Molecular Immunology 47 (2010) 2545–2551

Contents lists available at ScienceDirect

Molecular Immunology

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

# Binding of human antigen R (HuR) to an AU-rich element (ARE) in the 3′untranslated region (3′UTR) reduces the expression of decay accelerating factor (DAF)

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ARTICLE INFO

*Article history:*
Received 26 April 2010
Received in revised form 1 July 2010
Accepted 5 July 2010
Available online 9 August 2010

*Keywords:*
Complement
Decay accelerating factor (DAF/CD55)
AU-rich element

ABSTRACT

We have investigated the role of the 3′untranslated region (3′UTR) in the expression of decay accelerating factor (DAF), one of the major membrane regulators of Complement activation. We show here that the 3′UTR of DAF contains an adenylate uridine rich element (ARE) AUUUAUUUAUAUUUAUUUA, which belongs to Class II Cluster 4 of the AU-rich element-containing mRNA (ARED) database. Enhanced Green Fluorescent Protein (EGFP) Reporter constructs containing the DAF 3′UTR showed reduced levels of expression when transfected into a variety of cell lines compared to 3′UTR reporter constructs without the ARE sequence. Furthermore, the inhibitor of mRNA transcription Actinomycin D had a much stronger effect on mRNA half-life of the ARE-containing 3′UTR demonstrating that this ARE destabilises the mRNA. Electrophoretic Mobility Shift Assays (EMSA) using biotinylated RNA probes, demonstrated that cytoplasmic Human antigen R (HuR) bound to the DAF ARE. Transfection experiments using HuR specific siRNA increased DAF expression whilst plasmids containing the coding sequence of HuR had the opposite effect, demonstrating that HuR reduced the stability of DAF mRNA and suggesting that it is of importance in regulating the expression of DAF. These data suggest that modulators of HuR could potentially be used to alter DAF expression and therefore increase the susceptibility of malignant cells to immunotherapy.



## 1. Introduction

Monoclonal antibodies are becoming more and more widely used in cancer immunotherapy. One of the most well established is Rituximab, a monoclonal antibody targeted against human CD20 and used to treat B-cell lymphoma. One of the mechanisms by which this antibody kills malignant B-cells is through Complement-mediated cytotoxicity (reviewed Johnson and Glennie, 2003). Problems associated with antibody therapies of this sort relate to the resistance of nucleated cells to Complement attack, which is mediated by membrane bound regulators of complement (CRegs; reviewed in Fishelson et al., 2003; Gancz and Fishelson, 2009). One of the main membrane bound CRegs is Decay Accelerating Factor (DAF/CD55), a widely expressed 65 kDa protein, attached to the cell membrane through a glycosylphosphatidylinositol (GPI) anchor (Lublin and Atkinson, 1989). DAF protects cells from complement attack by inhibiting the assembly of and accelerating the dissociation of C3/C5 convertases. Increased expression of CRegs is frequently observed on malignant cells, thus reducing the efficacy of antibody-mediated immunotherapies (reviewed in Fishelson et al., 2003; Mikesch et al., 2006). High levels of DAF expression in tumours is also suggested to contribute to tumourigenesis (Mikesch et al., 2006). *In vitro* experiments have shown that blocking or downregulation of DAF expression at the tumour cell surface can have a beneficial effect in terms of increased Complement-susceptibility and increased chemosensitivity (reviewed in Fishelson et al., 2003; Yan et al., 2008).

