A Biochemical Analysis Linking APOBEC3A to Disparate HIV-1 Restriction and Skin Cancer

Phuong Pham, Alice Landolph, Carlos Mendez, Nancy Li & Myron F. Goodman
From the Departments of Biological Sciences and Chemistry, Molecular and Computational Biology Section, University of Southern California, Los Angeles, California 90089-2910, USA
Address correspondence to: Myron F. Goodman, Tel (213) 740-5190, Fax (213)-740-8631; E-mail: mgoodman@usc.edu.

Running title: Roles of Apo3A in HIV-1 restriction and skin cancer

CAPSULES
Background: Apo3A restricts HIV-1 in myeloid cells and mutates genomic DNA.
Results: Optimal Apo3A activity and narrow deamination specificity occur at acidic pH; weak activity and broad specificity, featuring CC→TT mutations, occur at physiological pH.
Conclusion: Apo3A’s pH-dependent catalytic properties reflect targeting of HIV-1 cDNA and “off-targeting” of genomic DNA.
Significance: Apo3A enzymology provides a molecular basis to elucidate viral exclusion and cancer induction.

SUMMARY
Human deoxycytidine deaminase APOBEC3A (Apo3A) acts as an HIV-1 restriction factor in cells of myeloid lineage yet functions separately as a potent mutator for genomic DNA. Apo3A activity and C motif deamination specificity exhibit a striking dependency on pH that reflects these two distinct biological processes. Upon infection of macrophages, HIV-1 induces the formation of autophagosomes, requires autophagosomes for replication, while inhibiting lysosomal fusion indicative of late stage autophagy. Here we show that Apo3A has optimal activity and a strict 5'YYCR motif specificity in the pH range 5.8 to 6.1, characteristic of enclosed autophagosomal membrane compartments, and reflective of the mutation pattern of HIV-1. In contrast to Apo3A’s high activity and narrow specificity at acid pH, a 13- to 30-fold reduction in specific activity is accompanied by relaxed C deamination specificity at pH 7.4 to 8. Notably, Apo3A is also expressed in keratinocytes, and is upregulated in skin lesions. At pH 7.9, we show that Apo3A generates transcription-dependent CC→TT tandem mutations on the non-transcribed strand, a hallmark signature of skin cancer. The biochemical data taken in conjunction with the biological upregulation of Apo3A in skin lesions suggests that enzyme-catalyzed deaminations at adjacent C sites followed by normal replication generating CC→TT mutations provides an alternative molecular basis for the initiation events in skin cancer in contrast to well-established pathways in which CC dimers formed in response to UV radiation either undergo nonenzymatic spontaneous deaminations or aberrant replication.

Activation-induced deoxycytidine deaminase (AID) and APOBEC3G (Apo3G), two extensively studied members of the APOBEC family of C deaminases, catalyze the deamination of C → U on single-stranded (ss)DNA (1,2), AID to initiate
somatic hypermutation and class-switch recombination in B cells during transcription of immunoglobulin variable genes and switch regions (3,4). Apo3G to initiate hypermutation on HIV-1 cDNA in T cells lacking the viral infectivity factor vif (5-7). More generally, the APOBEC deaminases can act to provide innate resistance against retroviruses and endogeneous retroelements in a variety of cells (2).

Although most studies on antiretroviral activity of APOBEC proteins have been centered on Apo3G, recent data show that Apo3A is an important restrictive factor for HIV-1 and other primate lentviruses. While Apo3G functions most effectively in T-cells, Apo3A has antiviral activity in blood cells of myeloid lineage (8-10), and is highly expressed in myeloid cells, monocytes, dendritic cells and macrophages (8-10). Apo3A is likely to be functionally important in humans based on two salient observations: first, siRNA-mediated downregulation of Apo3A in monocytes and macrophages render them considerably more vulnerable to infection by HIV-1 (8,9); second, treatment of myeloid cells with interferon-alpha (IFN-α) markedly enhances cell resistance to HIV-1 infection (11,12) coincident with an induction of Apo3A expression by as much as three orders of magnitude (8,13-16).

Apo3A functions not just in the cytoplasm of myeloid cells, but when overexpressed in a variety of cultured cells it mutates nuclear and mitochondrial DNA (17-20). Although Apo3A acts against HIV-1 in its natural myeloid cellular setting, its overexpression inhibits replication of many viruses including papilloma (21), aden-associated (22), Rous sarcoma (23), T-lymphotropic type 1 (24), degrades foreign DNA (25), and blocks retrotransposition of LINE-1, Alu and TLR (13,26-28). Apo3A when overexpressed can be cytotoxic by inducing strand breaks in genomic DNA that activate a damage response causing cell-cycle arrest (17,18,29-31). It has recently been reported that endogenous Apo3A can be prevented from entering nuclei, but a mechanism has not been identified (18). If entry into nuclei does occur, then to protect against potential inadvertent action of Apo3A, an interaction between TRIB3 protein and Apo3A prevents deamination of genomic DNA, promoting enzyme degreadation, thereby eliminating cytotoxicity (17).

APOBEC proteins require tight cellular regulation to prevent C deaminations in DNA from occurring at the wrong place or time; “off targeted” mutations by AID can cause B-cell lymphoma (32,33), while inadvertent action of Apo3B has recently been implicated in breast cancer (30). Apo3A is expressed in skin keratinocytes (21,34,35) possibly to counteract viral infections. However, its overexpression in inflamed skin tissues, acne or psoriasis (34,35), by the “law of unintended consequences” has the potential to turn normally benign skin lesions into precancerous lesions, leading to full-blown cancer (21).

