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Large-Scale Overexpression and Purification of ADARs from *Saccharomyces cerevisiae* for Biophysical and Biochemical Studies

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Abstract

Many biochemical and biophysical analyses of enzymes require quantities of protein that are difficult to obtain from expression in an endogenous system. To further complicate matters, native adenosine deaminases that act on RNA (ADARs) are expressed at very low levels, and overexpression of active protein has been unsuccessful in common bacterial systems. Here we describe the plasmid construction, expression, and purification procedures for ADARs overexpressed in the yeast *Saccharomyces cerevisiae*. ADAR expression is controlled by the Gal promoter, which allows for rapid induction of transcription when the yeast are grown in media containing galactose. The ADAR is translated with an N-terminal histidine tag that is cleaved by the tobacco etch virus protease, generating one nonnative glycine residue at the N-terminus of the ADAR protein. ADARs expressed using this system can be purified to homogeneity, are highly active in deaminating RNA, and are produced in quantities (from 3 to 10 mg of pure protein per liter of yeast culture) that are sufficient for most biophysical studies.

1. Introduction

Adenosine deaminases that act on RNA (ADARs) convert adenosine to inosine in regions of RNA that are largely double stranded (Bass, 2002; Keegan *et al.*, 2004; Valente and Nishikura, 2005). Since inosine is recognized as guanosine, the result of editing by an ADAR is an A-to-G transition mutation. ADARs are found in every metazoan animal (not plants) and are essential for proper neuronal function. This is supported by evidence showing that knockouts of ADARs lead to various behavioral abnormalities (Higuchi *et al.*, 2000; Palladino *et al.*, 2000a; Tonkin *et al.*, 2002) and that several known pre-mRNA substrates code for neuronal receptors and ion channels (Burns *et al.*, 1997; Higuchi *et al.*, 1993; Lomeli *et al.*, 1994). Though the effects of editing coding sequences are dramatic, these editing events are rare relative to the levels of editing in noncoding regions, especially untranslated regions (UTRs) (Blow *et al.*, 2004; Levanon *et al.*, 2004; Morse and Bass, 1999; Morse *et al.*, 2002). The extensive editing of noncoding regions has been proposed to affect expression of a gene posttranscriptionally, although how this might occur is unclear. Editing of double-stranded RNAs, such as those regions found in UTRs, creates I-to-U mismatches from A-to-U base pairs, and results in an RNA that is less double stranded. Although the biological consequences are not yet understood, an RNA made more single stranded by A-to-I modification can affect recognition by the RNAi machinery (Knight and Bass, 2002; Scadden and Smith, 2001).

ADARs are composed of an N-terminal RNA-binding domain and a C-terminal catalytic domain. The RNA-binding domain consists of one to three double-stranded RNA-binding motifs (dsRBM). The catalytic domain consists of a deaminase motif that resembles

Escherichia coli cytidine deaminase and binds zinc, as well as a motif that coordinates the small molecule *myo*-inositol hexakisphosphate (IP₆, Maas *et al.*, 2003; Macbeth *et al.*, 2005). In addition, human and *Xenopus* ADAR1 contain two Z-DNA-binding motifs N-terminal to their three dsRBMs (Maas *et al.*, 2003). ADARs are modular, and for hADAR2, the isolated dsRBMs will each bind RNA in the absence of the catalytic domain. Further, the catalytic domain will deaminate dsRNA in the absence of the dsRBMs, albeit poorly.

As the number of ADAR enzymes and substrates from various species rapidly increased over the past several years (Chen *et al.*, 2000; Palladino *et al.*, 2000b; Patton *et al.*, 1997; Tonkin *et al.*, 2002), it became apparent that our understanding of ADAR biochemistry was struggling to keep pace. There are several reasons for this, the most prominent being a dearth of active, pure enzyme. Purification of native enzymes was tedious and yielded low amounts of protein (Hough and Bass, 1994; O'Connell and Keller, 1994; O'Connell *et al.*, 1997). Indeed, the initial purification of *Xenopus* ADAR yielded only a few micrograms from over 4 liters of eggs (Hough and Bass, 1994).

