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The nuclear DNA deaminase AID functions distributively whereas cytoplasmic APOBEC3G has a processive mode of action

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ABSTRACT

AID deaminates cytosine in the context of single stranded DNA to generate uracil, essential for effective class-switch recombination, somatic hypermutation and gene conversion at the B cell immunoglobulin locus. As a nuclear DNA mutator, AID activity must be tightly controlled and regulated, but the genetic analysis of AID and other DNA deaminases has left unstudied a number of important biochemical details. We have asked fundamental questions regarding AID's substrate recognition and processing, i.e. whether AID acts distributively or processively. We demonstrate that in vitro, human AID exhibits turnover, a prerequisite for our analysis, and show that it exhibits a distributive mode of action. Using a variety of different assays, we established that human AID is alone unable to act processively on any of a number of DNA substrates, i.e. one AID molecule is unable to carry out multiple, sequential deamination events on the same substrate. This is in contrast to the cytoplasmically expressed anti-viral DNA deaminase APOBEC3G, which acts in a processive manner, possibly suggesting that evolutionary pressure has altered the ability of DNA deaminases to act in a processive or distributive manner, depending on the physiological need.

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1. Introduction

Activation induced cytidine deaminase (AID) was recently identified as a B cell specific protein necessary for antibody maturation during the adaptive immune response [1–4] and one of the evolutionary founder members of the new DNA deaminase family [5]. Deamination by AID of cytosine generates uracil and genetic analysis of AID function indicated the generation of uracil in DNA to be the key step in initiating immunoglobulin diversification [6–8]. Subsequently it was shown that in vitro, DNA deaminases including AID, preferred single stranded DNA as their substrate [9–13], and by genetic as well as in vitro assays that each of the DNA deaminases studied to date has a preferred target motif [9–17]. AID's preferred target motif, WRC̲ (A/T, A/G, C̲), has long been identified to be of importance in the immunoglobulin (Ig) locus during somatic hypermutation (SHM), class-switch recombination (CSR) and gene conversion (GC) (reviewed [18]). This intrinsic target preference is also evident when AID acts as an epigenetic reprogramming factor by deaminating 5-methylcytosine in DNA [19].

In somatic hypermutation if the uracil is replicated, the consequence is a C → T transition. In contrast, if the uracil lesion is excised, creating an abasic site and this is replicated,

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Abbreviations: AID, activation induced cytidine deaminase; hAID, human activation induced cytidine deaminase; Ig, immunoglobulin; SHM, somatic hypermutation; CSR, class-switch recombination; GC, gene conversion; ung, uracil N-glycosylase; MBP, maltose binding protein; WRC, (A/T), (A/G), C; RPA, replication protein A; ACF, APOBECl complementation factor

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both transition and transversion mutations result. Mutations at A:T base pairs are introduced via the second phase of SHM as a potential consequence of mismatch repair of uracil. This second phase is thought to contribute to a greater extent to class-switch recombination, in which DNA breaks forming during repair of the uracil lesion enable DNA recombination of switch regions to take place [7,20–26].

While AID functions in the nucleus, another member of the DNA deaminase family, APOBEC3G, is confined to the cytoplasm [27]. Also able to mutate DNA in a bacterial over-expression assay [28], human APOBEC3G was shown to inhibit HIV replication [29]. In vitro, APOBEC3G has a preferred sequence motif of T/CCC̲ [14,15,17,30,31]. While an AID induced nuclear DNA deamination event at the Ig locus may lead to the recruitment of a number of different repair pathway proteins, cytoplasmic DNA deamination events have a very different aim and outcome: APOBEC3G inhibits retroviral infection by deaminating first strand cDNA, generating viral DNA mutations and potential viral elimination [15,32–34]. In this context, DNA repair is of little consequence, therefore regulation of APOBEC3G's substrate specificity is thought to be less stringent (i.e. any cDNA in the cytoplasm may be regarded as foreign). Indeed, the identification of APOBEC3G induced hypermutation of retroviral DNA [34] could suggest a mechanism by which once APOBEC3G acts on one molecule of viral DNA, it would attempt to induce as much damage (deamination) as possible. This concept has been supported by the recent observation that APOBEC3G acts in a processive manner, i.e. moves along the DNA, catalysing deamination events [30].

