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The Vif Protein of HIV Triggers Degradation of the Human Antiretroviral DNA Deaminase APOBEC3G

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Summary

APOBEC3G is a human cellular enzyme that is incorporated into retroviral particles and acts to restrict retroviral replication in infected cells by deaminating dC to dU in the first (minus)-strand cDNA replication intermediate [1–5]. HIV, however, encodes a protein (virion infectivity factor, Vif [6, 7]), which overcomes APOBEC3G-mediated restriction but by an unknown mechanism. Here, we show that Vif triggers APOBEC3G degradation by a proteasome-dependent pathway and that an 80 amino acid region of APOBEC3G surrounding its first zinc coordination motif is sufficient to confer the ability to partake in an interaction involving Vif. Inhibitors of this interaction might therefore prove therapeutically useful in blocking Vif-mediated APOBEC3G destruction.

Results and Discussion

While the mechanism by which Vif overcomes APOBEC3G-mediated restriction of viral infection is unknown, it is evident that Vif (like APOBEC3G) needs to be expressed in the viral-producer cell (rather than just in the infected cell target) in order to exhibit an effect on viral replication [8–11]. One could imagine that Vif might mediate its effect by inhibiting the catalytic activity of APOBEC3G, by blocking APOBEC3G incorporation into virions, by triggering APOBEC3G degradation, or by protecting viral replication intermediates from access by APOBEC3G. Therefore, to discriminate between some of these possibilities, we started by asking whether Vif might have an effect on APOBEC3G in the absence of the viral genome and its products.

A plasmid was constructed that directs the expression of a fusion protein of APOBEC3G with GFP (green fluorescent protein). This construct, which is effective in restricting retroviral infection in the previously described MLV-based assay ([2]; data not shown), was transfected into 293T cells together with a plasmid encoding either a functional or mutated version of HIV Vif. The GFP-APOBEC3G gene was well expressed but the stability of the fluorescence was greatly diminished in cells that had been cotransfected with Vif (Figure 1A). A similar destabilizing effect was not observed when vif was cotransfected with a nonchimaeric GFP target (Figure 1B). Cotransfecting target cells with a plasmid encoding a red fluorescent protein (RFP) together with plasmids encoding GFP-APOBEC3G and Vif (or ΔVif) revealed that the red and green fluorescence diminished with similar kinetics when using the nonfunctional ΔVif; the fluorescence of most cells was therefore distributed along the diagonal in the 2D fluorescence plots (Figure 1C). However, when coexpressing Vif itself, a greatly accelerated disappearance of the green (GFP-APOBEC3G) but not the red (control RFP) fluorescence was observed.

Pulse-chase experiments showed that coexpression of Vif causes accelerated degradation of GFP-APOBEC3G but not of a nonchimaeric GFP internal control (Figure 2A). While both the fluorescence (Figure 1) and biosynthetic labeling experiments (Figure 2A) reveal that Vif accelerates GFP-APOBEC3G turnover, there is an apparent difference in the kinetics observed in the two assays. This presumably reflects the fact that whereas the pulse-chase experiments monitor the disappearance over a shortish time (up to 8 hr) of a bolus of radiolabeled GFP-APOBEC3G that was synthesized in a short pulse, the fluorescence assay monitors the dynamic change in total GFP-APOBEC3G as analyzed over a long period (96 hr) during which the Vif:GFP-APOBEC3G ratio will be continuously increasing.

Furthermore, while chloroquine (an inhibitor of the lysosomal pathway) did not suppress the Vif acceleration of GFP-APOBEC3G degradation, the Vif enhancement of GFP-APOBEC3G turnover was largely blocked by ALLN (a calpain I/proteasome inhibitor) as well as by lactacystin, MG132, and proteasome inhibitor I (more specific proteasome inhibitors) (Figure 2A).

