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Cloning, expression, purification and preliminary crystallographic studies of the adenylate/uridylate-rich element-binding protein HuR complexed with its target RNA

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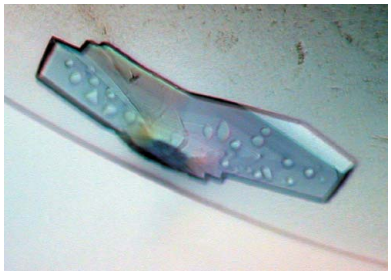
Adenylate/uridylate-rich elements (AREs), which are found in the 3′-untranslated region (UTR) of many mRNAs, influence the stability of cytoplasmic mRNA. HuR (human antigen R) binds to AREs and regulates various genes. In order to reveal the RNA-recognition mechanism of HuR protein, an RNA-binding region of human HuR containing two N-terminal RNA-recognition motif domains bound to an 11-base RNA fragment has been crystallized. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 42.4, b = 44.9, c = 91.1 Å. X-ray diffraction data were collected to 1.8 Å resolution.

1. Introduction

In eukaryotic cells, gene expression is controlled at various levels such as activation of chromatin structure, initiation of transcription, processing of the transcript and translation of mRNA. Transport of mRNA from the nucleus to the cytoplasm and its turnover are important control points in post-transcriptional regulation. The mRNA decay rate controls the level of cellular mRNAs and affects the expression of specific genes. Adenylate/uridylate-rich elements (AREs) are found in the 3′-untranslated region (UTR) of many mRNAs. The AREs influence the stability of cytoplasmic mRNA and regulate specific gene expression at the post-transcriptional level (Wilusz *et al.*, 2001).

HuR (human antigen R) is a ubiquitously expressed member of the Hu-protein family (which includes HuB, HuC and HuD) which contains three highly conserved RNA-recognition motif (RRM) domains (Chen & Shyu, 1995). This protein selectively binds AREs and stabilizes ARE-containing mRNAs coding for various proteins (Myer *et al.*, 1997; Fan & Steitz, 1998; Peng *et al.*, 1998; Marco *et al.*, 2001; Loflin & Lever, 2001; Goldberg-Cohen *et al.*, 2002; Yeap *et al.*, 2002; Chen *et al.*, 2002; Jones *et al.*, 2007; Wilusz & Wilusz, 2007; Dong *et al.*, 2007; Fialcowitz-White *et al.*, 2007; Dormoy-Raclet *et al.*, 2007; Casolaro *et al.*, 2008). Recently, the target mRNAs of HuR were identified by cDNA array hybridization and sequence analysis led to the identification of a 17–20 base RNA motif that is recognized by HuR (Lopez de Silanes *et al.*, 2004). In order to reveal how HuR protein recognizes and stabilizes various kinds of mRNA, the three-dimensional structure of the RNA-binding region complexed with RNA is essential, as well as analysis of the target RNA motif for HuR. A structural comparison with HuC (PDB code 1fnx; M. Inoue, M. Hirao, K. Kasashima, I.-S. Kim, G. Kawai, T. Kigawa, H. Sakamoto, Y. Muto & S. Yokoyama, unpublished work) and HuD (Wang & Tanaka Hall, 2001), which are neuron-specific members of the Hu-protein family and have sequence identities of 68 and 72%, respectively, to HuR, would also be useful in order to understand the structural features of HuR in RNA binding. Here, we report the crystallization and preliminary X-ray crystallographic study of the HuR RNA-binding region, which contains two N-terminal RRM domains, complexed with an 11-base RNA within the c-fos ARE.



2. Experimental procedures

2.1. Cloning, expression and purification

The overall HuR gene was amplified from a human liver cDNA library (Invitrogen) by PCR with specific primers (forward, 5′-AG-

CATCGCCATATGTCTAATGGTTATGAAG-3′; reverse, 5′-ATACTCTGCTCGAGTTATTTGTGGGACTTG-3′) and then cloned into pET22b vector (Novagen). The pET22b vector containing the full-length gene of HuR was used as a template to amplify the fragment coding for an RNA-binding region of HuR (amino-acid residues 18–184) with specific primers (forward, 5′-AGCATCGCCATATGGGGAGAACGAATTTGA-3′; reverse, 5′-ATACTCTGCTCGAGTTATGCAAACTTCACT-3′). The amplified DNA fragment was digested with *Nde*I and *Xho*I and ligated into pET22b vector, resulting in an expression vector for the RNA-binding region of HuR containing two N-terminal RRM domains without any tag.

Escherichia coli strain BL21 (DE3) competent cells were transformed with the recombinant plasmid and grown to an OD_{600} of 0.6 at 310 K in 800 ml LB broth containing 100 mg l^{-1} ampicillin. The cells were then induced with 1 m*M* IPTG and expression was continued for 3.5 h at 310 K.