Protein expression is regulated both at the level of transcription and translation. The activity of the DAF promoter has been investigated and key regions identified which may regulate DAF gene expression (Ewulonu et al., 1991; Shao et al., 2008; Thomas and Lublin, 1993). It has also been known for some time that the 3′untranslated regions (3′UTR) of mRNAs can regulate protein expression. In particular certain sequences containing adenylate-uridylate rich elements (ARE) have been shown to bind proteins which affect the stability of the mRNA transcript, thus changing the half-life of the mRNA and consequently affecting protein synthesis (Bakheet et al., 2001, 2003, 2006; Kuersten and Goodwin, 2003). We analysed the 3′UTR of DAF mRNA (Caras et al., 1987) (Genbank No. M64356) and found 4 overlapping pentameric sequences

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0161-5890/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2010.07.002

AUUUA (position 1909–1930), characteristic of sequences involved in the regulation of mRNA stability. Based on these sequences, DAF was grouped in Class II Cluster 4 of the AU-rich element-containing mRNA (ARED) database assembled by Bakheet et al. (2001, 2006) (http://rc.kfshrc.edu.sa/ARED). The role of this 3′UTR in the regulation of expression of DAF has not been investigated previously.

The aim of our study was to investigate if the 3′UTR of DAF has an effect on DAF expression and to study the interaction of regulatory proteins with this 3′UTR. Understanding the mechanism of regulation of expression of DAF may aid future developments for the treatment of malignancies. Enhanced Green Fluorescent Protein (EGFP) is frequently used as a reporter to measure the expression and sub-cellular localisation of proteins, but has also been used as a reporter molecule to measure the function of promoter and 3′UTR regions in the expression of proteins (Caballero et al., 2004; Hellweg et al., 2003; Kuwano et al., 2010). A destabilised form of EGFP was chosen as the reporter protein in our studies, which enabled analysis of the function of the DAF 3′UTR after stable expression in two B-cell lines. We show here that the presence of the ARE in the 3′UTR of DAF causes reduction of reporter protein expression, mediated by the binding of Human antigen R (HuR), a member of the Hu/ELAV family of RNA-binding proteins, which are known to play a role in posttranscriptional regulation of protein expression.

## 2. Materials and methods

### 2.1. Plasmid constructs

All reporter constructs were made using the promoterless pd2EGFP-1 plasmid (Clontech). Table 1 shows the primers used for amplification reactions. Either the DAF promoter (bp −796 to −1; Genbank No. M64356) or EF1α promoter (bp −1208 to −1; Genbank No. J04617) were inserted in the multiple-cloning site of pd2EGFP-1 (Fig. 1A). For the 3′UTR constructs the SV40polyA region of the promoter containing plasmids was removed by excision using NotI and AflII, and replaced by the DAF 3′UTR (bp 1146–2040; Genbank No. M31516; Fig. 1B) or the DAF 3′UTR minus the ARE sequence (DAF 3′UTR ΔARE; Fig. 1C: removed sequence: ATTTATTTATATTTATTTA residues 1909–1930). The DAF 3′UTR ARE was removed by 2-step splice overlap PCR. The regions either side of the ARE were amplified using DAF 3′UTR 'A/B' primer pairs. The two PCR products were mixed in equal ratio and used as template for the final PCR using the DAF 3′UTR 'A' primers resulting in the deletion of residues 1909–1930.

### 2.2. Cell culture

Non-Hodgkin's lymphoma cell lines Raji (DAF/CD59 positive subline, Harris and Morgan, 1995) and Ramos (DAF/CD59 positive clone, generated by limiting dilution from original cell line obtained from Martin Glennie, Southampton, UK), and the Cervical carcinoma epithelial cell line HeLa (obtained from Dr. R. Donev, Cardiff University) were all cultured in RPMI1640, 10% foetal calf serum, penicillin G (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), sodium pyruvate (2 mM). Media and supplements were all from Invitrogen (Paisley, UK). $5 \times 10^5$ cells were treated for 1–7 h with either 2 μM Actinomycin D or the equivalent DMSO (0.1%) as a vehicle control and cell were analysed for fluorescence on the FACSCalibur.