Thus, it would seem likely that Vif stimulates APOBEC3G degradation by a ubiquitin-dependent pathway. Indeed, Western blot analysis of GFP-APOBEC3G that has been immunoprecipitated from cells in which it has been coexpressed with an HA-tagged ubiquitin together with Vif or ΔVif reveals a somewhat smeary ladder characteristic of multi/polyubiquitination [12]. The intensity of this ladder is enhanced in the presence of Vif (Figure 2B).

Vif has been reported to interact with APOBEC3G [5]. We were therefore interested to ascertain whether this interaction correlated with the induction of APOBEC3G degradation. We first asked which region of APOBEC3G was needed for Vif interaction. The predicted amino acid sequence of APOBEC3G reveals the presence of two likely zinc-coordination motifs which, given that the related deaminases APOBEC1 and AID contain a single such motif, suggests that the APOBEC3G polypeptide might possess two deaminase active sites [13]. Immunoprecipitation experiments performed using extracts of 293T cells that had been cotransfected with a Vif-expressing plasmid together with vectors directing the expression of different GFP-APOBEC3G chimaeras indeed provided evidence of an interaction between Vif and APOBEC3G; the results indicated that the amino-terminal (but not

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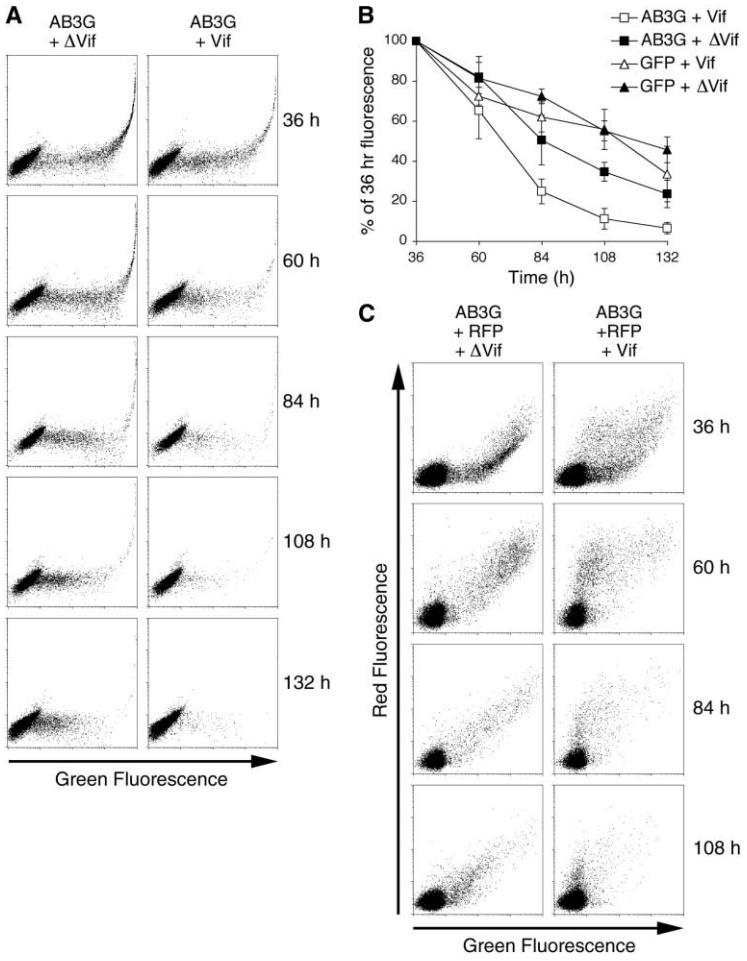

Figure 1. HIV Vif Accelerates Degradation of APOBEC3G Fusion Proteins

(A) Flow cytometric analysis of GFP fluorescence in 293T cells that have been cotransfected with plasmids directing the expression of GFP-APOBEC3G (AB3G) and either a functional or mutated Vif (Vif or ΔVif, respectively) shown at various times after transfection. Green fluorescence is indicated on the *x* axis. The fluorescence in FL2 (*y* axis) is due to very bright green fluorescent cells, whose fluorescence leaks into FL2.

