A real-time biochemical assay for AID-catalyzed DNA deamination

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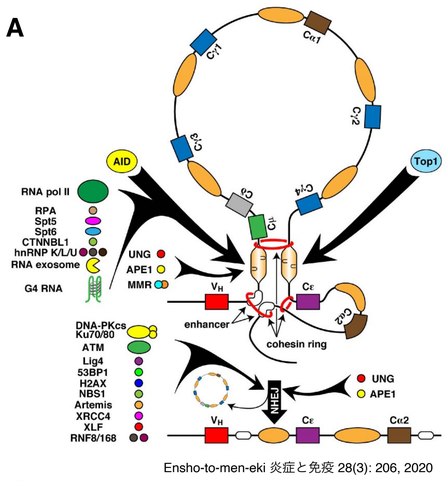
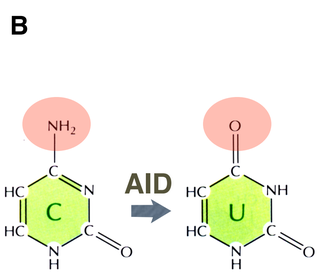
Activation-induced cytidine deaminase (AID), a member of the APOBEC cytidine deaminase family, plays a central role in immunoglobulin gene diversification through class-switch recombination and somatic hypermutation in mammals. However, AID's off-target activity on non-immunoglobulin genes, including cancer-related ones, has been implicated in tumorigenesis. Therefore, the development of specific AID inhibitors could be a promising strategy for treating antibody-mediated diseases such as allergies and autoimmune disorders, as well as certain malignancies, by suppressing its off-target effects.

To this end, we adapted the RADD assay, originally developed by Hideki Aihara's group at the University of Minnesota for APOBEC3 inhibitor screening, and optimized the substrates for AID. A 20-nucleotide synthetic DNA was selected as the optimal substrate, containing a 5'-end fluorophore, a single cytosine in the AID-preferred context, and dual quenchers positioned centrally and at the 3' end. Our assay utilizes EndoQ, a recently characterized DNA repair enzyme from *Pyrococcus furiosus*, which cleaves DNA adjacent to 2'-deoxyuridine generated by AID. Unlike conventional methods requiring uracil removal by uracil-DNA glycosylase followed by alkaline heating, EndoQ enables direct DNA cleavage at 37 °C, facilitating high-throughput screening in a multi-well plate system.

This method provides a sensitive and efficient assay for AID inhibitor screening.

1. Principle of the assay and substrate optimization

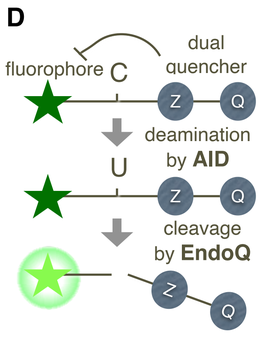




C

human Sμ switch region (60 nt part)
T2T-CHM13v2.0 14:100130138-100130197 (-)
GRCh38 14:105858572-105858631 (-)

AGCTgggctaagccgAGCTgAGCTgAGCTg-
AGCTgggatgatctgggctgggctgggctg



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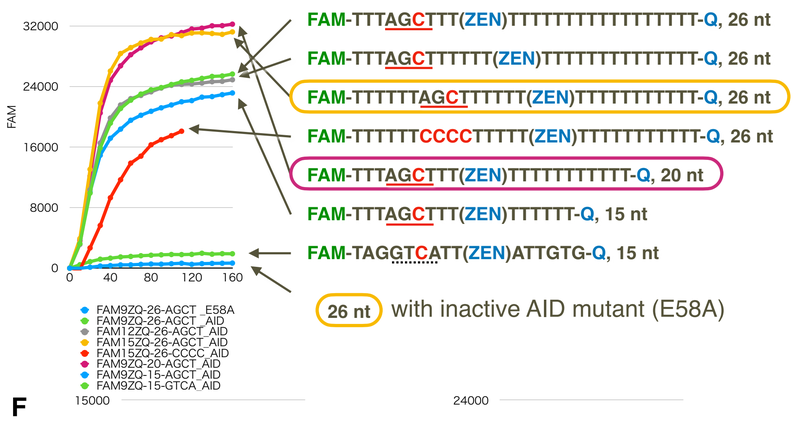

