50 and 101–150).for	*TAATACGACTCACTATAGG*ATGAAGATCAAAAGCTTATTC
GloFor141_T7	*TAATACGACTCACTATAGG*GCAGGCTGCTGGTTGTCTAC

albumin 3′UTR or its respective deletions. A T7 minimal promoter sequence was included in the forward primers. ^{32}P-labelled and non-labelled RNA transcripts were produced using 0.8 μg of purified PCR products (Qiagen). Reaction mixtures were prepared in an RNase-free microcentrifuge tube at room temperature according to the manufacturer's instructions and incubated for 3 h. To remove the template, 1 μl of turbo DNase (Ambion) was added and mixed well. The reaction was incubated at 37 °C for 15 min at room temperature. Electrophoretic mobility shift assays (EMSA) were performed with 1–3 μg of S-100 CHO cell protein extract (hereafter termed CHO cell protein extract) and 12 fmol of ^{32}P-labelled RNA transcript in binding buffer (40 mM NaCl, 4 mM $MgCl_2$, 1 M dithiothreitol, 30 mM Tris–HCl, pH 7.6 with ½ tablet EDTA-free complete protease inhibitor) in a total volume of 8 μl at room temperature for 15 min. For competition assays, labelled and unlabelled transcripts were added simultaneously. After incubation, 40 U of RNase T1 was added and the samples were incubated for a further 5 min at room temperature. The native polyacrylamide gel was pre-run in 0.5× TBE buffer at 120 V. Next, 2 μl of 20% (w/v) Ficoll was added to samples and electrophoresis was carried out in 0.5× TBE buffer for 2 h at 120 V. The gel was then dried and exposed to Kodak Biomax XAR film. UV cross-linking experiments were performed with 50 fmol of ^{32}P-labelled transcript and 2.5–3 μg of S-100 CHO cell protein extract in 40 mM NaCl, 5 mM $MgCl_2$, 30 mM Tris–HCl, pH 7.6 (lysis buffer). The total volume was made up to 13 μl with lysis buffer and incubated at room temperature for 15 min. In competition experiments labelled transcripts and excess unlabelled transcripts were added simultaneously. The reaction mixture was cross-linked for 12 min in SpectroLinker XL 1000 V Cross-Linker on ice and digested with 10 μg of RNase A at 37 °C for 60 min. The samples were analysed by SDS–PAGE.

2.3. Protein isolation using streptavidin-coated paramagnetic particles

RNA-binding proteins were isolated using biotinylated albumin 3′UTR linked to paramagnetic particles [9]. The biotinylated albumin sequence was produced in an *in vitro* transcription reaction using the albumin 3′UTR sequence generated by PCR (see Section 2.2) with the exception of adding 2 μl of Biotin-UTP16 (Roche). The synthesised biotinylated transcripts were subjected to phenol/chloroform extraction and quantified by spectrophotometry. Twenty micrograms of biotinylated transcript were heated at 70 °C for 5 min, then at 40 °C for 20 min and then allowed to cool down at room temperature. The MagneSphere Streptavidin-Coated Paramagnetic Particles (SA-PMP; Promega) were incubated with 100 μl of 0.5× SSC buffer, 10 μg of BSA and 10 μg of yeast tRNA for 60 min at room temperature with shaking. The SA-PMP were washed twice with 300 μl of 0.5× SSC buffer (containing 4.38 mg/ml of NaCl, 2.205 mg/ml of sodium citrate, pH 7.0) and incubated with 20 μg of biotinylated transcript in 300 μl of 0.5× SSC buffer for 10 min at room temperature. Particles were washed with 0.3 ml of 0.5× SSC buffer and incubated with 1 mg of CHO cell protein extract in 500 μl of 40 mM lysis buffer with an additional 25 μg of yeast tRNA, 10 μg of BSA and 800 U/ml of RNasin at 4 °C for 60 min with shaking. Particles were pelleted magnetically by placing the tubes onto MagnaRack™ (Life Technologies). The supernatant fluid was removed and particles washed 5 times with 1 ml of 40 mM lysis buffer. Particles were then re-suspended in 25 μl of 40 mM lysis buffer. Ten microlitres of this re-suspension was mixed with 2× dissociation buffer (90 mM Tris–HCl, pH 6.8, 20% [w/v] glycerol, 2% [w/v] SDS, 2% [w/v] β-mercaptoethanol, 0.02% [w/v] Bromophenol Blue). After denaturation by heating for 5 min at 95 °C the resuspended protein–particles mixture was loaded onto a 10% SDS–PAGE gel. After electrophoresis, the gel was visualised by Novex Colloidal Blue (Life Technologies).

2.4. Proteomic analysis and Western blotting

For protein identification bands in Colloidal Blue stained gels were excised for in-gel trypsin digestion followed by Liquid Chromatography Mass Spectrometry analysis (performed by NEPAF, Newcastle-upon-Tyne). In addition, for Western blotting proteins were transferred to a PVDF membrane (Roche) by a semi-dry transfer method and incubated with a polyclonal antibody to CUG-BP1 (Abcam, 1:1000 dilution) following the manufacturer's instructions. After washing and incubation with anti-rabbit IgG antibody linked to horseradish peroxidase (Sigma – Aldrich, 5000 dilution) bands were visualised using an ECL detection system (GE Healthcare) and Kodak Biomax XAR film (Sigma – Aldrich).

2.5. siRNA transfection

The Chinese hamster CUG-BP1 gene sequence is not known and therefore a consensus sequence was constructed from the CUG-BP1 mRNA sequence in *Rattus norvegicus* (Accession number: NM_001025421) and sequence alignment with isoforms of CUG-BP1 from *Mus musculus* (Accession numbers: NM_198683.1 & NM_017368.2), *Homo sapiens* (Accession numbers: NM_006560, & NM_198700 & NM_001025596) and *Canis familiaris* (Accession numbers: XM_533186 & NM_855451). A specific CUG-BP1 siRNA (sense AAACCUUGGCAGACACGACAUUCCC, antisense GGGAAUGUCGUGUCUGCCAAGGUUU) was then designed using the consensus sequence and Blok-iT™ RNAi designer software (Invitrogen). Cells (5×10^5) were seeded in a 6 well plate 24 h before transfection in order to reach 30–50% confluency. On the day of transfection, the growth medium was replaced with fresh medium containing only 5% FCS. Eighty pmol of CUG-BP1 specific siRNA or negative control siRNA (Life Technologies) in a volume of 4 μl water were added to 246 μl of OptiMEM medium for each well and incubated for 10 min at room temperature. Separately, for each well, 5 μl of Lipofectamine™ 2000 and 245 μl of OptiMEM were mixed and incubated for 10 min at room temperature. The siRNA and lipofectamine OptiMEM solutions were mixed and incubated for further 25 min before being gently added to the cells in the 6 well plate. The medium was replenished the next day and cells were collected 1–4 days after siRNA transfection.

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