(B) Quantitation of GFP fluorescence as a function of time, taking fluorescence at 36 hr as 100%. The results are shown for experiments in which GFP-APOBEC3G (AB3G) fusion protein or GFP alone (GFP) were expressed in the presence of functional or mutated versions of HIV Vif. The results are the average ± standard deviation of between five and nine independent experiments.

(C) Flow cytometric analysis of both GFP (*x* axis) and RFP (*y* axis) fluorescence in 293T cells that have been cotransfected with plasmids directing the expression of GFP-APOBEC3G (AB3G), red fluorescent protein (RFP), and either a functional or mutated Vif shown at various times after transfection.

the carboxy-terminal) portion of APOBEC3G was sufficient to mediate this interaction (Figures 3A and 3B). While APOBEC3G can act directly on single-stranded DNA [2] and Vif has been shown to be able to bind nucleic acids [14–16], it is notable that the Vif/APOBEC3G interaction is not abolished by treating the extracts with RNase and DNase, although we still cannot conclude that the interaction is necessarily direct. Analysis of further chimaeras revealed that an 80 amino acid sequence encompassing the first putative zinc-coordination motif of APOBEC3G is sufficient to confer the ability to partake in the interaction with Vif (Figures 3A and 3B).

We then asked whether the regions of APOBEC3G that conferred the ability to interact with Vif were also able to confer Vif-dependent destabilization. Pulse-chase (Figure 3C) as well as immunofluorescence (data not shown) experiments revealed that GFP fusions containing the C-terminal (non-Vif-interacting) domain of APOBEC3G were stable and not subject to Vif-dependent degradation. In contrast, GFP fusions with the N-terminal (Vif-interacting) domain of APOBEC3G were inherently unstable, not exhibiting any evident additional destabilization in the presence of Vif. Indeed, so far, we have not been able to identify GFP-APOBEC3G fusions comprising subregions of the APOBEC3G N-terminal domain that exhibit Vif-dependent degradation. Either they are intrinsically stable and do not interact with Vif; alternatively, they are intrinsically unstable with or without Vif (data not shown).

While our results concerning a physical interaction involving Vif and APOBEC3G agree with and extend upon those of Mariani et al. [5], our findings that Vif triggers APOBEC3G degradation results differ from those of these authors who, while noting diminished abundance of APOBEC3G in Vif-expressing cells as well as the presence of APOBEC3G degradation products, did not find that Vif coexpression affected the half-life of APOBEC3G. The explanation for this discrepancy remains a matter for speculation. We do not believe our results are artifacts peculiar to our fusion proteins: during the review of this manuscript, others have described experiments that use different APOBEC3G expression constructs but which have led them to similar conclusions [19–21]. Rather, we note that the Vif antagonism of the antiretroviral effect of APOBEC3G appears to be critically sensitive to the Vif:APOBEC3G ratio [1–3, 5, 17, 18]. Indeed, we have found that the effect of Vif on APOBEC3G turnover as monitored in the cotransfection assay is sensitive to the ratio of Vif to APOBEC3G expression as well as to the time course used to monitor APOBEC3G turnover (data not shown).

Thus, Vif (in the absence of other viral components) is able to trigger degradation of APOBEC3G through a proteasome-dependent pathway. This correlates with