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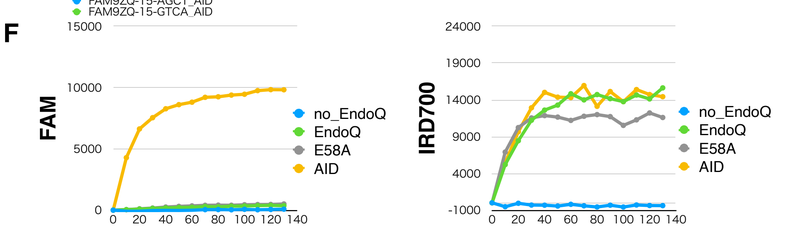

FAM-TTTAGCTTT(ZEN)TTTTTTTTTT-Q, 20 nt IRD700-TAGGTUATT(ZEN)ATTGTG-Q, 15 nt

AID

FAM-TTTAGUTTT(ZEN)TTTTTTTTTT-Q

EndoQ

FAM-TTTAG-3'

5'-UTTT(ZEN)TTTTTTTTTT-Q

EndoQ

IRD700-TAGGT-3'

5'-UATT(ZEN)ATTGTG-Q

Figure 1. *(left bottom) a* (**A**) The looping-out model of class-switch recombination. AID converts cytosine into uracil in both upstream and downstream switch regions (orange ovals), triggering cleavage (center). Subsequently, double-strand DNA breaks are repaired by non-homologous end joining (NHEJ). (**B**) Deamination of cytosine by AID, producing uracil. (**C**) AGCT motif in switch regions that are composed of repetitive sequences, spanning 1-10 kb preceding each constant region genes. (**D**) Assay principle showing cytosine (C) containing oligonucleotide deaminated by AID, followed by EndoQ cleavage, releasing quencher, and increment of fluorescence (1). (**E**) Comparison of FAM-labeled substrates for AID, in terms of fluorescence increase during reaction with AID and EndoQ. AGCT containing 26-nucleotide (nt)- (yellow box) or 20-nt-long oligonucleotides (purple box) gave the highest fluorescence increase. F. Dual assay using FAM-labeled C-containing oligonucleotide and IRD700 (infrared dye)-labeled uracil (U)-containing oligonucleotide is (2). The former reports combined activity of AID and EndoQ, while the latter reports only EndoQ activity. The sequence of IRD700 oligonucleotide were taken from a previous report (1).

2. Preparation of AID protein

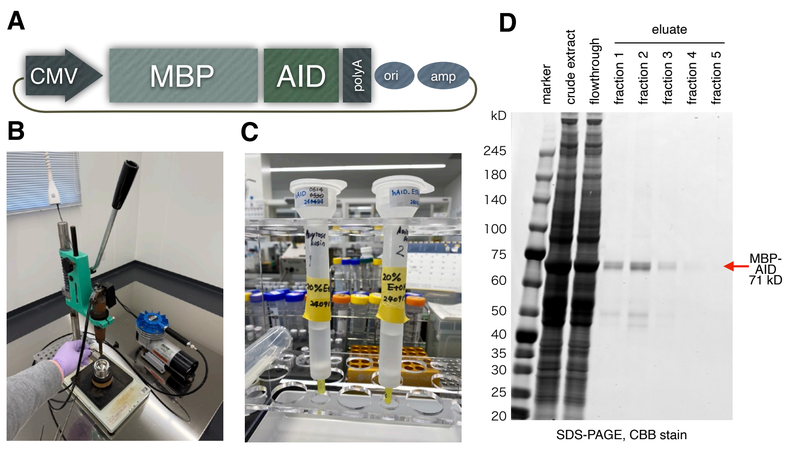

Figure 2. (**A**) A plasmid vector with cytomegarvirus (CMV) promoter which drives expression of fusion protein of maltose-binding protein (MBP) and full-length human AID. This plasmid was transiently introduced into adherent culture of Expi293F cells using PEI-MAX lipofection reagent (24765, Polysciences), based on a literature (3) with modifications. Cells were collected after 2 days. Liquid-nitrogen-frozen cell suspension in a lysis buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM PMSF, 0.2 mg/ml DNase I, 1 mM DTT) was pulverized with a Cryopress device (Microtech Nichion, Japan) (**B**). Cleared lysate by centrifuge was applied to 2.0 ml (bed) of amylose-resin (E8021S, New England Biolabs) packed in a column (732101, Bio-rad: **C**). After washing with a buffer (20 mM Hepes pH7.5, 150 mM NaCl, 10% glycerol, 1 mM DTT), MBP-AID protein was eluted sequentially with 0.5 ml of buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 10 mM maltose, 1 mM DTT). (**D**) The presence of MBP-AID in each fraction was verified with SDS-PAGE gel stained with Coomassie Brilliant Blue (CBB) and western blotting using anti-AID monoclonal antibody MAID-2 (not shown). Fractions 2-5 were pooled and supplemented with 10 μM $ZnCl_2$ and 0.01% Tween-20. The protein concentration was estimated with SDS-PAGE after CBB stain using serial dilutions of known concentration of human IgG1 as a reference (not shown). Fifty μg of MBP-AID protein was obtained per 7 x 10^7 cells (5 x 15-cm dishes).

