Biochemical Analysis of ADAR1 Mutant to Characterize Potential Dimerization Events in ADAR1

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ABSTRACT: Adenosine Deaminase Acting on RNA (ADAR) is a base-editing enzyme that post-transcriptionally modifies the structure of an Adenosine on a double-stranded RNA substrate to an Inosine. Dysregulation or dysfunction of ADAR has been linked to several disorders; indicating ADAR proteins serve a key role in certain biological pathways linked to tumor sensitivity and diseases such as Aicardi-Goutières syndrome (AGS) [5,6]. A recently solved crystal structure of ADAR2 R2D revealed a helix involved in protein-protein interaction. Considering the sequence in the helix is conserved between ADAR1 and ADAR2, we hypothesize that ADAR1 shares the dimerization mechanism of ADAR2 within the same RNA loop. To begin answering our hypothesis, we a performed preliminary study where we introduced an alanine to position D1023, a residue in the helix involved in protein-protein interaction through an essential hydrogen bond interaction. This mutation is analogous to a mutation in ADAR2 R2D that impaired its dimerization mechanism. Initial results from size exclusion chromatography (SEC) revealed that the ADAR1 dsRBD mutation has minimal effect in protein-protein interaction in contrast to ADAR2 R2D mutation.

INTRODUCTION

Adenosine Deaminase Acting on RNA (ADAR) is a base-editing enzyme that post-transcriptionally modifies the structure of an Adenosine on a double-stranded substrate to an Inosine (Figure 1a**)**. ADAR's hydrolytic deamination alters the hydrogen bond specificity of adenosine to resemble a guanosine base pair. This A to G modification caused by ADAR1 recodes specific codon in the human transcriptome, regulates microRNA-mediated modulation of specific genes and regulates splicing events [1-4]. ADAR1, an isoform of the ADAR family, has been linked to several diseases. A recent study has revealed that the loss of ADAR1 genes increases tumor sensitivity to therapeutic agents. Reducing the presence of ADAR1 would reduce A-to-I editing of interferon-inducible RNA species which are important for antigen presentation in anti-tumor checkpoints[5]. Therefore, their results showed that the reduced presence of ADAR1 allowed inducible RNA species to function efficiently, allowing the tumor to become sensitized to immunotherapy. Loss of function mutations in ADAR1 have been linked with Aicardi-Goutières syndrome (AGS). AGS related mutations in ADAR1 indicate its potential role as a suppressor of type I interferon signaling. This suggests ADAR1 prevents abnormal upregulation of interferon-stimulated genes observed in AGS patients[6]. With the growing interest in ADAR1, structural information of the protein is becoming increasingly sought after as it may provide key insight on its stability and catalysis.

Despite our limited knowledge of ADAR1's structure, recent progress on ADAR2, a close member of ADAR1 in the ADAR family (Figure 1b), has revealed an essential dimerization interface through a resolved ADAR2-R2D crystal structure produced by Alexander et al[7]. Furthermore, the structure identified a conserved dimerization interface consisting of the homologous sequence TWDG. Alexander et al. observed D503 had three potentially essential