A biochemical analysis of Apo3A activity and motif deamination specificity at acidic pH, corresponding to enclosed autophagosomal membrane compartments, and at physiological pH, corresponding to cell nuclei, indicates the presence of a dual mutagenic mechanism that might link disparate HIV-1 inactivation in myeloid cells and susceptibility to skin cancer in precancerous skin lesions.

**EXPERIMENTAL PROCEDURES**

**Materials** - DNA substrates were synthesized using an Applied Biosystem 3400 DNA synthesizer and purified by denaturing PAGE. T7 RNA polymerase was purchased from Promega and ultrapure rNTPs from GE Healthcare. M13mp2 gapped substrate DNA was prepared as described (36). M13mp2T7 closed circular dsDNA substrate containing a T7 promoter at the 5’-side of lacZα reporter gene was constructed by site directed mutagenesis. Reaction buffers (pH 5.1 to 7.4) used for Apo3A deamination activity and DNA binding were as follows: 10 mM Sodium cacodylate (pH 5.1, 5.5, 6.5 or 7.4), 1 mM dithiothreitol, 0.5 mM EDTA and 25 mM NaCl. For the reaction buffer at pH 8.0, 10 mM Tris pH 8.0 was used instead of 10 mM Sodium cacodylate.

**Apo3A Purification** - A plasmid for expression of Apo3A protein in baculovirus infected Sf9 insect cells was constructed by cloning the coding portion of Apo3A into the pAcG2T vector (BD Biosciences). Recombinant virus was obtained by co-transfection of pAcG2T- Apo3A and linearized baculovirus DNA (BD Biosciences) into Sf9 insect cells (Invitrogen). Apo3A was expressed in Sf9...
insect cells following infection with the recombinant baculovirus at an MOI of 3 and harvested after 72 h. Cells harvested from 2 L cultures were suspended in 50 ml insect cell lysis buffer (10mM HEPES pH 7.5, 150 mM NaCl, 0.1 % Triton X-100, 10 mM NaF, 10 mM NaH2PO4, 10 mM Na2PO4, 1mM EDTA, 1 mM dithiothreitol, 50 µg/ml RNaseA, 10% glycerol and protease inhibitor) and lysed by sonication. The crude lysate, containing soluble GST-Apo3A was collected after centrifugation at 12,000 rpm for 30 min followed by incubation with Glutathione Sepharose resin (GE healthcare) at 4 °C overnight. After extensive washing with PBS buffer, 120 units of Thrombine (Calbiochem) were incubated for 6 hr at room temperature to cleave the GST-tag. Apo3A, containing extra six amino acids (GSPGID) at its N-terminus, was eluted and concentrated using an Ultracel-10K filter unit (Millipore) and applied to Superdex 75 gel filtration column (GE Healthcare), equilibrated with GF-buffer (20mM Tris pH 7.5, 250 mM NaCl, 1 mM dithiothreitol and 0.5 mM EDTA). Fractions containing chromatographically homogeneous Apo3A were pooled (Figs. 1A, B), dialyzed in GF buffer containing 10% glycerol, concentrated to 10 to 30 mg/ml using Ultracel-10K, and stored at -80 °C.

**Deamination Assay to Measure Apo3A Specific Activity on ssDNA** – The C-deamination activity of Apo3A was measured at different values of pH using a 65 nt Fluorescein labeled (FdT) ssDNA substrate, 5’GAGGTGTTCATAATGTGTGGTT(FdT)ATGTAGAGTTGCTCATATGTGTGTGTGTGT GTAGAAAAG-3’. For reactions at pH 5.1, 5.5 and 6.5, 500 nM labeled ssDNA was incubated with 5 nM Apo3A in the reaction buffer (30 µl) at 37 °C. For reactions at pH 7.4 and 8.0, a higher concentration (50 nM) of Apo3A was used. After incubations for 1, 2, 5, and 10 min, the reactions were quenched by a double extraction with phenol:chloroform:isoamyl alcohol (25:24:1) followed by a buffer exchange to 10 mM Tris pH 7.4 using Micro Bio-spin P-6 column (BioRad). Deaminated products were separated on a 16% denaturing PAGE gel, visualized and quantified using an FX fluorescence scanner (BioRad). Products of single deamination at the 5’-side (5’), the 3’-side (3’), target or double deaminations at both sites (5’& 3’) were detected as 54 nt, 43 nt and 32 nt cleaved products, respectively. Specific activity, defined as pmol of deaminated C per min per µg enzyme was calculated in the linear range of Apo3A concentration and time, using 1 to 2 min incubation times.

**Steady-state Rotational Anisotropy Binding Assay**– Apo3A binding to ssDNA was monitored by changes in steady-state fluorescence depolarization (rotational anisotropy) (37). Fluorescein labeled (F) ssDNA (5 to 65 nt), dsDNA (37 nt) and duplex DNA containing single-stranded “bubble” regions (1, 3, and 7 nt) were used as substrates for Apo3A binding. Reaction mixtures (200 µl), containing fluorescein-labeled DNA (50 nM), at the pH values indicated, were incubated with Apo3A, at room temperature, at the enzyme concentrations indicated. Rotational anisotropy was measured using a QuantaMaster QM-1 fluorometer (Photon Technology International) with a single emission channel. Samples were excited with vertically polarized light at 494 nm, and both vertical and horizontal emission was monitored at 520 nm (5 nm bandwidth). The microscopic dissociation constant ($K_a$) for cooperative binding is defined as the concentration of Apo3A at which half the input F-labeled DNA is bound. The $K_a$ and Hill coefficient parameters were calculated by fitting the data to a sigmoidal curve for cooperative binding using Sigma Plot 10.0 software. For non-cooperative binding, the data were fit to a rectangular hyperbola to obtain the apparent dissociation constant $K_D$. The binding constants were determined from three independent experiments.