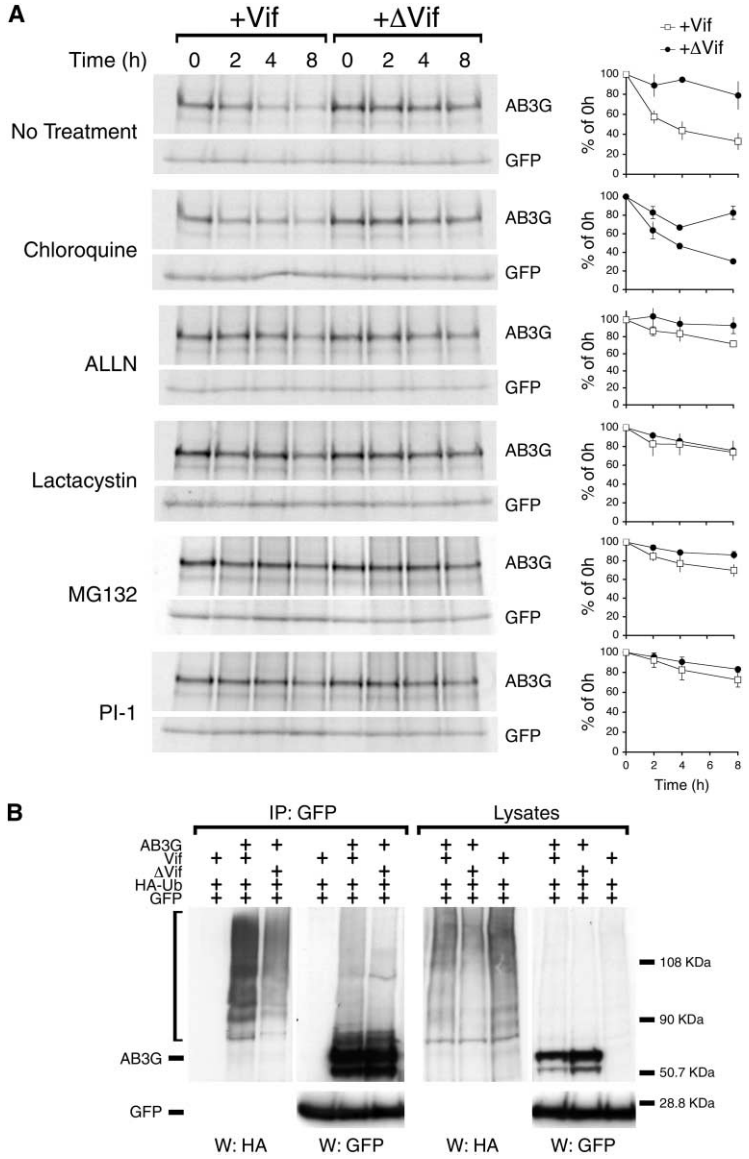

Figure 2. APOBEC3Gs Degraded through a Proteasome-Dependent Pathway

(A) Pulse-chase analysis of GFP-APOBEC3G degradation in the presence or absence of Vif. 293T cells expressing GFP-APOBEC3G (AB3G), GFP (GFP), and either a functional or mutated HIV Vif (Vif or ΔVif, respectively) were pulsed for 30 min with [L-^{35}S]Cys-Met and then chased for the indicated number of hours in the absence or presence of the specified inhibitor. A representative gel is shown together with a graph summarizing the results (means ± standard deviations) of three to six independent experiments. The graphs show the amount of labeled protein after various lengths of chase expressed as a percentage of the radioactivity at time zero.

(B) Polyubiquitination of GFP-APOBEC3G. 293T cells were cotransfected with GFP-APOBEC3G, GFP, HA-ubiquitin, and functional or mutated Vif as indicated. The bulk (90%) of the cell extracts were subjected to immunoprecipitation with anti-GFP antibodies. These immunoprecipitates (or aliquots [2%] of total lysates) were then subjected to SDS/PAGE, and the Western blots were probed with anti-HA or anti-GFP antibodies. The migration of bands corresponding to GFP-APOBEC3G fusion protein (AB3G) and the GFP internal control for protein loading (GFP) are indicated; the bands presumed to correspond to multi/polyubiquitinated GFP-APOBEC3G are indicated by a bracket. The intensity of the multi/polyubiquitinated GFP-APOBEC3G smear (after normalization for total GFP-APOBEC3G amounts [as determined in the anti-GFP Western blot] and correction for the total level of ubiquitination [as monitored in the anti-HA-tag Western blots of total lysates]) was increased by 1.9 fold (SD 0.39 from five independent experiments) in the presence of Vif.

the observation that a region in the vicinity of the first zinc binding domain of APOBEC3G is sufficient to confer the ability to partake in an interaction involving Vif—information which might prove useful in designing peptide inhibitors. While we do not know the details of the mechanism by which Vif-induced destabilization of APOBEC3G occurs, the data are consistent with a model in which formation of a complex involving Vif causes a conformational shift in APOBEC3G that favors its degradation.