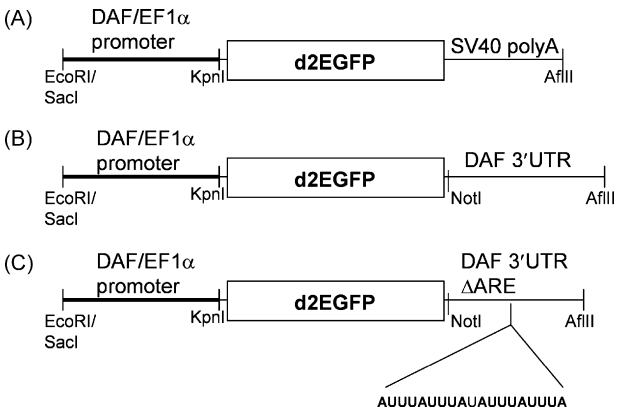


**Fig. 1.** Schematic diagram of pd2EGFP-1 (Clontech) derived plasmids containing DAF and EF1α promoters and SV40 polyA tail (A) or the DAF 3′UTR (B) and DAF 3′UTR ΔARE (C).

### 2.3. Cell transfections

Raji and Ramos cells were transfected with EGFP reporter constructs by electroporation as described (Powell et al., 1997). Stable transfectants were selected using 1–1.5 mg/ml G418 (Invitrogen). Single positive cell populations were obtained by flow cytometry sorting.

Gene silencing was performed using small interfering RNAs (siRNAs) for human HuR (sc-35619) or control siRNA (sc-44238) (Santa Cruz). Transfection of HeLa cells was performed using the Lipofectamine reagent and 40 pmol of each siRNA following the manufacturer's instructions for plasmid transfection (Invitrogen). Two days after transfection cells were harvested and DAF expression was analysed by flow cytometry.

The HuR coding sequence (Genbank No. NM_001419) was cloned into pDR2-EF1α (Charreau et al., 1995) expression plasmid. HuR encoding plasmid and control plasmid were transiently transfected into HeLa cells using Lipofectamine and Plus reagent (Invitrogen). Two days after transfection cells were harvested and DAF expression was analysed by flow cytometry.

### 2.4. RNA EMSA

Cell extraction methods were adapted from: (Lee et al., 1988; Ramji et al., 1993). Cellular protein was extracted by differential salt lysis in a core buffer containing 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml I-S soybean trypsin inhibitor) added prior to extractions. Cytoplasmic extraction buffer additionally contained 10 mM KCl and whole cell extraction buffer additionally contained 400 mM NaCl and 5% glycerol. Cytoplasmic extracts were prepared as follows: 1 volume of cytoplasmic extraction buffer was added to a cell pellet and left on ice for 15 min. Cells were lysed by pipetting up and down with a Hamilton syringe and spun at maximum speed in a microfuge for 20 s 4 °C. The supernatant (cytoplasmic extract) was stored at

**Table 1**
Primer sequences used for the amplification of promoter and 3′UTR regions and used for the deletion of the ARE sequence from the 3′UTR of DAF and for the EMSAs. Restriction enzyme site are indicated in bold and underlined. Sequences specific for the amplified regions are on the 3′ side of the restriction enzyme sites. ARE probe: AU-rich sequence is in italics.

| Primer | Sequence |
|---|---|
| DAFprom5′ | GC**GAATTC**CGCGCGCACACACACACACA |
| DAFprom3′ | GC**GGTACC**CGCGCCGGGTTAGAACAAGG |
| EF1α 5′ | GC**GAGCTC**GTACCGAATTCAAGCTTCGT |
| EF1α 3′ | GC**GGTACC**CACGTGTTCACGACACCTGA |
| DAF 3′UTR 5′A | GC**GCGGCCGC**C AAAGAAGAGTTAAGAAGA |
| DAF 3′UTR 3′A | GC**GCCTTAAG**ACCAAAAAATGGGATCATTT |
| DAF 3′UTR 3′B | GTTCACTGTCAATTACAAAAGAATAAGATTTA |
| DAF 3′UTR 5′B | CTTTTGTAATTGACAGTGAACATTCTGATT |
| ARE probe | UCUUUUGUAAU*AUUUAUUUAUAUUUAUUUA*AUGACAGUGAA |
| Scrambled probe | UCUGCCGUCAUAGCUAUGUACAUCCACGCAGGACAGCGAA |