**Analysis of Apo3A Deamination Specificity on ssDNA** – Apo3A motif specificities were determined by measuring mutational spectra in a lacZα reporter contained in a ssDNA gapped region of M13mp2 dsDNA, as described previously (36). M13mp2 gapped DNA (500 ng) was incubated with Apo3A (50 fmol at pH 5.5, 100 fmol at pH 6.5, and 1000 fmol at pH 8.0) in a 30 µl reaction volume, for 5 min at 37°C.
reactions were quenched by a double extraction with phenol:chloroform:isoamyl alcohol (25:24:1). The DNA product was transfected into ung E. coli competent cells and plated on α-complementation host cells. Conversions of C→U on the DNA substrate were detected as C→T mutations in a lacZα target gene (36).

Deamination Activity and Motif Specificity for APOBEC Enzymes on Transcribed dsDNA - Transcription-dependent deamination on dsDNA was measured using a closed circular M13mp2 dsDNA substrate containing a T7 promoter. In a typical reaction (50 μl), 100 fmol of Apo3A, GST-AID or Apo3G was incubated with 50 ng dsDNA substrate, 10 units of T7 RNA Polymerase (Promega) and four rNTPs (250 μM each) in a reaction buffer containing 40 mM Tris-HCl (pH 7.9), 6 mM MgCl2, 10 mM dithiothreitol, 10 mM NaCl, 2 mM spermidine, 0.05% Tween-20. Reactions were carried out for 30 min at 37 °C, and were quenched by extracting the product DNA twice with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was transfected into ung E. coli competent cells and plated on α-complementation host cells. Conversions of C→U on the DNA substrate were detected as C→T mutations on the non-transcribed strand and G→A mutations on the transcribed strand in a lacZα target gene; mutants were scored by counting white or light blue M13 plaques; see e.g., Ref. (38).

RESULTS
As Apo3A functions biologically in significantly different environments, presumably at acidic pH in autophagosomes, and at physiological pH in nuclei, we set out to determine if the enzyme’s response to pH might reflect its biological roles in a pH range spanning 5.1 to 8.0. Using ssDNA substrates, we have investigated the dependence on pH of Apo3A specific activity and substrate binding, motif specificity, and processivity. We have also measured transcription-dependent C deamination-initiated mutation spectra at pH 7.9 to determine if a mutational signature linked to skin cancer might be observed.

Influence of pH on Apo3A activity and binding to ssDNA – Recombinant Apo3A containing six amino acids (GSPGID) at its N-terminus was expressed in S9-infected insect cells and purified to chromatographic homogeneity (Fig. 1A). The enzyme behaves as a monomer in solution with an apparent molecular mass of 23 kDa (Fig. 1B).

A 65 nt ssDNA oligomer containing an internal fluorescein (F) label situated between two 5'-TC target motifs (Fig. 1C, sketch at top) was used a substrate to measure Apo3A activity (Figs. 1C, D) and binding (Fig. 2), at different pH. The specific activity for Apo3A is optimal at pH 5.5 (626 pmol/min/μg), which is about 13-fold higher than at pH 7.4 (49 pmol/min/μg) (Fig. 1D). Along with single deaminations occurring at either 5' or 3' motifs, double deaminations are also observed at both 5' and 3' motifs at pH 5.5 and 6.5 (Fig. 1C). Notably, for the 1 and 2 min data points, less than 10% of the initial “intact” substrate is converted to product demonstrating, in accord with Poisson statistics, that a single bound Apo3A is responsible for catalyzing both deamination events prior to dissociation (39). Therefore, Apo3A is able to act processively as reported previously for AID and Apo3G (36,39,40). However, owing to the 13- to 30-fold reduction in Apo3A activity, 5'&3' correlated double deamination bands were not observed at pH 7.4 and 8.0 (Fig. 1C). Apo3A is indeed processive at these at these higher pHs, as we discuss in the next subsection and shown in Figure 3.

The higher activity at acidic pH correlates with tighter binding (~ 4-fold), comparing the apparent affinities, Kd = 330 nM, pH 5.5 with Kd = 1400 nM, pH 7.4 (Fig. 2). Apo3A has a net positive charge +7.1 at pH 5.5 and a net negative charge -2.5 at pH 7.4, which clearly accounts for its favored binding at acid pH (Fig. 2).

Although Apo3A behaves as a monomer in solution (Fig. 1B), a least squares fit of the binding data to a sigmoidal curve (Hill coefficients 1.5 to 1.6) is better than to a rectangular hyperbola indicating the possibility of weakly cooperative binding of Apo3A to ssDNA (Fig. 2), as was recently observed for wild type AID (37). Thus, despite purifying as monomer in solution, it’s possible that Apo3A might be active on ssDNA as dimer, which might also be the case for AID (37) and Apo3G (41). Binding of Apo3A at pH 5.5 to dsDNA, to dsDNA containing 1, 3, 5 and 7 nt stretches of ssDNA “bubbles” and to short ssDNA...
(5 nt and 7 nt), showed a considerably reduced affinities and no indication of cooperative binding (Table 1).