Experimental Procedures

Plasmid pEGFP-C3 (BD Biosciences) was used as the expression vector for GFP; plasmids directing expression of GFP-APOBEC3G fusion proteins were derived from pEGFP-C3 by inserting PCR-amplified APOBEC3G fragments downstream of the EGFP coding sequence, between the XhoI and PstI sites. Oligonucleotides used for amplifying APOBEC3G (or portions thereof) were APOBEC3G-N (AAActcgagGCCACCATGAAGCCTCACTTCAGA; AAACTGCAGCGT CACCTAGTCAGAGCTCNNNNNN), APOBEC3G-C (AATctcgagATG GATCCACCCACATTCAC; AAGctgcagTCAGTTTTCCTGATTCTG GAG), APOBEC3G-N1 (AAActcgagATGAAGCC-TCACTTCAGA; AAGctgcagTCATTCGGAATACACCTGGCCTC), APOBEC3G-N2 (AAA ctcga-gATGTTTCGAGGCCAGGTGTATT; AAGctgcagTCAGTAGAG GCGGGCAACGAAGA), APOBEC3G-N3 (AAActcgagATGGCCCGC CTCTACTACTTCT; AAGctgcagTCACCAA-CAGTGCTGAAATTC GTC), and APOBEC3G-N4 (AAActcgagATGGAATTTCAGCACTGTT GGAG; AAGctgcagTCAATCCATCGAGTGTCTGAGAATC). Plasmids expressing Vif and ΔVif were as used previously [2], the plasmid directing the expression of an HA-tagged ubiquitin was kindly provided by J. Sale, and the plasmid encoding the red fluorescent protein was pHcRed1-C1 (BD Biosciences).

Fugene 6 (Roche) was used to transfect plasmids into 293T cells via lipofection, and fluorescence was analyzed via flow cytometry (Becton-Dickinson) at the peak of the expression (36 hr after transfection) and every 24 hr thereafter. For biosynthetic labeling, cells (at 36 hr after transfection) were preincubated in DMEM without cysteine and methionine (Sigma); 4 mM L-glutamine; 0.1% FCS for 2 hr prior to a 30 min pulse with medium containing L-[^{35}S]Cys-Met (0.1 mCi/ml) (Redivue Pro-Mix; Amersham) followed by a chase in complete medium. Where required, chloroquine (100 μM; Sigma), N-Acetyl-Leu-Leu-Norleu-Al (ALLN; 100 μM; Sigma), lactacystin (10 μM), MG-132 (50 μM), or proteasome inhibitor I (50 μM; Calbiochem) were added during the preincubation, incubation, and chase.