Various expression systems have been employed to increase the yield and specific activity of ADAR proteins. Standard *E. coli* overexpression systems are useful for expressing the dsRBMs, but constructs that contain the catalytic domain are largely insoluble; any soluble protein (typically <1%) lacks deaminase activity (M. R. Macbeth and B. L. Bass, unpublished observations). The reason for this is unclear, but might be explained by the lack of IP₆ in *E. coli*, which is required for ADAR editing activity and perhaps proper folding. Eukaryotic expression systems such as *Pichia pastoris* and insect cell culture harboring baculovirus have been utilized with good results (Cho *et al.*, 2003; Ring *et al.*, 2004). However, each of these systems has its drawbacks. For the *Pichia* system, induction times are several days, and, in our experience, yields are low. We believe the poor yield is due to the toxic nature of ADAR overexpression, which may lead to downregulation. The baculovirus–insect cell system is tedious and requires tissue culture facilities and expensive media.

We have achieved good success using a *Saccharomyces cerevisiae* overexpression system for generating full-length and truncated ADAR proteins that contain the catalytic domain. There are many benefits to using this system: (1) There is a short (~6 h) induction time that is beneficial if the enzymes are unstable or toxic. (2) The yeasts are easy to genetically manipulate, which allows studying the effects of other factors (i.e., IP₆) on ADAR function. (3) There is no contamination from native editing activity, as *S. cerevisiae* does not encode an ADAR. (4) The medium is relatively inexpensive.

The ADAR proteins expressed in this system are produced in substantial quantities, are highly active, and are >99% pure. They have been used for several biophysical and biochemical assays including equilibrium sedimentation, dynamic light scattering, gel shift analyses, RNA editing, and X-ray crystallography (Macbeth *et al.*, 2004, 2005). The details of the expression and purification, as well as some of the protein analyses, are discussed in Chapter 16.

2. Methods

2.1. General considerations

Unless stated otherwise, all media and chemicals are from Sigma-Aldrich. Large-scale yeast expression cultures are grown at 30° using a 15-liter Bellco bioreactor that allows for stable regulation of oxygen levels at 22%. All media and purification buffers are either autoclaved or filter sterilized. Smaller cultures can be grown in shaker flasks with proportionate amounts of media components; however, expect disproportionately less cell mass and purified protein, an effect, we believe, that is due to unregulated air/O₂ levels. Before attempting a large-scale

preparation, it is prudent to perform a small-scale growth (50–100 ml cultures) and test for expression by Western blotting using an appropriate antibody.

All purification steps are performed at 4° or on ice, and after each step the protein is analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Many of the purification steps described here use a pump-driven AKTA system (GE Healthcare); however, many columns can be eluted using gravity flow or a peristaltic pump. It is imperative that the time from cell lysis to protein storage be minimized for the highest specific activity of the enzyme.

2.2. Expression constructs and yeast strains

The vector used for overexpression of ADARs is based on the YEpTOP2P-GAL1 vector used to express DNA topoisomerase (Giaever *et al.*, 1988). The vector has a pBR322 backbone and contains a URA3 gene, a 2 μ m origin of replication, an ampicillin resistance marker, and a GAL1 promoter immediately upstream of the *Bam*HI (5') cloning site (Fig. 15.1A). If the ADAR gene contains a *Bam*HI or *Xho*I site, several other restriction enzymes are available that will cleave different recognition sites yet leave *Bam*HI and *Xho*I compatible ends. We have successfully used *Bsm*BI (New England Biolabs) for this purpose.

The ADAR insert construct generated by polymerase chain reaction (PCR) consists of an N-terminal 10-histidine tag followed by a tobacco etch virus (TEV) protease recognition site (Fig. 15.1B). The 5' end of the PCR product is of the sequence GGGGGG GGATCC GTAACC ATGTCA (CACCAT)₅ GAGAACCTCTATTTCCAGGGA (X)₂₀. The first six nucleotides provide space from the DNA end for the *Bam*HI recognition site (which is the second six nucleotides). The GTAACC sequence allows for efficient translation initiation, and it is followed by the initiator methionine codon (ATG) and a +2 serine codon (TCA) that also promote efficient translation. A 10 $\times$ -histidine coding sequence, (CACCAT)₅, is followed by a sequence that codes for TEV protease recognition, and the remaining ~20 nucleotides (X) code for the N-terminus of the ADAR gene of interest. Note that after cleavage of the His-tag with TEV, the protein will contain an N-terminal glycine, a remnant of the TEV recognition site, prior to the start of the ADAR sequence. A primer that encodes this entire sequence is exceptionally long, and we have found that splitting the primer in half and performing tandem PCR led to more efficient cloning. For example, the first round of PCR would incorporate the TEV site onto the N-terminus of the ADAR gene, while the second round would incorporate the restriction site, start codon, and histidine tag. The 3' primer is of the sequence GGGGGG CTCGAG TCA (X)₂₀. The six Gs provide a spacer from the end of the DNA for *Xho*I site recognition, and this is followed by a stop codon and 20 (X) of the 3' residues of the ADAR gene of interest.