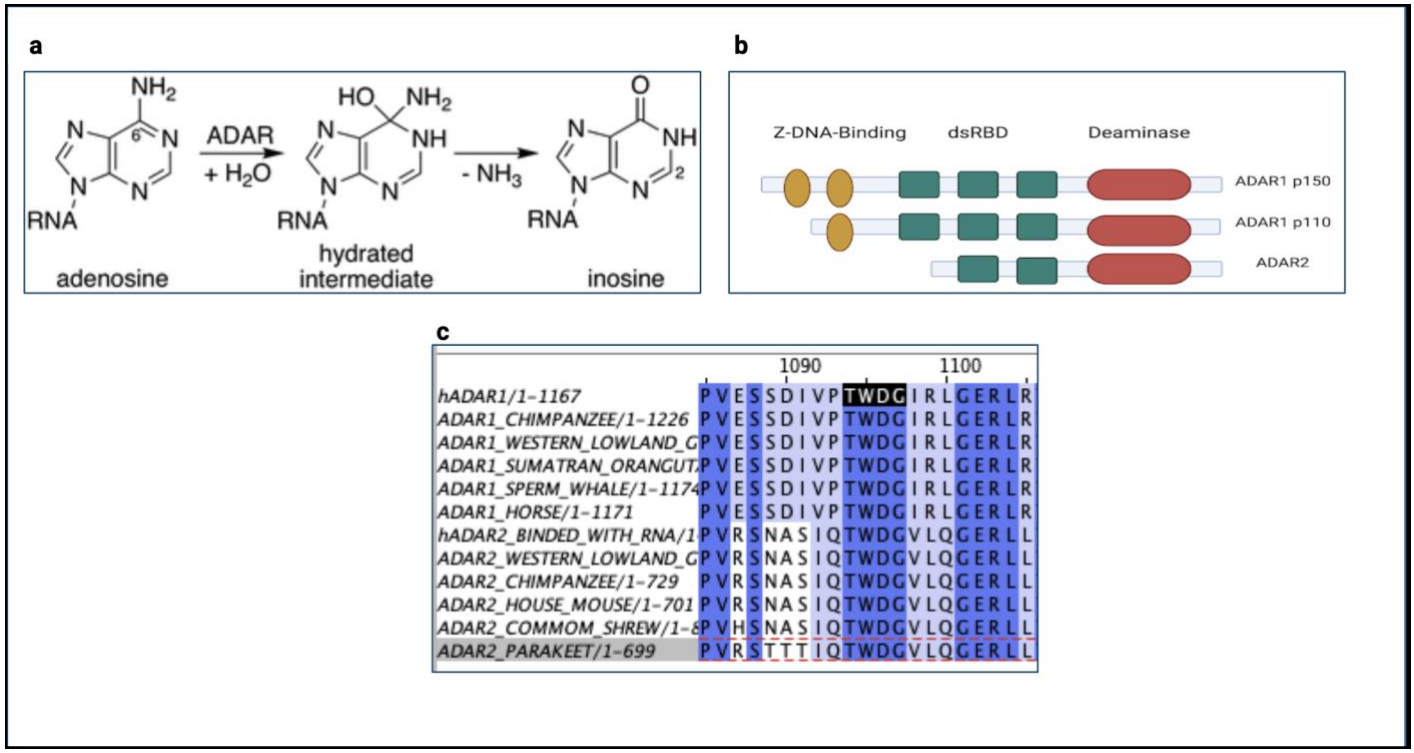


Figure 1. a Mechanism of ADAR's hydrolytic deamination. **b** ADAR1 and ADAR2 are closely related and belong to the same ADAR family. **c** close alignment of ADAR1 and ADAR2 from various species reveal a conserved sequence essential to ADAR2 R2D dimerization and deamination.

hydrogen bonds to the attached monomer that wasn't directly involved in the editing of the RNA substrate. The sidechain of D503 residue of the active monomer was in hydrogen bond distance from the main chain of G452 and the side chain of T490, where the main chain of D503 was in hydrogen bond distance from the side chain of R455; mutation of the D503 residue to alanine resulted in a loss of dimerization and decrease in editing in dimerization dependent substrate 5-HT_{2C}R. TWDG sequence is conserved in ADAR1, leading us to hypothesize that ADAR1 shares a similar dimerization mechanism (Figure 1c). Here, we use the structural knowledge of ADAR2-R2D to mutate our MBP-ADAR1 dsRBD construct at D1023 residue to alanine and analyze the effects of this mutation through size exclusion chromatography.

MATERIALS & METHODS

Site-Directed Mutagenesis

D1023A mutant was introduced using site-directed mutagenesis using the Quick-Change II mutagenesis kit. The primers designed contained the mutation of interest. Mutant plasmids were confirmed by sanger sequencing.

Expression and purification of ADAR1 double-stranded RNA binding domain and deaminase domain carrying D1023A mutation (ADAR1-dsRBD D1023A)

The expression and purification performed on the mutated protein closely followed a previously reported protocol[8]. The yeast strain *Saccharomyces cerevisiae* BCY 123 cells were transformed with a pSc-ADAR plasmid containing our MBP-ADAR1-dsRBD D1023A sequence using lithium acetate. Following transformation, the cells were streaked onto minimal media without Uracil) CM-ura. Individual colonies were selected inoculated in a 15ml media containing CM-ura and glucose. The culture was grown overnight (~18 hrs) at 30°C shaken at 300rpm. 6ml of starter culture was transferred to 750ml of media containing CM-ura and galactose and grown overnight for 21 hours until cells reached OD_{600} of 1.5. Cells were induced for expression by adding 82.5 ml of 30% galactose into each flask at which point the protein was left to express for ~6 hrs. The cells were then pelleted for 8 min at 5000xg and stored at -800°C.