**A3A motif specificity dependence on pH** - The presence of a wide variety of tri- and tetranucleotide motifs that contain C permits the “lengthy” lacZα reporter gene (365 nt) to serve as an ideal target to ascertain APOBEC motif specificities (Fig. 3) (36). Here is a brief synopsis of the assay. Apo3A is incubated with phage M13mp2 circular DNA containing an single-stranded gap region containing the mutation reporter, which, upon transfection in ung E. coli, produces either C→T mutated white and light-blue plaques or non-mutated dark blue plaques. The concentrations of enzyme, DNA, and incubation times are arranged so that the mutated targets comprise less than 5% of the total target DNA, which ensures that the C→T mutations determined by sequencing individual DNA clones, originate from C→U deaminations catalyzed by just a single Apo3A molecule. The key point is that from two to about 30 mutations are present in > 80% of the mutated clones (Fig. 3A), which means that Apo3A catalyzes processive deaminations, including at pH 8.0, where double deamination bands had not been detected in the F-labeled target motif cleavage assay (Fig. 1C).

5′-YYC (Y = pyrimidine) is by far the predominant favored motif for deamination by Apo3A irrespective of pH, accounting for nine of ten most favored motifs at pH 8.0, 6.5 and 5.5, the exceptions being TAC at pH 8.0 and GTC at pH 6.5 and 5.5 (Table 2). A YYC motif preference is consistent with reported higher activities of Apo3A at physiological pH on short oligonucleotide substrates containing a pyrimidine at the 5′-side of C (42-44). And yet the analysis of motif specificity yields the surprising observation that there are roughly half as many mutations per clone at pH 5.5 compared to pH 8.0 (Fig. 3B), even though Apo3A is 30-fold more active at pH 5.5 (Figs. 1C, D). At pH 5.5 the large majority of clones (~ 65%) contain 2 to 5 mutations compared to about 35% of the clones at pH 6.5 and 8.0 (Fig. 3A). Approximately 10% of the clones at pH 6.5 and 8.0 have more than 15 mutations, compared to none at pH 5.5 (Fig. 3A).

The explanation for these results appears to be that there is a significant reduction in the number of YYC consensus motifs that are deaminated with relatively high efficiency by Apo3A at pH 5.5 compared to pH 6.5 and 8.0 (Fig. 3B, Table 2). At pH 8.0 and 6.5, there are ten YYC motifs that have undergone deamination in about 25% of the clones. In contrast, at pH 5.5, there are only three such motifs. Specifically, the non-YYC motif TAC, which is the second most favored motif at pH 8.0, undergoes deamination in 54% of the clones (Table 2), and, while not in the top ten, is still deaminated in 18% of the clones at pH 6.5 (Fig. 3B); in contrast, deamination at TAC occurs in less than 2% of the clones at pH 5.5 (Fig. 3B). There is therefore a considerable narrowing in the range of most favored motifs at acidic pH, both YYC and non-YYC, which appears to be the explanation for the reduction in numbers of mutations per clone at pH 5.5 compared to pH 6.5 and 8.0.

There is an additional constraint on the consensus YYC at pH 5.5, in which nine out of the ten most favored motifs contain a 3′ purine (R) compared to six of ten at pH 8.0, and there are only fifteen YYCR sites in the mutational target sequence further accounting for the reduction in mutations per clone at pH 5.5. Despite having reduced numbers of deaminations at pH 5.5, the range of enzyme motion covers the entire lacZα target to a similar extent because at least 40% of the clones at each pH have mutations that span virtually the entire lacZα target; i.e., during a single Apo3A – ssDNA encounter each of these clones has a mutation near the 5′-end of lacZα and a mutation near the 3′-end of lacZα that is separated by between 300 and 365 nt (Fig. 4).

**Linking Apo3A motif specificity at pH 8.0 to a tandem CC→TT skin cancer signature** - The upregulation of Apo3A expression in precancerous keratinocytes (21,34,35) suggests that its ability to deaminate C in ssDNA could be directly responsible for generating the types of tandem CC→TT mutations found in skin cancer. We have investigated whether Apo3A generates excess mutations at consecutive C sites using, as before, the lacZα target, which has ten motifs containing from two to five consecutive C residues (Table 3). Consecutive deaminations in the lacZα target are
frequently present at pH 6.5 and 8.0, with 78% and 63% of the clones having at least one CC→TT tandem double mutation at pH 6.5 and 8.0, respectively (Table 3). In contrast, very few clones (<10%) undergo deaminations at consecutive C’s at pH 5.5, which provides another clear indication of a strong dependence of motif specificity on pH.

While Apo3A is likely to be working on HIV-1 cDNA, it is probable that its action on genomic DNA occurs during transcription, and indeed the CC→TT mutations found in skin cancer occur exclusively on the non-transcribed strand (45,46), even though there are numerous potential sites for mutation on the transcribed strand. We have used the M13-lacZα reporter assay to measure T7 RNA polymerase transcription-dependent Apo3A catalyzed deamination (Fig. 5). Apo3A is active on DNA undergoing deamination based on a 26-fold increase in M13 mutant phage frequency (124 × 10⁻⁴) over the background mutant frequency (4.8 × 10⁻⁴) (Table 4). Every one of the more than two hundred C→T mutations detected by sequencing eighty mutant clones occurs on the non-transcribed strand; G→A mutations, which would result from deaminations on the transcribed strand, are absent from the spectrum (Fig. 5A).