3. Preparation of EndoQ protein

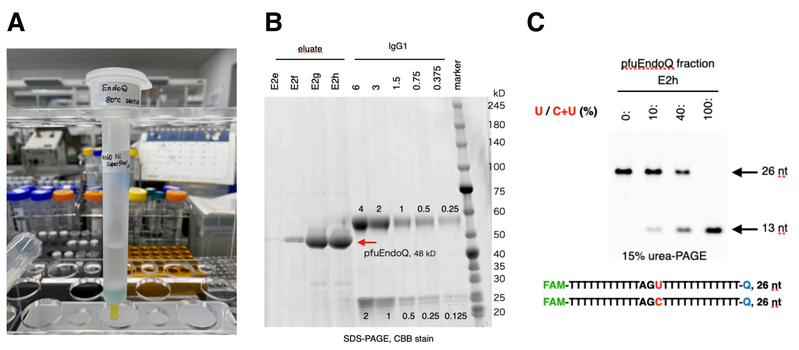

Figure 3. pET24a_pfuEndoQ plasmid, encoding 6xHis-tagged archaeal EndoQ endonuclease specific for not only uracil but also xanthine, hypoxanthine and abasic site (4), was introduced into Rosetta (DE2) *E. coli*. Expression of EndoQ was induced by 0.5 mM IPTG for 24 hours at 18 °C. The cell pellet was lysed in a buffer L (20 mM Tris-HCl pH8.0, 500 mM NaCl, 5 mM imidazole, 5 mM 2-ME) with 1 mg/ml lysozyme and was sonicated on ice. The lysate was heated at 80 °C for 30 min before centrifuge at 15,000 x g. Cleared lysate was 0.22-μm filtrated and diluted with the lysis buffer L before application to a 1-ml bed of His60 Ni Superflow Resin (Takara-bio, Japan) packed in a column (**A**). After washing with buffer L, the protein was eluted sequentially with 0.5 ml of buffer (20 mM Tris-HCl pH8.0, 500 mM NaCl, 400 mM imidazole, 5 mM 2-ME). The presence of EndoQ in each fraction was verified with SDS-PAGE gel stained with CBB (**B**) and western blotting using anti-His monoclonal antibody (not shown). Fractions 3 and 4 were pooled. The protein concentration was estimated with SDS-PAGE after CBB stain using serial dilutions of known concentration of human IgG1 as a reference. Four hundred μg of EndoQ was obtained per 10 ml of LB culture. U-specific endonuclease activity of pfuEndoQ was verified using mixtures of FAM-labeled C- or U-containing oligonucleotides and urea-PAGE (**C**).