Recently, there have been reports that AID may also act in a processive manner [35,36]. However, observations from our and other labs [37] do not correlate with AID acting in a processive manner during deamination of cytosine on single stranded DNA. Furthermore, consistent with the majority of observed SHM in vivo, when the pattern of SHM in $Msh2^{-/-}$ $Ung^{-/-}$ knockout mice (i.e. in the absence of mutation repair) was investigated, there was little evidence of processivity, in that if a mutation occurred at one target WRC̲, there was no greater likelihood of a subsequent, proximal mutation occurring ([38] and C. Rada, personal communication). In addition, controlled distributivity of a nuclear deaminase such as AID would decrease the danger of this already oncogenic protein. We therefore felt that it was important to accurately determine how AID and other deaminases behave during substrate deamination; whether they were capable of acting processively, or whether the deaminases themselves were controlled, distributive mutators.

We previously demonstrated the fine target specificity of AID, APOBEC1 and APOBEC3G using an in vitro oligonucleotide based assay, in which the deaminases were shown to exhibit a sequence specificity closely mirroring that observed in vivo [12,14,15,19,39]. We have adapted this basic assay to address, in two different ways, the question of whether AID acts in a processive or distributive manner on a variety of oligonucleotide substrates. While we can show that the cytoplasmic deaminase APOBEC3G exhibits a $3' \rightarrow 5'$ like processivity, which may have evolved as part of the innate immune response to fight viral infection, our findings suggest that although AID exhibits enzymatic turnover, it displays clear and consistent evidence of distributivity. We propose that the inherent distributive action of the nuclear deaminase AID, may act as an intrinsic control mechanism that ensures that a measured mutational load is introduced during antibody affinity maturation and limits its oncogenic potential in the absence of further regulatory cofactors.

2. Materials and methods

2.1. *Preparation of recombinant AID and APOBEC3G*

Recombinant his tagged proteins were partially purified as described previously [12]. Human AID in a pET30 derived vector with a C-terminal his tag was constructed as described previously [19]. N-terminal his tagged hAID was also constructed using the same pET30 vector background. MBP-chicken AID was cloned using the Invitrogen Gateway system with the pMAL vector (New England Biolabs, MA, USA) and purified according to the manufacturers protocol. C-terminal his tagged hAID was cloned into a Baculovirus host with an Invitrogen Gateway pDEST10 background and used to inoculate Sf21 cells for 4 days. N-terminal his tagged human APOBEC3G was cloned as described previously [12,14]. Quantitation of protein was carried out by both Coomaisse and Western Blot analysis.

2.2. *Determination of enzyme turnover*

In order to determine the % active recombinant protein, an active site titration was carried out using multiple concentrations of protein and calculating % conversion of DNA oligo SPM179 (5′-biotin-ATTATTGTTATTAGCTATTTGTTTATTTGTTTATTTATTT-fluorescein-3′) to product, as described previously [14,19,39]. From this, the μM product concentration was calculated and plotted as a function of time for different enzyme concentrations. According to the principle described by Fersht et al. [40], assuming that $k_1 \gg k_2$, the concentration of active sites can be determined by taking the linear portion of the chart and extrapolating back to the y-intercept which is equivalent to $n[E]_0$. Percentage activity of the recombinant protein was then determined by calculating (active $[E]_0$/total $[E]_0$) × 100. Enzyme turnover was then established by calculating the pmol product/pmol active protein. One cannot exclude the unusual possibility that AID exhibits $k_2 > k_1$, in which case the active site titration would generate an underestimate of the active protein. If this were the case, one could not observe turnover, and as an extension of this, processivity.

2.3. *Heterodimer block assay*

Twenty pmol (HC1187/HC1188) or 10 pmol (HC1189) RNA and 5 pmol DNA oligonucleotides were allowed to slowly anneal in 1× reaction buffer [40 mM Tris pH8, 40 mM KCl, 50 mM NaCl, 1 mM DTT, 10% glycerol, 3 mM $MgCl_2$]. (All DNA oligos were obtained from Sigma-Genosys, UK and RNA oligos from Cure Vac, Germany). Oligonucleotide sequences; HC1186 DNA 5′-biotin-ATTGTTATTAGCTTATTGTTGTTA-TAGCTATTGTTATTGT- fluorescein-3′. HC1187 RNA 5′-ACAAUAACAAU-AGCUAUAAC-3′. HC1188 RNA 5′-AACAAUAAGCUAAUAACAAU-3′. HC1189 RNA 5′-AACAAUAGCUAUAACAACAAUAAGCUAAUA-3′). We determined that in order to maintain optimal oligonucleotide heteroduplexes, the assay would need to be carried out at 18 °C. We therefore confirmed that hAID was active and exhibited turnover at 18 °C, and in the presence of RNA or RNase H and degraded RNA (data not shown). An estimated 1.2 pmol active recombinant hAID–his was then added to generate a final volume of 20 μl and the whole reaction incubated at 18 °C for 10 min. The reactions were then temporarily placed on ice while 500 pmol unlabelled, competing DNA oligo

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