The deamination specificity during transcription at pH 7.9 shows the same prominent 5’-TACα motif, which was present on ssDNA at pH 8.0 (Fig. 3B, Table 2). Most importantly, tandem mutations at CC sites are clearly present in the spectrum in three runs of C, TTCCG, ACCCA, and most avidly in TCCCCC, which occurs in 16% of the clones (Fig. 5A, Table 3). There is one mutation in 30% of the clones; 30% with two mutations, 18% with three mutations 11% with four mutations and about 11% with at least five mutations going out to eight (Fig. 5B). The presence of mutations near the 5’- and 3’-ends of lacZα occurring in the same clone shows that Apo3A is able to remain bound and acts processively during the time that the entire lacZα target is being transcribed (Fig. 5C). Deaminations might be occurring either in a moving transcription bubble, or perhaps more likely in a bubble that’s transiently stalled. We find that Apo3A has about the same deamination activity on a 7 nt bubble substrate compared to ssDNA, and retains small but measurable activity on 3 and 1 nt bubbles (Fig. 6).

**DISCUSSION**

This study makes a key connection linking the enzymatic properties of the ssDNA-dependent C deaminase Apo3A together with two seemingly disconnected biological events, the inactivation of HIV-1 in myeloid cells and the potentiation of skin lesions toward cancer. Apo3A is normally expressed in myeloid cells and has the potential to inactivate HIV-1 by deaminating C→U on cDNA (8-10), presumably analogous to the “attack” by Apo3G on HIV-1 cDNA in T cells (5-7). In skin lesions, e.g., acne and psoriasis, Apo3A is upregulated with the possibility for causing serious “mutator” damage to cellular DNA (21,34,35). We have obtained biochemical evidence ascribing positive and negative biological roles to Apo3A, positive as an HIV-1 restriction factor in cells of myeloid lineage at acid pH, and negative as a potent mutator on genomic DNA in damaged keratinocytes at above neutral pH, which could portend skin cancer.

*A biochemical basis for inactivation of HIV-1 in myeloid cell autophagosomal compartments at acid pH* - A physiological pH range, 7.0 to 7.8 is characteristic of nuclei and cytosolic regions for diverse cell types, with subtle differences such as a slightly more basic nuclear environment (47). Proteins such as those involved in nucleic acid transactions often mirror this environment, having optimal activities in a near-neutral pH range. In contrast, not so subtle differences occur in compartments of the endocytic pathway, which are acidic and where the pH decreases from about 6.5 in early endosomes to < 6.0 in late endosomes, down to < 5.5 in lysosomes (48). We have shown that Apo3A activity and motif recognition are strongly modified by pH, where the C deamination activity is optimal and motif specificity narrowest in acidic aqueous surroundings (Figs. 1 & 3, Table 2).

Cellular studies support an important role for Apo3A in restricting HIV-1; knockdown of Apo3A expression by siRNA or miR-shRNA is accompanied by ~ 5-fold increase in myeloid cell susceptibility to infection by HIV-1 (8,9). Myeloid cells, composed of circulating monocytes, differentiated macrophage and dendritic cells, are prime targets for HIV-1 replication. Apo3A is expressed in these cells, and most significantly is upregulated concurrently with macrophage
infection by HIV-1 (9). HIV-1 replication in macrophages requires the formation of autophagosomal enclosed membrane compartments (pH ~ 5.8 to 6.1) (49-51).

Viral cDNA is reduced in interferon-treated myeloid cells while concomitantly accumulating mutations in 5'TC motifs, a clear indication that the induced Apo3A functions during an early phase of HIV-1 infection by deaminating C in reverse transcribed cDNA (9,10,12), the same as the action of Apo3G in T-cells (5-7). Yet there seems to be a basic distinction regarding where, and therefore perhaps how, Apo3A and Apo3G work because Apo3A shows no antiviral activity when overexpressed in HeLa and HEK293T cells, whereas Apo3G does (9,52,53).

But the different actions of Apo3A in myeloid cells and Apo3G in T-cells needs to be viewed in conjunction with the differing responses of the two types of HIV-1-infected cells to autophagy, which is the primary mechanism for degrading no longer functional cellular molecules and foreign substances. Autophagy is initiated by the formation of autophagosomes. Autophagosomes are double membrane vacuoles that can engulf deleterious cytoplasmic materials, which are then digested following fusion of autophagosomes with lysosomes (54). Autophagosomes in macrophages are characterized by their acidic environment pH ~ 5.8-6.1, before fusion with the lysosomes (55).

There are contrasting responses of the two cell types to viral infection: whereas the presence of HIV-1 downregulates autophagy in CD4+T-cells (50,56), the opposite occurs in macrophages where HIV-1 causes a marked increase in the numbers of autophagosomes in the cytoplasm, but inhibits the late proteolytic stage of autophagy involving lysosome fusion (49-51). The inhibition of autophagy in virus-infected macrophages substantially reduces HIV-1 production (50) suggesting that autophagy is needed for viral replication. Conversely, the induction of autophagy in macrophages is accompanied by increased virus production (49), suggesting that HIV-1 actually exploits autophagy enabling it to replicate more efficiently in the host myeloid cells.