After PCR, the ends of the insert product are trimmed with *Bam*HI and *Xho*I and inserted into a vector linearized with the same enzymes. The insert in the resulting plasmid should be sequenced entirely using primers internal to the subcloned ADAR gene. The expression plasmid is then transformed, by electroporation or lithium acetate, into the haploid (type a) yeast strain BCY123, which has the genotype *pep4::HIS3 prb::LEU2 bar1:HISG lys2::GAL1/10-GAL4 can1 ade2 ura3 leu2–3,112*. This strain has several defective proteases, allows selection of URA3 auxotrophs, and expresses additional Gal4 transcriptional activator during induction with galactose.

2.3. Yeast growth

After transformation, the yeasts are plated on agar plates containing 1 $\times$ minimal media without uracil [6.7 g/liter yeast nitrogen base containing (NH₄)₂SO₄ and lacking amino acids (Fisher), 10 g/liter succinic acid, 6 g/liter NaOH, 1.92 g/liter yeast synthetic dropout media without

uracil, 22 mg/liter adenine hemisulfate] supplemented with 1 M sorbitol and 2% dextrose. Colonies take 2–3 days to appear. We have found it beneficial to transform the expression plasmid freshly, prior to attempting a large-scale growth, and avoid using glycerol stocks of yeast harboring the plasmid. As mentioned, prior to attempting a large-scale growth for ADAR expression, small-scale cultures should be used to test for expression of the ADAR.

The cells are grown in three stages in which the expression of the ADAR gene is repressed, derepressed, or activated. In the repressed state, the cells are grown in the presence of dextrose (glucose) in the starter culture. The cells are then switched to a media containing glycerol and lactic acid. The dextrose is depleted, and the gene is then ready for induction by the addition of galactose, which activates transcription of the ADAR gene from the GAL1 promoter.

For ADAR expression, a 10 ml starter culture consisting of 1× minimal media without uracil supplemented with 2% dextrose is inoculated with one colony and grown overnight at 30° while shaking at 300 rpm. The following morning, the entire culture is used to inoculate 200 ml of the same media. After 24 h, the 200 ml culture is aseptically pumped into a 15-liter Bellco bioreactor vessel containing 13.5 liters 1× minimal media without uracil and 2% glycerol/3% lactic acid as the carbon source. The oxygen levels are calibrated according to the manufacturer's instructions, set at 22%, and the culture is grown at 30° with vigorous stirring. After 24 h, the culture is induced by the addition of 1.5 liter of 2× minimal media and 1.5 liter 30% galactose (final concentration of 2.7%). The final volume of the culture is 16.5 liters. Induction is for 5–8 h, but should be optimized for each construct by performing a time course and measuring protein production by Western blotting. The cells are harvested by centrifugation at 6000 rpm for 5 min in a Beckman JA-20 centrifuge equipped with a JLA 8.100 6L fixed-angle rotor. The cells are washed in a solution containing 20 mM Tris-HCl, 100 mM NaCl, 5% glycerol, 25 mM NaF, 1 mM sodium bisulfite and then centrifuged again. The wet mass of the cell pellet is determined prior to storage at -80°. The typical yield is approximately 10–12 g wet cells/liter of culture. To test for expression, we routinely lyse 0.1 g of cells by vortexing with glass beads for 30 min at 4° in 500 µl of a solution containing 20 mM Tris-HCl, 100 mM NaCl, 5% glycerol, 0.5 mM dithiothreitol (DTT), and 0.01% Triton X-100. The lysate is clarified by centrifugation at 14,000 rpm in a microfuge, and expressed proteins are detected by western blotting using the Penta-His antibody (Qiagen).

