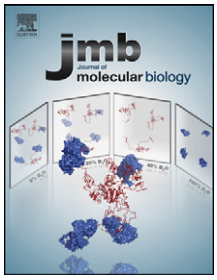

The *in vitro* Biochemical Characterization of an HIV-1 Restriction Factor APOBEC3F: Importance of Loop 7 on Both CD1 and CD2 for DNA Binding and Deamination

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Abstract

APOBEC3F (A3F) is a member of the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like (APOBEC) family of proteins that can deaminate cytosine (C) to uracil (U) on nucleic acids. A3F is one of the four APOBEC members with two Zn-coordinated homologous cytosine deaminase (CD) domains, with the others being A3G, A3D, and A3B. Here we report the *in vitro* characterization of DNA binding and deaminase activities using purified wild-type and various mutant proteins of A3F from an *Escherichia coli* expression system. We show that even though CD1 is catalytically inactive and CD2 is the active deaminase domain, presence of CD1 on the N-terminus of CD2 enhances the deaminase activity by over an order of magnitude. This enhancement of CD2 catalytic activity is mainly through the increase of substrate single-stranded (ss) DNA binding by the N-terminal CD1 domain. We further show that the loop 7 of both CD1 and CD2 of A3F plays an important role for ssDNA binding for each individual domain, as well as for the deaminase activity of CD2 domain in the full-length A3F.

The APOBEC family members contain one or two homologous cytosine deaminase (CD) domains that share a conserved sequence motif (H-X-E-X23–28-P-C-X-C) for Zn coordination at the putative catalytic center [1,2,3,4,5]. Four of the 11 family members (A3B, A3D, A3F, A3G) contain two homologous CD domains, and the others have only one CD domain. Nine of the 11 members are shown to catalyze deamination of cytosine to uracil on single-stranded (ss) DNA or RNA [1,4,5,6]. Most of the APOBEC3 family members constitute parts of the important immune system to safeguard the potential damage from the intrinsic retroviral elements and infectious retroviral and some other viral pathogens [7,8,9,10,11,12,13,14,15,16,17]. However, some of these members are also implicated in genome-wide accidental mutations that may result in significant repercussions and biological consequences such as epigenetic remodeling, cancer, and even evolution [18,19,20,21,22,23,24].

Among the four APOBEC3 members containing double CD domains, A3F and A3G show potent anti-HIV activities [25,26,27,28,29,30], which is through incorporation/encapsidation of the APOBEC3 enzymes into HIV virions [31,32,33,34]. The incorporation/encapsidation into HIV virions, which is mainly mediated through RNA binding by the A3 proteins, requires both CD1 and CD2 domains of A3F, whereas only CD1 of A3G is needed [32,34,35,36,37,38,39]. On the other hand, while HIV Vif targets A3G for E3 ubiquitin ligase-mediated degradation through binding to the CD1 of A3G, Vif targets A3F by binding to its CD2 domain [25,26,40,41,42,43].

The anti-HIV activity is thought to be mostly deamination dependent, with some reports for deamination independent activity as well [44,45,46,47,48,49,50].

Regardless, both CD1 and CD2 domains of A3F and A3G are required to display anti-HIV activity. For A3F and A3G, the CD1 is previously shown to be catalytically inactive, whereas CD2 is the catalytically active domain for both proteins [42,51,52,53]. Here we report *in vitro* biochemical studies of A3F and various mutant constructs containing loop 7 mutations on either the CD1 or CD2 domains using purified recombinant proteins from an *Escherichia coli* expression system.