These cells were harvested by centrifugation at 4000*g* for 15 min, suspended in 50 m*M* sodium phosphate buffer pH 7.3 containing 100 m*M* NaCl, 1 m*M* DTT, 1 m*M* EDTA and 0.2 m*M* PMSF, and disrupted using a French pressure cell. The lysate was centrifuged at 35 000*g* for 30 min to remove crude debris. The supernatant was treated with 1% polyethyleneimine to remove nucleic acids and subjected to cation-exchange chromatography using a HiTrap SP HP column (GE Healthcare) equilibrated with 50 m*M* sodium phosphate buffer pH 6.5 containing 1 m*M* EDTA and 1 m*M* DTT. Proteins were eluted using a linear gradient of 0–500 m*M* sodium chloride. Fractions containing the RNA-binding region of HuR were further purified to homogeneity using a HiLoad Superdex 75 pg gel-filtration column (GE Healthcare) equilibrated with 50 m*M* sodium phosphate buffer pH 6.5 containing 200 m*M* NaCl, 1 m*M* EDTA and 1 m*M* DTT. The final fractions were dialyzed against 10 m*M* HEPES buffer pH 7.0 containing 1 m*M* DTT, 0.1 m*M* EDTA and 5% glycerol and concentrated to 8 mg ml^{-1}. The protein concentration was determined using the Bradford assay. The purity of the RNA-binding domain of HuR was examined on a 15% SDS–PAGE gel (Fig. 1).

Figure 1
SDS–PAGE (15% polyacrylamide gel) of the purified RNA-binding domain of HuR.

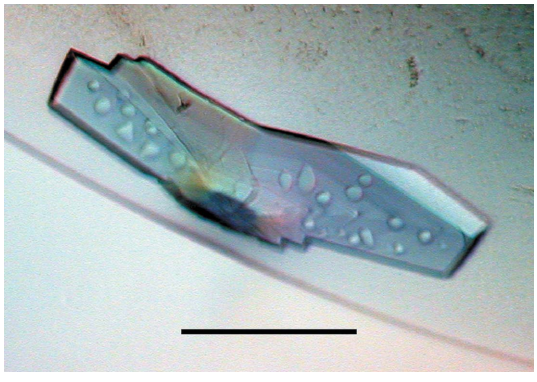


Figure 2
A single crystal of the RNA-binding region of HuR complexed with an 11-base RNA. The scale bar represents 0.2 mm.

Table 1
Summary of data collection.

Values in parentheses are for the outermost resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 42.4, b = 44.9, c = 81.1
Resolution (Å)	50.0–1.80 (1.86–1.80)
No. of observed reflections	94125
Unique reflections	14907
Completeness (%)	99.5 (99.4)
Averaged redundancy	6.3 (6.4)
R_{merge}† (%)	7.2 (37.5)
$\langle I/\sigma(I)\rangle$	46.9 (6.2)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the mean intensity of all observations of reflection hkl.

2.2. Crystallization

The synthesized oligoribonucleotide (5′-AUUUUUAUUUU-3′) used for complex formation was purchased from Hokkaido System Science Co. Ltd (Hokkaido, Japan). Oligoribonucleotide solution containing 0.1 m*M* EDTA was added to the HuR solution, yielding a final RNA:protein ratio of 1.2:1.0. Crystal Basic Kit and Extension Kit (Sigma) were used to determine the crystallization conditions. Cocrystals were screened at 293 K using the sitting-drop vapour-diffusion method, in which 0.8 µl complex solution was mixed with 0.8 µl reservoir solution and allowed to equilibrate against 0.1 ml reservoir solution. There was no need to optimize the conditions found during screening. Crystals suitable for X-ray diffraction measurement grew to approximate dimensions of 0.05 × 0.10 × 0.50 mm within a week from 0.1 *M* sodium acetate pH 4.6, 30%(*v*/*v*) polyethylene glycol 400 and 0.1 *M* cadmium chloride (Fig. 2).

2.3. X-ray diffraction analysis

X-ray diffraction data were collected using an ADSC Quantum 210 CCD detector and synchrotron radiation (1.000 Å wavelength) on beamline BL41XU at SPring-8 (Hyogo, Japan) under cryocooling conditions (in a 100 K nitrogen-gas stream) without any need for additional cryoprotectant. The oscillation angle was 0.8° and the exposure time was 1.0 s per frame. A total of 225 diffraction images were recorded at a camera distance of 140 mm and were processed using *HKL*-2000 (Otwinowski & Minor, 1997). The space group was determined to be $P2_12_12_1$, with unit-cell parameters a = 42.4, b = 44.9, c = 81.1 Å. Data-collection statistics are summarized in Table 1.

3. Results and discussion

A V_M calculation indicated that the crystals contained one protein–RNA complex per crystallographic asymmetric unit (V_M of 1.75 Å^3 Da^{-1}; 30% solvent content), based on an estimated molecular mass of 22 000 Da for the protein–RNA complex (Matthews, 1968).

The molecular-replacement method was performed with the program *AMoRe* (Navaza, 1994) in the resolution range 20.0–3.0 Å using the crystal structure of HuD protein complexed with RNA (PDB code 1fnx) as a search model. A molecular-replacement solution giving one protein–RNA complex per asymmetric unit was found in space group $P2_12_12_1$ with a correlation factor and R factor of 35.9 and 51.5%, respectively. An RNA molecule was observed in the initial electron-density map, providing evidence that our crystals contained the HuR–RNA complex. The structure is currently under refinement.

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