2.4. Purification

Typically, cells from 2.75 liters of the culture (~25–30 g) will yield enough pure protein for most applications, including X-ray crystallography, while the remainder of the cells can be stored at -80° indefinitely. The cells are resuspended in Buffer A (20 mM Tris-HCl, pH 8.0, 5% glycerol, 1 mM 2-mercaptoethanol) containing 750 mM NaCl, 35 mM imidazole, and 0.01% Triton X-100.¹ The cells are lysed by three passes through a Gaulin homogenizer (APV) at 20,000 psi² and the extract is clarified by centrifugation at 100,000×g for 1 h. The supernatant is mixed with 5 ml of Ni-NTA agarose (Qiagen) equilibrated with the same buffer used to lyse the cells. The slurry is gently rocked at 4° for 15 min and poured into a 2.5 × 20-cm column, while collecting the flowthrough by gravity. The washes are performed in four 40-ml steps: the first is with the same buffer used to lyse the cells; the three subsequent washes use Buffer A containing 35 mM imidazole and either 750 mM, 300 mM, or 100 mM NaCl, consecutively, in order to decrease the NaCl concentration. The protein is eluted in 5 × 5-ml fractions of Buffer A containing 400 mM imidazole and 100 mM NaCl. The flowthrough, wash, and elution fractions are analyzed by SDS-PAGE, and the protein is visualized by staining the gel with

¹If the protein is to be analyzed by mass spectrometry, the Triton X-100 should be omitted, as detergents strongly interfere with ionization.

²For smaller-scale preparations, i.e., 1-liter cultures or less, a French pressure cell is used. Other methods for lysing the yeast, such as sonication and vortexing in glass beads, have been inefficient in our hands, compared to lysing the cells under high pressure.

Coomassie blue. Figure 15.2A shows the purification of an hADAR2 protein construct after the first Ni-NTA column.

An AKTA FPLC system (GE Healthcare) is used to load the eluted protein onto a 5-ml Hi-Trap heparin column (GE Healthcare) equilibrated with Buffer A containing 100 mM NaCl. The column is washed with 50 ml Buffer A containing 100 mM NaCl and eluted with a gradient of 100 mM to 1 M NaCl in Buffer A. ADAR proteins typically elute between 300 and 500 mM NaCl, and fractions are analyzed by SDS-PAGE. At this stage, the hADAR2 protein is approximately 85–90% pure (Fig. 15.2B).

The fractions containing the ADAR protein are pooled, and TEV protease (Invitrogen) is added to cleave the His-tag and TEV recognition site from the ADAR protein. Typically, 100 units of TEV are used to cleave 5 mg of ADAR, but quantities of TEV added should be optimized empirically. The entire TEV-ADAR reaction is dialyzed against Buffer A containing 200 mM NaCl overnight at 4°. The following day, the reaction contents are bound to 5 ml of equilibrated Ni-NTA agarose for 1 h with gentle rocking. The slurry is poured into a 2.5 × 20-cm column and washed with 80 ml of Buffer A with 200 mM NaCl. Uncleaved protein and the cleaved His-tag (as well as several other contaminants) remain bound to the resin. The flowthrough and wash (which contain the cleaved ADAR protein) are concentrated to ~1 ml using an Amicon-Ultra 15 centrifugal concentrator tube with a 30,000 Da molecular weight cut-off. If the majority of the ADAR remains bound to the resin and is not in the flowthrough or wash, it is likely the TEV reaction was inefficient. The major factor affecting cleavage by TEV is the presence of secondary structure at the N-terminus of the ADAR, which may inhibit recognition of the TEV site. If this is a problem, the N-terminus should be redesigned to include three glycine residues to act as a “spacer” between the cleavage site and the N-terminus of the ADAR.

The concentrated protein is then injected onto a Superdex 200 26/60 gel filtration column (GE Healthcare), and eluted with Buffer A containing 200 mM NaCl. One milliliter fractions are collected and analyzed by SDS-PAGE (Fig. 15.3A). Typically, our hADAR2 protein construct (calculated MW ~76.6 kDa) elutes as a symmetrical peak with a molecular weight of ~100 kDa, relative to a standard curve of proteins of a known size. The “shoulders” of the peak often contain minor contaminants and are discarded. The main peak fractions are pooled, concentrated, and dialyzed against storage buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 20% glycerol, and 1 mM 2-mercaptoethanol. We quantify the protein using two methods. The first is by the Bradford method using a kit available from Bio-Rad; the second is by SYPRO-Red (Molecular Probes) staining of an SDS-PAGE gel and visualization using a Molecular Dynamics Storm Phosphor Imager. Yields of pure protein vary between construct, but typically range from 3 to 10 mg/liter of original yeast culture. The protein is stored as aliquots at –80°.

3. Analysis of Purified hADAR2

Various ADAR proteins purified using this scheme have been used for kinetic analyses, RNA binding assays, and X-ray crystallography (Haudenschild *et al.*, 2004; Macbeth *et al.*, 2004, 2005). Here we present an analysis of the oligomerization state of hADAR2, as purified from *S. cerevisiae*, using analytical gel filtration and equilibrium sedimentation.