And so for Apo3A to eliminate the virus, it would presumably have to inhibit HIV-1 replication at the earliest stage of autophagy. Our speculative best guess is that Apo3A-mediated inactivation of HIV-1 is taking place in the enclosed acidic environment of the autophagosome (55), wherein, as our biochemical data reveal, Apo3A is optimally active (pH 5.1 to 6.5, Fig. 1D). The 13- to 30-fold increase in Apo3A specific activity at acidic compared to near neutral pH values (pH 7.4 to 8.0) is accompanied by a significant narrowing of favored motif deamination specificity (Table 2). A rather strict 5'YCR motif specificity in the pH range 5.5 to 6.1 is consistent with the mutation pattern of HIV-1 (9,10,12), which also reflects the favored motif preference for Apo3G acting on HIV-1 cDNA in T cells (5-7). It has been reported recently that Apo3G has a more modest 4-fold increase in activity at acidic pH 5.5, compared to pH 7.4 (57). Thus, the Apo3A-catalyzed inactivation of HIV-1 in macrophages is fully compatible with its biochemical behavior. Notably, it has been shown that overexpression of Apo3A in HeLa or HEK293T cells shows no significant antiviral activity (9,52,53), which reinforces the biological relevance of the biochemical data – Apo3A’s strong activity at pH 5.1 to 6.5 is highly effective in eliminating HIV-1, whereas its weak activity at pH 7.4 to 8.0 is ineffective.
differently for different malignant cell types but typically share an APOBEC-like 5'-TC favored deamination motif signature (58). Based on protein expression levels taken in conjunction with a 5'-TC→5'-TT mutational signature, it's been proposed that genomic off-targeting by Apo3B is, in part, responsible for the occurrence of breast cancer (30). When expressed individually in yeast, AID, Apo3A, Apo3B, and lamprey APOBEC generate similar looking mutational clusters (31,59,60), which we would attribute to two features of APOBEC deaminases, first, a very simple shared motif recognition sequence, and second, the shared property of processivity ensuring that several and often many deaminations can be catalyzed by the same enzyme during a single APOBEC-ssDNA binding event. Processive catalysis by APOBECs has been shown biochemically to generate single along with short and long clusters of mutations for AID, Apo3G (36,38,61) and in this study for Apo3A.

The upregulation of Apo3A in damaged skin lesions suggests that it might be implicated early on in initiating skin cancer. The key biochemical data supporting a fundamental role for Apo3A in initiating skin cancer is its telltale skin cancer signature, CC→TT tandem mutations, in the pH range 7.4 to 8.0 (Table 3). Apo3A, especially when overproduced, could gain access to transient regions of single-stranded genomic DNA generated during DNA repair, or more likely perhaps during active gene transcription. Our data show the substantial presence of Apo3A-catalyzed CC→TT mutations on ssDNA (Table 3), and most importantly on dsDNA undergoing transcription (Fig. 5A, Table 3). We suggest that a large increase in the range of Apo3A motif preferences at basic compared to acidic pH, including most prominently mutations in runs of consecutive C’s at pH 6.5 and 8.0, virtually absent at pH 5.5 (Table 3), enables Apo3A to generate off-target genomic mutations despite its 30-fold reduction in activity and ~ 4-fold weaker binding to DNA at basic pH (Figs. 1D and 2).

Apo3A is highly processive when acting on ssDNA in acidic and basic milieu (Figs. 3A, B). Similar to AID, Apo3A maintains strong processivity during transcription of dsDNA (Figs. 5A, B), reinforced by the observation that mutations occurring during transcription cover the entire lacZ reporter gene, immediately downstream from the promoter to the end of gene (Fig. 5). Thus, in a single binding event, Apo3A appears to track along with a moving transcription bubble, while deaminating C→U on the non-transcribed strand exclusively (Fig. 5A). The biochemical data are consistent with the biological data showing that 100% of the CC→TT tandem mutations in skin cancer cells occur on the non-transcribed strand (45). The presence of substantial numbers of CC→TT mutations in skin cancer (45,46) suggests to us that to the extent that tandem mutations are taking place in actively transcribed genes, that Apo3A is probably not acting on a moving transcription bubble, because of the extremely small likelihood to have consecutive deaminations occurring in a rapidly moving bubble, ~100 nt/sec. Instead, we speculate that perhaps most of the deaminations, single and especially double deaminations, take place on a transiently stalled bubble. The activity of Apo3A on a 7 nt bubble substrate is indistinguishable from its activity on ssDNA (Fig. 6).

The biological and biochemical linkage of Apo3A to skin cancer suggests a reevaluation of the longstanding model that the cyclobutane CC dimers that result from exposure of skin to sunlight undergo spontaneous deamination at both C residues (45,46,62). Perhaps a substantial fraction of the deaminations at C, and especially at neighboring Cs, are instead enzymatically caused by overproduced Apo3A in precancerous skin lesions acting on normal C residues. There is, for example, evidence suggesting that the spontaneous deamination of UV-induced DNA photoproducts may be insufficient to explain the formation of the tandem mutations in mammalian cells (46). In cells exposed first to UV light and then to oxidants (H2O2) or antioxidants (N-acetylcysteine, trolox) undergo substantially increased levels of CC→TT mutations that was attributed to a putative inducible “factor” (46). Apo3A off-targeting of genomic DNA could provide a plausible enzymatic basis to explain how commonly occurring non-cancerous skin lesions are converted to precancerous lesions that subsequently become cancerous.
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**Footnote:** 1The abbreviations used are: AID, activation induced deoxycytidine deaminase; Apo3A, APOBEC3A; Apo3G, APOBEC3G; ssDNA, single stranded DNA; dsDNA, double stranded DNA; nt, nucleotide; polyacrylamide gel electrophoresis, PAGE.
FIGURE LEGENDS