The role of CD1 and CD2 for A3F deaminase activity

To assess the *in vitro* biochemical activities of A3F and the role of loop 7 residues, we first cloned and purified wild-type and mutant A3F proteins as MBP fusions to near homogeneity (Fig. 1a and b). Four constructs were tested, including the wild-typefull-length (fl) A3F (A3F-fl) construct, as well as the mutants A3F-fl E67A (with the CD1 catalytic residue mutation at E67), A3F-fl E251A (with the corresponding CD2 catalytic residue mutation), and A3F-fl E67/E251A (with both catalytic residue mutations). These purified MBP fusion proteins of A3F were assayed for deaminase activity using a 30 nucleotide (nt) ssDNA substrate (Fig. 1c). As expected, the *in vitro* activity assay result showed obvious deaminase activity for both A3F-fl and A3F-fl E67A, whereas no deamination activity was detected for A3F-fl E251A and A3F-fl E67/E251A (Fig. 1d; Table 1). This *in vitro* assay result confirms that CD1 has no deaminase activity, with only CD2 acting as the catalytically active deaminase domain, and is consistent with previous finding. In addition, quantification of the dose–response deamination activity (Fig. 1e and f) revealed that the CD1 mutant A3F-fl E67A activity is more or less comparable to that of the wild-type A3F-fl, suggesting that the E67A mutation within the CD1 domain did not have a significant impact on the deaminase activity of the full-length protein. This is similar to what is observed for A3B and A3G, where the mutation of the CD1 Glu residue appears to have no obvious effect on the fl A3B and A3G deaminase activity [51,54].

The effect of CD2 loop 7 mutations on A3F deaminase activity

Since previous studies suggest a role of loop 7 for DNA binding and deaminase activity for some APOBEC3 proteins, such as A3G-CD2, A3B-CD2, and A3A [29,55,56,57], we want to assess the functional role of specific residues on loop 7 of both the CD1 and CD2 of A3F. We made point mutations around the tip of CD2 loop 7 on the constructs of both A3F-fl and A3F-CD2 alone, including W310A, D311A, D311K, and T312P, which are located next to the Zn-coordinated active center (Fig. 2a and b). All constructs were expressed as MBP fusions in *E. coli* and purified to near homogeneity (Fig. 2c). For the four mutants in A3F-fl constructs, subsequent deamination assays revealed that the W310A mutant showed the most reduced activity over the tested protein concentration range (Fig. 2d), with about 57% reduction of deaminase activity when compared to wild type (Fig. 2e; Table 1). The A3F-fl D311A and A3F-fl T312P mutants showed very little effect on deaminase activity. Interestingly, the A3F-fl D311K mutant showed significantly increased activity, with approximately 38% increased activity over wild-type A3F-fl.

The purified wild-type A3F-CD2 alone also showed obvious deamination activity (Fig. 2f and g; Table 1). However, specific deaminase activity of the A3F-CD2 construct by itself [~0.501 nM/μM/h (product/substrate/h)] is more than 10-fold lower than that of A3F-fl or A3F-fl E67A (Table 1), suggesting that the presence of CD1 on the N terminus of CD2 greatly enhanced the activity of CD2. For the four loop 7 mutations on this A3F-CD2 construct, we observed a similar trend of deaminase activity changes as with the A3F-fl construct, with the exception of a dramatic activity drop on D311A (Fig. 2f and g; Table 1). Again, the mutant A3F-CD2 W310A showed the most significant impact, reducing the deaminase activity to background level, followed by the A3F-CD2 D311A mutant, which showed about an 80% reduction of deaminase activity compared to wild-type A3F-CD2. As with the full-length construct, A3F-CD2 T312P had little effect on deamination activity, and quite interestingly, the D311K mutation once again showed an increased activity, with approximately 22% higher activity than wild-type A3F-CD2 (Fig. 2g; Table 1). To summarize, the above result showed that the four mutations of the three CD2 loop 7 residues showed similar effects on deaminase activity in the context of either the A3F-fl or the A3F-CD2 construct. Generally speaking, the W310A mutation had a significant negative impact on deaminase activity, whereas D311K had a positive impact.

The effect of CD2 loop7 mutations on substrate DNA binding

In order to examine whether the CD2 loop 7 mutations on A3F-fl and A3F-CD2 have altered substrate binding affinity, we performed the ssDNA binding assay for each protein construct using a gel shift assay as described in previous studies [58,59], and the result is shown in Fig. 2h and i and Table 2. For the A3F-fl constructs, the A3F-fl and A3F-fl D311A, D311K, and T312P constructs all had similar binding affinity, with a K_d of around 1.0 μM (Table 2). However, the A3F-fl W310A construct showed a greater than 3-fold reduction of the binding affinity