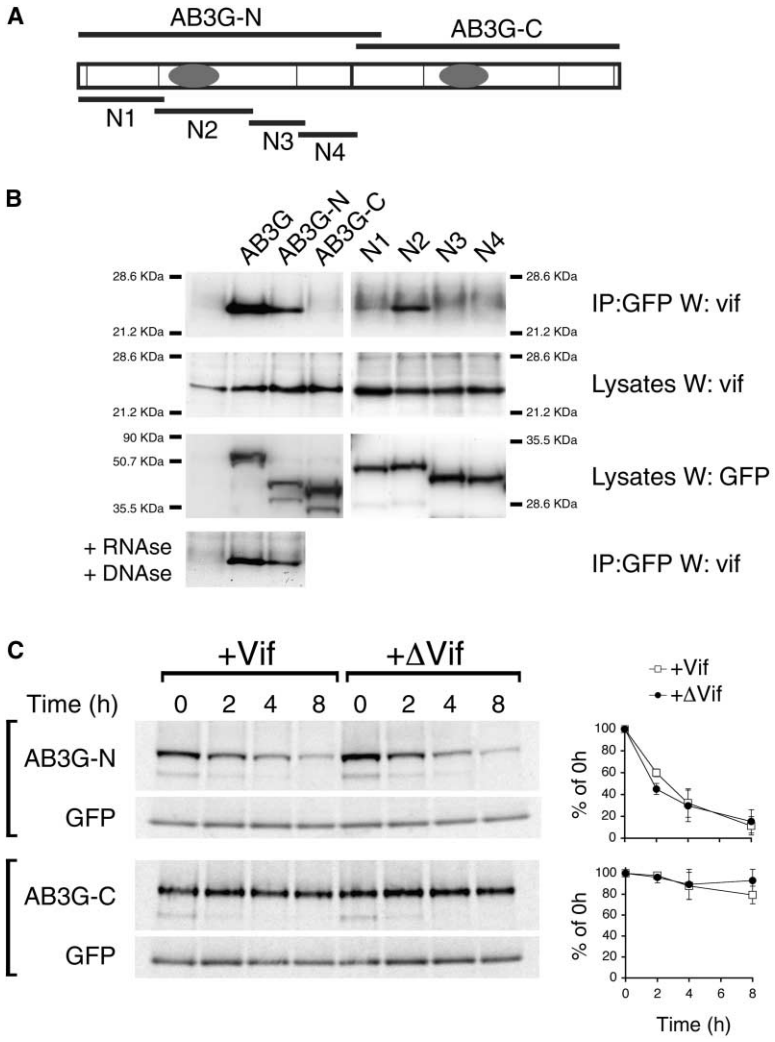

Figure 3. The N-Terminal Domain of APOBEC3G Confers Instability and the Ability to Partake in a Vif Interaction

(A) Schematic representation of APOBEC3G polypeptide. The gray ellipses represent the positions of the putative zinc-coordination domains; the locations of corresponding exon junctions in the mRNA structure are shown as thin vertical lines. The constructs that were analyzed are depicted as horizontal lines above and below the APOBEC3G structure. AB3G-N (amino acid residues 1–215 of APOBEC3G), AB3G-C (residues 197–384), N1 (residues 1–61), N2 (residues 54–124), N3 (residues 121–161), and N4 (residues 156–198).

(B) Interaction of Vif with APOBEC3G and its subdomains. Extracts from cells that had been transfected with plasmids encoding Vif together with plasmids encoding fusion proteins of GFP with subdomains of APOBEC3G were immunoprecipitated with anti-GFP antibodies and immunoblots were probed with anti-Vif antibodies. Aliquots (4%) of the total cell extracts were also loaded and blotted with anti-GFP or anti-Vif antibodies as controls for expression. Where specified, RNaseA (0.5 mg/ml) and DNaseI (130 U/ml) treatment was carried out for 15 min at 37°C before the addition of anti-GFP antibodies.

(C) Pulse-chase analysis of the stability of GFP fusion proteins containing the first domain or second domain of APOBEC3G. 293T cells coexpressing either GFP-APOBEC3G-N (AB3G-N) or GFP-APOBEC3G-C (AB3G-C) together with GFP and either a functional or mutated HIV Vif (respectively, Vif and ΔVif) were pulsed for 30 min with [^{35}S]Cys-Met and then chased for the indicated lengths of time (hours). A representative gel is shown together with a graph summarizing the results from three independent experiments.

For protein analysis, cells were lysed in 10 mM Tris (pH 8), 150 mM NaCl, 10% glycerol, and 1% Nonidet P-40 supplemented with protease inhibitors (Roche). GFP fusion proteins were immunoprecipitated using rabbit anti-GFP (5 μg/ml; BD Bioscience and ABCAM) together with protein A-Sepharose beads (Amersham). GFP-chimeras were visualized by Western blot with HRP-conjugated anti-GFP antibody (1:1000 dilution; ABCAM), whereas Vif was detected with a 1:1000 dilution of a monoclonal anti-Vif antibody (NIBSC, South Mimms, UK). A monoclonal anti-HA antibody (1:1000; Roche) was used to detect HA-ubiquitinated proteins.

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