3.1. Analytical gel filtration

For an analytical gel filtration analysis, 50 µl of purified, concentrated (6.6 mg/ml) hADAR2 is injected onto a Superdex 200 10/300 gel filtration column using an AKTA FPLC system (GE Healthcare). The protein was eluted with 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5% glycerol, and 1 mM 2-mercaptoethanol, and the elution profile was analyzed with the

PrimeView software package (GE Healthcare, Fig. 15.3B). The elution volume of the hADAR2 peak was determined to be 14.4 ml. Relative to the elution of standard molecular weight markers, hADAR2 has an observed molecular weight of 98.1 kDa and appears to be a monomer under these conditions (Fig. 15.3B).

3.2. Equilibrium sedimentation

To observe the oligomeric state of hADAR2, equilibrium sedimentation is performed as it allows an accurate determination of the molecular weight of a macromolecule that is independent from its shape. Purified hADAR2 was dialyzed against a buffer containing 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 1 mM 2-mercaptoethanol and diluted to 1.3, 3.3, and 6.6 μ M. The protein was centrifuged in a Beckman XLA analytical ultracentrifuge, equipped with the AN-60 Ti rotor, at 16,000 and 18,000 rpm. Figure 15.3C shows data, fits, and residuals for hADAR2 spun at the three concentrations. The best overall fit of the data was to a single species molecule with an observed molecular weight of 75,320 Da. The residuals are relatively small and randomly distributed, indicating a good fit of the data. The MW_{obs}/MW_{calc} is 0.98, suggesting hADAR2 exists as a monomer under these concentrations.

4. Future Perspectives

We have developed an overexpression protocol and rapid purification scheme to generate ADAR protein that is untagged and contains native sequence except for an N-terminal glycine. The protein is >99% pure, exists as a single species, and is highly active in deaminating dsRNA. The protein purified using this system can be used for many biophysical and structural studies (such as calorimetry, crystallography, and single turnover kinetics) that require large amounts of pure protein.

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Figure 15.1.

The expression vector and 5' sequence of the construct used to tag and purify ADARs. (A) The pSc-ADAR plasmid. The empty vector (lacking an ADAR gene) is approximately 8 kb. The *Bam*HI and *Xho*I restriction sites are unique in the empty vector. Transcription of the ADAR gene is controlled by the GAL1 promoter (indicated by the arrow). (B) A schematic of the ADAR construct after PCR of an ADAR gene using the 5' primers described in the text. The translation start and stop sites are indicated. Below the schematic are the DNA and translated protein sequences. Note that after cleavage with TEV protease, there is a glycine residue N-terminal to the first methionine of the ADAR protein of interest.

Figure 15.2.

SDS-PAGE analysis of the first two purification steps of hADAR2. (A) The Coomassie blue-stained gel after the first Ni-NTA column. The lanes from left to right are molecular weight standard (M), flowthrough (FT), washes 1–3 (W1–W3), and elution fractions (E1–E5). hADAR2 migrates as a band of 80–90 kDa. (B) The stained gel containing fractions eluted from a heparin column. Every third fraction from a 0.1 to 1M NaCl elution gradient is loaded on the gel.

Figure 15.3.

Analysis of hADAR2 purified from *S. cerevisiae*. (A) A Coomassie blue-stained gel containing every third fraction from the hADAR2 peak eluted from a Superdex 200 26/60 preparative gel filtration column. (B) The UV trace of hADAR2 eluted from a Superdex 200 10/300 analytical gel filtration column. The elution volume of hADAR2 is 14.4 ml. The elution peaks of several molecular weight standards are indicated and numbered 1–4. Their sizes and retention volumes are (1) aldolase 158 kDa, 13.8 ml, (2) albumin 67 kDa, 14.7 ml, (3) ovalbumin 43 kDa, 15.7 ml, and (4) chymotrypsinogen A 25 kDa, 17.5 ml. The calculated molecular weight of hADAR2, determined from a standard curve of the known molecular weight standards, is 98.1 kDa. (C) The equilibrium sedimentation data for hADAR2 at concentrations of 1.3 ($\circ$), 2.6 (Δ), and 6.6 ($+$) μ M, respectively. The lower panels are the data fit to a species of MW 75,320 Da. The upper panels show the residuals corresponding to each fit.