The cells were then lysed in 750mM NaCl lysis buffer (20mM Tris-HCl, pH 8.0, 5% glycerol, 1 mM BME, 0.1mM $ZnCl_2$ and 0.05% Triton X-100) with a microfluidizer. The lysate was centrifuged at 18500 rpm for 50 mins. The supernatant was separated from the pellet and mixed with 2.5ml of amylose equilibrated with Buffer A (20mM Tris-HCl, pH 8.0, 5% glycerol, 1 mM BME, 0.1mM $ZnCl_2$). The slurry was gently rocked for 30 min at 4ºC and then poured into a 2.5 x 20 -cm column packed with 3ml amylose resin for a gravity flow purification. Three washes were performed using 12 column volumes of the following buffers, Wash I (20 mM Tris–HCl, pH 8.0, 5% glycerol, 1M NaCl, 1mM 2 -mercaptoethanol, 0.1mM $ZnCl_2$), Wash II (20 mM Tris–HCl, pH 7.5, 5% glycerol, 500mM NaCl, 1mM 2 -mercaptoethanol, 0.1mM $ZnCl_2$), Wash III (20 mM Tris–HCl, pH 7.0, 5% glycerol, 75mM NaCl, 1mM 2 -mercaptoethanol, 0.1mM $ZnCl_2$). The protein was eluted using wash III + 10mM Maltose. Eluted fractions were analyzed by staining samples with blue dye and running an SDS-PAGE gel.

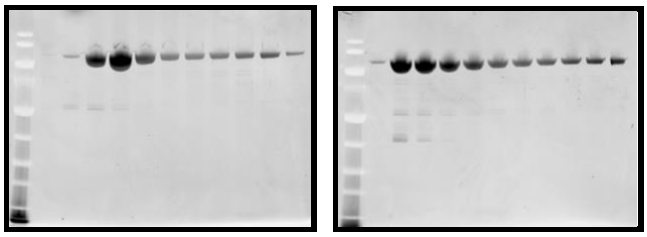
Fractions containing protein were pooled, diluted to 55 ml and purified in a 5ml Hi-Trap heparin column (GE Healthcare) column using an AKTA FPLC system (GE Healthcare). The column was equilibrated with 50 ml of buffer A (20mM Bis-tris, 50mM NaCl, 5% glycerol, 1mM 2-mercaptoethanol, pH 7). The protein sample was then loaded with 0-100% buffer B [20mM Tris, 1M NaCl, 5%glycerol pH 8] over 8CV at a flow rate of 1.3 ml/min. The eluted fractions were pooled and analyzed using an SDS-PAGE gel from which fractions containing the protein were pooled and dialyzed against the final dialysis buffer (50mM Tris-HCl pH 8.0, 200mM KCl,0.01% NP-40,10% glycerol, 1mM DTT, 0.1mM $ZnCl_2$. After dialysis, final centrifugation was performed followed by BSA serial dilutions to determine final concentrations visualized by SYPRO orange staining on a final SDS-PAGE gel.

Analytical gel-filtration: size exclusion chromatography of ADAR1 dsRBD D1023A

The size exclusion chromatography analysis of ADAR1 dsRBD D023A was performed by injecting 25u1 of 10uM ADAR1 dsRBD D1023A onto a superdex 5/150 gel filtration column using an AKTA FPLC system (GE healthcare). The protein was eluted with 20mM Tris-HCl pH 8.0, 350 mM NaCl, 5% glycerol. The elution profile was analyzed using the software Unicorn 7.4 package.

RESULTS

Protein analysis was done throughout the time-sensitive purification process to ensure the viability of our protein. Once purified, the protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The first SDS-PAGE gel analysis was performed after our amylose gravity flow through purification - the gel revealed bands that indicated we had successfully purified our mutated protein. The second SDS-Page gel was performed on eluted fractions from the 5-ml Hi-Trap heparin column (GE Healthcare) from which the gel revealed clear bands that indicated our ADAR1 dsRBD D1023A protein was purified.

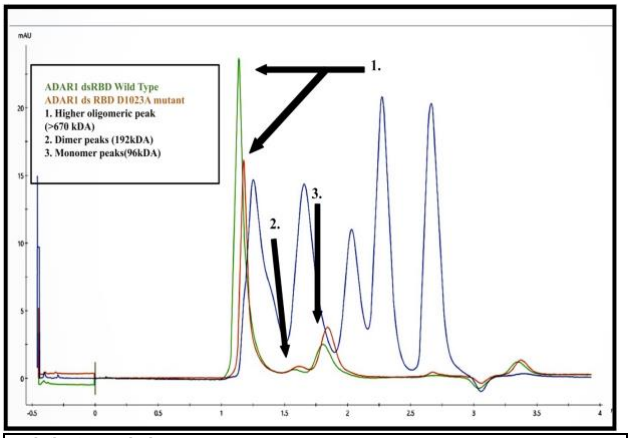


Amylose **Heparin**

Figure 2. SDS-PAGE Gels confirms purified ADAR1 dsRBD D1023A in post-gel analysis for amylose and heparin purifications.