Figure 1. pH-dependent activity of Apo3A. A, Denaturing PAGE chromatography of human Apo3A expressed and purified from baculovirus infected Sf9 cells. Apo3A was purified as described in “Experimental Procedures”. The Apo3A gel band shown at the right was obtained by loading 2 μg protein obtained from the final purification step, gel-filtration with Superdex-75. Molecular mass marker bands are shown on the left. B, The Superdex-75 elution profiles for Apo3A and standard molecular marker proteins are shown, with each elution peak and molecular mass (kDa) indicated by arrows. C, The deamination activity of Apo3A was measured as a function of pH using a fluorescein-labeled (F) 65nt ssDNA containing two favored TC motifs, situated toward the 5'- and 3'-ssDNA ends (sketch at top). Apo3A (5 nM) was incubated with the substrate ssDNA (500 nM) for 1, 2, 5 or 10 min. The ssDNA was treated with UDG and hot alkali, and the products separated by 16% denaturing PAGE. Products of single deamination at the 5’site (5’), 3’site (3’) or double deaminations at both sites (5’ & 3’) were detected as 54 nt, 43 nt and 32 nt cleaved products, respectively. D, Comparison of Apo3A specific activity (pmol min⁻¹ μg⁻¹) acting on ssDNA substrates at pH 5.1, 5.5, 6.5, 7.4 and 8.0. Error bars represent ± S.E. obtained from 3 independent measurements. The chromatographic gel in (A) was stained with Coomassie brilliant blue.

Figure 2. pH-dependent binding of Apo3A to ssDNA. Apo3A binding to a fluorescein-labeled 65 nt ssDNA at pH 5.5, 6.5, 7.4 and 8.0 was measured by rotational anisotropy. The changes in rotational anisotropy with increasing Apo3A concentrations were fit to a sigmoidal binding curve (pH 5.1, 6.5 and 7.4) or a rectangular hyperbola binding curve (pH 8.0). Values for each data point represent the mean ± S.E, determined from 3 independent measurements.

Figure 3. Apo3A C→U deamination spectra determined in a lacZα mutational reporter gene at pH 8.0, 6.5 and 5.5. A, Percentage of clones containing 1 to 29 mutations. Apo3A-catalyzed C→U deaminations, are detected as C→T mutations in lac2α (white or light blue M13 plaques). B, Apo3A deamination spectra were obtained by sequencing DNA from ~ 100 individual mutant M13 clones at each pH. Colored bars represent the percentage of clones with C→T mutations at the position indicated on the lacZα sequence (-217 to +149). Red bars identify C deaminations occurring in 5’TCC motifs, blue bars represent 5’CC motifs, and green bars represent 5’RC motif (R = A or G). Arrows, shown at top and bottom, identify C sites, including the surrounding sequence context, exhibiting the largest pH-dependent change in deamination frequency.

Figure 4. Range of Apo3A C deaminations in a lacZα mutational target. The Apo3A scanning range on ssDNA occurring during a single enzyme-ssDNA binding event is defined as the largest distance between deaminated sites on individual clones containing ≥2 mutations. The mutations span the entire lacZα gene, and the range is similar for all values of pH. The lacZα mutational reporter gene contains 365 nt.

Figure 5. Apo3A transcription-dependent deaminations occur exclusively on the non-transcribed strand, featuring CC→TT tandem mutations a hallmark of skin cancer. A, Apo3A deamination spectrum on dsDNA transcribed by T7 RNA polymerase. Apo3A was incubated with a M13 dsDNA substrate (sketch at top) in the presence of T7 RNA polymerase and four rNTPs at pH 7.9. C→U deaminations, are detected as C→T mutations in lacZα if deaminations occurred on the non-transcribed strand or as G→A mutations for deaminations on the transcribed strand. All 203 lacZα mutations were C → T, shown as stars (*). B, Percentage of clones containing 1 to 8 mutations. C, The Apo3A scanning range on transcribed dsDNA occurring during a single enzyme-dsDNA binding event is defined as the largest distance between deaminated sites on individual clones containing ≥2 mutations.
Figure 6. Comparison of Apo3A deamination activity on 37 nt ssDNA and 37 bp duplex DNA containing a 7 nt bubble, 3 nt bubble or 1 nt bubble. Apo3A (1 to 8 pmol) was incubated with 10 pmol of a $^{32}$P-labeled ssDNA or bubble DNA substrates for 10 min at 37 °C at pH 8.0. Deamination products were separated by 16% denaturing PAGE, visualized and quantified by PhosphoImaging.
Table 1. Apo3A binding to dsDNA, dsDNA containing an ssDNA “bubble”, and to ssDNA oligonucleotides, measured at pH 5.5

| Substrate* | $K_D$ (μM)** |
|------------|--------------|
| dsDNA      | 5.40 ± 1.20  |
| 1 nt bubble| 2.81 ± 0.78  |
| 3 nt bubble| 1.22 ± 0.11  |
| 7 nt bubble| 0.62 ± 0.11  |
| 5 nt ssDNA | 2.95 ± 0.17  |
| 7 nt ssDNA | 0.91 ± 0.09  |

* Apo3A binding was measured by rotational anisotropy using a Fluorescein-labeled 37 base-pair dsDNA; a 37 base-pair duplex DNA containing 1nt, 3 nt or 7 nt ssDNA bubble regions; 5-nt and 7-nt ssDNA substrates.