4. Real-time AID-catalyzed DNA Deamination (RADD) assay

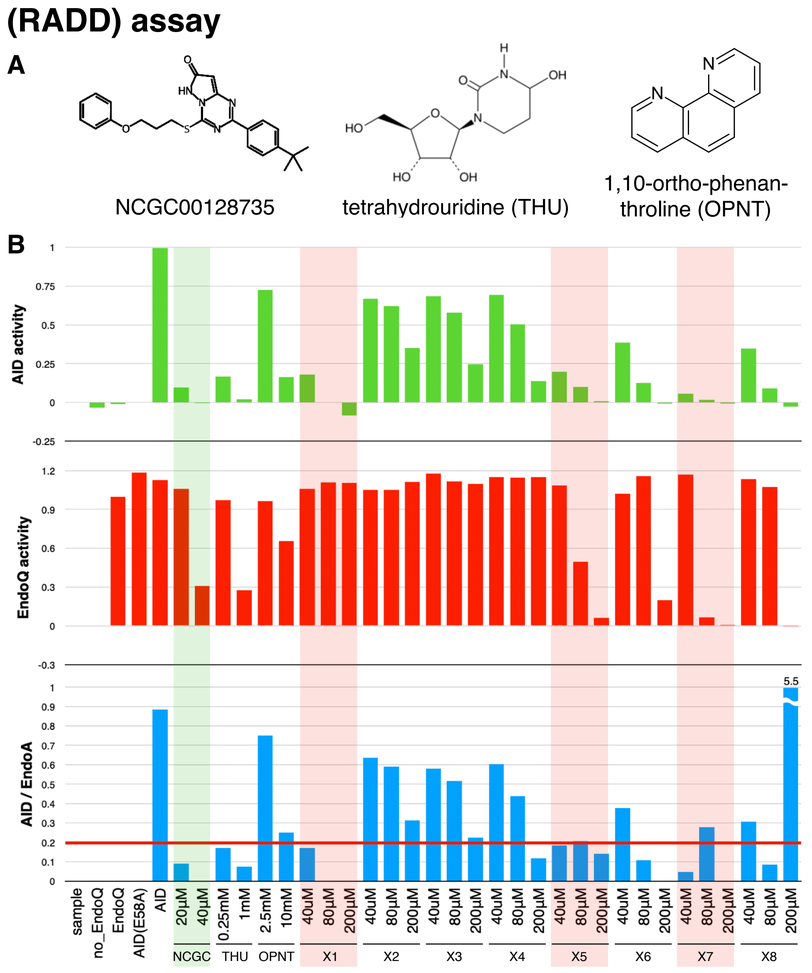

Figure 4. Inhibition of AID activity was tested for known AID inhibitors. (**A**) NCGC00128735 (Lifechemicals F9994-5218; ref. 2), tetrahydrouridine (THU; non-specific cytidine deaminase inhibitor; ref. 5), 1,10-ortho-phenanthroline (OPNT; a chelating agent for zinc and other metal ions; ref. 5). Eighty-one structural analogs of NCGC00128735 and other two compounds reported in ref. 2 were selected from a library at Fuji Pharma Valley Center (Shizuoka, Japan) and tested for AID inhibition at 200 μM using RADD assay (Figure 1F; data not shown). For 8 compounds that exhibited inhibition were further tested at 40, 80, and 200 μM (**B**) Reactions were performed in 45 μl in a well of a 384-well plate at 37 °C. The reaction buffer consisted of 60 mM Tris-HCl pH 8, 0.01% Tween 20, 10% DMSO, 1mM DTT, 0.1 mg/ml RNase A, 10 μM $ZnCl_2$, 80 nM FAM-labeled 20-nt AID substrate (Figure 1F), 20 nM IRD700-labeled EndoQ substrate (Figure 1F), 4 μg/ml (83 nM) EndoQ, 0.8 μg/ml (11 nM) MBP-AID. FAM or IRD700 fluorescence was measured using a SPARK instrument (Tecan, Switzerland) every 10 min for 130 min. The difference between values at 0 and 130 min were subtracted with that of a negative control [AID(E58A) mutant for FAM; no_EndoQ for IRD700]. The values were represented as ratios to a positive control (AID for FAM; EndoQ for IRD700), represented as AID (top) and EndoQ activity (middle), respectively. Degree of inhibition was calculated as AID activity divided by EndoQ activity (bottom), values of which at 40 μM are shown in a table (**C**).

C

compound	AID / EndoQ at 40 μM
NCGC00128735	-0.014
X1	0.170
X2	0.636
X3	0.580
X4	0.604
X5	0.184
X6	0.378
X7	0.048
X8	0.306

5. Conclusion

In addition to the previously reported AID inhibitor NCGC00128735, we identified three moderate inhibitors of AID: compounds X1, X5, and X8. Among them, X1 is unique in that it did not show inhibition of EndoQ at concentrations of 200 μM or lower, while NCGC00128735 showed moderate inhibition at 40 μM. Therefore, X1 is a promising lead compound for future exploration of stronger and more specific AID inhibitors.

The RADD assay, originally reported by Hideki Aihara's group (1) for the APOBEC3 enzymes, was coupled with dual-color monitoring of U-containing DNA cleavage activity, which was developed by Patricia Gearhart's group (2). With the use of the AID-optimized oligonucleotide substrate reported in this study, the application of the RADD assay has been expanded to include inhibitor screening for AID. Applications of AID inhibitors include treatment of antibody-mediated diseases (*e.g.*, allergy and autoimmunity) and cancer prevention, as well as regulation of base editors featuring AID.

6. Acknowledgment

We thank Dr. Hideki Aihara for a gift of the pfuEndoQ expression vector.

7. References

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I have no financial relationships to disclose.