The MBP-ADAR1 dsRBD construct equilibrated to three distinct peaks: monomer, dimer, and highly aggregated oligomeric state peaks in the size exclusion column. We calculated the area under the curves and calculated the percentages for each of these distinct peaks. In comparison to the wild-type protein, the D1023A mutant had a near two-fold increase in percent % monomer peaks and a decrease by a similar magnitude in higher oligomeric peaks (Figure 3). The dimer peaks exhibited a minimal shift in the dimer peak profile in comparison to ADAR2 R2D D503A peak profile. To confirm this observation, we took the area under the curves for each peak and found the ratio for each corresponding peak (Table 1). The ratios confirmed the notable drop in peak height in wildtype compared to D1023A mutant in region 1 peaks which correspond to

oligomeric species. We also observed an increase by a similar magnitude in region 3 peaks which correspond to monomer peaks.



Gel Filtration Standards					
Component	Thyroglobulin (Bovine)	Y-globulin (Bovine)	Ovalbumin (chicken)	Myoglobulin (horse)	Vitamin B12
Molecular Weight(kDa)	670,000	158,000	44,000	17,000	1,350

Figure 3. Size Exclusion Chromatography (SEC) profile of ADAR1 dsRBD D1023A reveals minimal disruption in comparison to ADAR1 dsRBD wildtype. Blue peaks correspond to Gel filtration standard components for reference.

Corresponding Peak [Wildtype: Mutant]	Peak Ratio of calculated percentages
Peak 1	1.73
Peak 2	0.842
Peak 3	0.869

Table 1. Ratios for the area under the peaks from the SEC produced by ADAR1 dsRBD WT and ADAR1 dsRBD D1023A.

DISCUSSION

Our study aimed to determine if ADAR1 dsRBD mutation D1023A had any effect on both the dimerization and deamination of ADAR1 dsRBD. The only comparable results obtained are from the SEC performed where we observed a reduction in oligomer peak as well as an increase in monomer peak by a similar magnitude regarding ADAR1 dsRBD D1023A. It should be noted that our SEC data is of an MBP fusion protein, meaning our SEC peaks may not be an accurate representation of true peak profiles; however, the shifts in the peaks we see are significant enough to believe this shift in peak profiles can be attributed to the disruption of important hydrogen bond contacts that Alexander et al. observed in D503 that are also present in D1023A.

FUTURE WORK

If ADAR1 dsRBD shares the same dimerization mechanism as ADAR2 R2D, we expect the D1023A mutation to interrupt its deamination activity in certain substrates. Although we haven't been able to perform this, we predict that protein-protein interaction may be required for the deamination of certain ADAR1 substrates. We plan to perform deamination assays to measure the editing efficiency of our mutation, allowing us to observe what kind of effects this has on ADAR1 dsRBD D1023A. It could be that there are more suitable mutations that could lead to a higher disruption in the same D1023 position. We mutated an aspartate, a negatively charged residue in physiological conditions, into a neutrally charged alanine; however, it could be that if we instead mutated aspartate into a positively charged residue such as arginine that we would create stronger disruption in the potential hydrogen bonds that form. In the future, we want to explore all amino acids in place of D1023, utilizing the SAT-FAC-Seq screening method developed by the Beal lab.

This method will allow us to individually explore any disruption caused by the other 19 amino acids through saturation mutagenesis. Saturation mutagenesis would then be followed by fluorescence-activated cell sorting (FACS) to sort yeast cells containing our fluorescent activity reporter based on observed fluorescence. We will then incorporate next-generation sequencing (Seq) to help us identify ADAR library mutants that will give us a better idea of editing efficiency[9]. Ultimately, this method will allow us to determine which residues will create a larger disruption for further investigation. Sat-Fat-Seq will include the other residues in the homologous TWDG sequence and other residues embedded in the dimerization helix of ADAR1. The importance of D1023 residue and any differences in residues involved in ADAR1 dimerization should be highlighted through the Sat-FACS-Seq screening method.

Once we identify suitable mutations that are not well tolerated in our locations of interest, we'll take a deeper analytical approach. We'll follow up with size exclusion chromatography, deamination kinetics and gel shift assays that will offer a preliminary glimpse of the dimerization mechanism in ADAR1 dsRBD.

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