** $K_D$ was calculated by fitting the changes in rotational anisotropy with increasing Apo3A concentrations to a rectangular hyperbola.
Table 2. Apo3A motif deamination preferences at pH 8.0, 6.5 and 5.5*

| Motif | pH 8.0 Frequency** | Motif | pH 6.5 Frequency | Motif | pH 5.5 Frequency |
|-------|-------------------|-------|-----------------|-------|-----------------|
| CTCG  | 0.73              | CTCG  | 0.64            | CCA   | 0.66            |
| TACA  | 0.54              | CCGA  | 0.64            | TCGG  | 0.45            |
| CTGT  | 0.50              | TCGG  | 0.61            | CTCA  | 0.30            |
| CTCC  | 0.42              | CTCT  | 0.36            | CTCA  | 0.19            |
| TCGG  | 0.33              | CTCA  | 0.33            | TCGG  | 0.15            |
| CCCG  | 0.33              | CCGA  | 0.33            | TTCA  | 0.14            |
| CCUG  | 0.31              | CCCG  | 0.29            | CCCG  | 0.12            |
| TTGG  | 0.31              | CTCA  | 0.26            | CTGG  | 0.12            |
| CTGG  | 0.27              | GTGG  | 0.25            | GTGG  | 0.11            |
| CCCG  | 0.25              | TTGG  | 0.23            | CTCG  | 0.10            |

YYC Consensus  YYC Consensus  YYCR Consensus

* Ten most mutated motifs are listed for each pH.
** Frequency represents fraction of clones containing deaminations in the indicated motifs.
Y = pyrimidine
R = purine
Table 3. A3A-catalyzed tandem deaminations in runs of consecutive C

| Sites*       | pH 8.0 | pH 6.5 | pH 5.5 | Transcription (pH 7.9) |
|--------------|--------|--------|--------|------------------------|
| GCCCG        | 0.0    | 0.0    | 1.4    | N/A**                  |
| ACCCT        | 0.0    | 0.0    | 1.4    | N/A                    |
| GCCCA        | 2.1    | 0.0    | 0.0    | N/A                    |
| TCCCCG       | 33.3   | 28.9   | 0.0    | N/A                    |
| TCCCG        | 2.1    | 4.4    | 0.0    | N/A                    |
| ACCCCCA      | 0.0    | 4.4    | 0.0    | 0                      |
| TTCCG        | 20.8   | 17.8   | 2.7    | 8.75                   |
| GCCG         | 0.0    | 1.1    | 0.0    | 0                      |
| ACCCA        | 2.1    | 7.8    | 4.1    | 3.75                   |
| TCCCCCT      | 2.1    | 13.3   | 0.0    | 16.25                  |
| **Total**    | 62.5   | 77.8   | 9.6    | 28.75                  |

* Mutated sites are underlined.
** Because of the placement of the T7 promoter, these sites are not accessible for deamination by Apo3A in a dsDNA template undergoing transcription.
Table 4. Comparison of Apo3A, AID and Apo3G activities in T7-RNA pol transcribed M13 dsDNA*

|                      | M13 mutant phage frequency (x 10⁻⁴) |
|----------------------|--------------------------------------|
| DNA alone            | 4.2 ± 1.4                            |
| DNA + T7 RNA Pol     | 4.8 ± 3.2                            |
| DNA + T7 RNA Pol + Apo3A** | 124 ± 12                           |
| DNA + T7 RNA Pol + AID | 35.5 ± 5.8                          |
| DNA + T7 RNA Pol + Apo3G | 5.4 ± 2.9                           |

* Deamination activity is detected by the presence of mutant M13 phage (light blue or white plaques) caused by APOBEC C → U deamination in \( lacZa \).

** Apo3A, AID or Apo3G (100 pmol each) were present in the reactions.
Figure 1
Figure 2

**pH = 5.5**

- $K_A = 330 \pm 26$ nM
- Hill coefficient = 1.5

**pH = 6.5**

- $K_A = 830 \pm 100$ nM
- Hill coefficient = 1.4

**pH = 7.4**

- $K_A = 1400 \pm 120$ nM
- Hill coefficient = 1.6

**pH = 8.0**

- $K_D = 17000 \pm 3100$ nM
Figure 3
Figure 4
Figure 5
Figure 6

Intact substrate

Deamination products

Apo3A (pmol)

Relative specific activity

1.0

1.0

0.06

0.05

ssDNA

TTCTCCCCG

7nt bubble

TTCTCCCCG

A

TTTTTTTT

3nt bubble

TTCTCCCCG

A

GGGGC

1nt bubble

TTCTCCCCG

A

AGGGGC

Apo3A (pmol) 0 1 2 4 8

Relative specific activity 1.0

0 1 2 4 8

1.0 0.06 0.05

0 1 2 4 8

0 1 2 4 8

0 1 2 4 8
A Biochemical Analysis Linking APOBEC3A to Disparate HIV-1 Restriction and Skin Cancer
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