A Single Amino Acid in Human APOBEC3F Alters Susceptibility to HIV-1 Vif

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Human APOBEC3F (huA3F) potently restricts the infectivity of HIV-1 in the absence of the viral accessory protein virion infectivity factor (Vif). Vif functions to preserve viral infectivity by triggering the degradation of huA3F but not rhesus macaque A3F (rhA3F). Here, we use a combination of deletions, chimeras and systematic mutagenesis between huA3F and rhA3F to identify E324 as a critical determinant of huA3F susceptibility to HIV-1 Vif-mediated degradation. A structural model of the C-terminal deaminase domain of huA3F indicates that E324 is a surface residue within the \( \alpha \)4 helix adjacent to residues corresponding to other known Vif susceptibility determinants in APOBEC3G and APOBEC3H. This structural clustering suggests that Vif may bind a conserved surface present in multiple APOBEC3 proteins.

Human APOBEC3 proteins including APOBEC3F (huA3F) and APOBEC3G (huA3G) are DNA cytidine deaminases that restrict the infectivity of HIV-1 in target cells following virion incorporation in producer cells [recently reviewed by (1-3)]. HIV-1 overcomes this restriction activity by utilizing its accessory protein virion infectivity factor (Vif) to facilitate the degradation of APOBEC3 proteins in producer cells, thus preventing particle incorporation and restriction.

Previously, several groups identified specific changes in the N-terminal deaminase domain (NTD) of huA3G that affect the ability of HIV-1 Vif to neutralize this restriction factor (4-10). The first of these studies sought to determine the basis for the observation that the Vif proteins of the lentiviruses infecting different species neutralize the A3G proteins of their natural host species but not the A3G proteins of other species (11). For example, african green monkey A3G (agmA3G) is susceptible to Vif from the simian immunodeficiency virus (SIV) that naturally infects Chlorocebus aethiops (agmSIV) but not to HIV-1 Vif, while huA3G is susceptible to HIV-1 Vif but not to agmSIV Vif. By substituting agmA3G residues into huA3G where the two differed, several groups identified D128 as a critical determinant of this species specificity (4-7). Subsequent mutational analyses have confirmed that huA3G D128 and surrounding residues including D130 impact HIV-1 Vif-mediated degradation (8-10).

More recently, two reports showed that, in contrast with huA3G, huA3F is recognized at its C-terminal deaminase domain (CTD) by HIV-1 Vif (9,12). One of these groups further narrowed the determinants of this recognition to amino acids 283-300, although individual amino acid changes critical for HIV-1 Vif susceptibility were not identified in a manner analogous to the huA3G studies cited above. Thus, the residues of huA3F critical for the ability of HIV-1 Vif to bind and degrade this restriction factor are presently unknown.

Here we identify a critical determinant of huA3F susceptibility to HIV-1 Vif by comparing huA3F with the closely related but HIV-1 Vif-resistant rhA3F (13,14). Using chimeras between these orthologs as well as single-domain studies, we confirm that Vif recognizes the CTD of huA3F. Through systematic replacement of selected C-terminal huA3F residues with their corresponding rhA3F residues, we further identify huA3F QE323-324EK as a critical determinant of this differential susceptibility. Additional mutagenesis between these two residues revealed that mutation of E324 to the rhA3F lysine or to alanine results in resistance to HIV-1 Vif-mediated degradation. To determine the three-dimensional context surrounding this residue, we created a model of the CTD of huA3F and found that E324
is a surface residue contained within the α4 helix that forms part of a broader surface shared with the linearly separate huA3F Vif interaction domain previously narrowed to residues 283-300 (9). Importantly, this analysis also revealed that the huA3F residues corresponding to three known Vif susceptibility determinants, D128 and D130 in huA3G and D/E121 in human APOBEC3H (huA3H), also cluster at this helix. These studies combine to suggest that a conserved structural surface is targeted by HIV-1 Vif en route to APOBEC3 neutralization and degradation.

**EXPERIMENTAL PROCEDURES**

**Plasmid DNA construction and site-directed mutagenesis** – All constructs were confirmed by DNA sequencing. huA3F and huA3G coding sequences correspond to those found in GenBank NM_145298 and NM_021822, respectively. rhA3F was provided by Dr. Theodora Hatzioannou (Aaron Diamond AIDS Research Center, New York) (13,14). Substitutions of rhA3F residues into huA3F were based on alignment between huA3F and rhA3F reference sequences NM_145298 and NM_001042373.1.

pcDNA3.1-V5, -huA3F-V5 and -huA3G-V5 have been described, and pcDNA3.1-huA3G-V5 D128K was similarly derived (15). A3F domain chimeras were made using overlapping PCR (16). PCR products were digested with KpnI/XhoI and ligated into similarly cut pcDNA3.1-V5.

Single-domains of huA3F and rhA3F were amplified using primers containing SacI/SalI sites and cloned into similarly cut pEGFP-N3 (Clontech). Full-length huA3F-GFP has been described (17); NTD = residues 1-191; CTD = residues 192-373.

huA3F PE281-282LD, NLT298-300KLA, T303A and D313H were introduced into the pcDNA3.1-huA3F-3xHA construct (17) by site-directed mutagenesis using Pfu polymerases (Stratagene). The 3xHA tag was subsequently replaced with a V5 tag (15). All other mutations were introduced directly into pcDNA3.1-huA3F-V5.

HIV-1_{hun} and SIV_{mac239} Vif as well as a vector derived from pVR1012 have been described (15,18). An untagged, codon-optimized version of HIV-1_{hun} Vif was made by PCR amplification and ligation of the coding region into the Sall/BamHI segment of the original construct. The codon-optimized translated Vif open reading frames are those of HIV-1_{hun} (GenBank EU541617) and SIV_{mac239} (GenBank AY588946).

Proviral plasmid HIV-1_{hun} is a nucleotide A200C derivative of pH1B (15). A Vif-deficient A200C pH1B derivative containing a previously described deletion in vif made by overlap extension PCR was used in spreading infections (19). A Vif-deficient pH1B derivative containing tandem stop codons at positions 26-27 of vif was used for all single-cycle infectivity experiments and has been previously described (15,20). Wildtype and Vif-deficient LAI-GFP were kindly provided by Dr. Mario Stevenson (University of Massachusetts, Worcester, MA).

**Cell lines** – 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum and, in some cases, penicillin/streptomycin. CEM-GFP reporter cells were maintained in RPMI supplemented with 10% fetal bovine serum, penicillin/streptomycin and β-mercaptoethanol (20,21).

**Stability of A3F chimeras in the presence of HIV-1 and SIV Vifs** – At 50% confluency in 6-well plates, 293T cells were transfected using Trans-IT transfection reagent (Mirus Bio) with 100 ng A3-V5 and 25 ng Vif-HA. After 48 hours, cells lysates were harvested and resuspended in 2x sample buffer 25 mM Tris pH 6.8, 8% glycerol, 0.8% SDS, 2% β-mercaptoethanol, 0.02% bromophenol blue, boiled for 10 minutes, and run on a 12% SDS-PAGE gel prior to transfer to a PVDF membrane (Millipore). Membranes were probed with mouse anti-V5 (Invitrogen), mouse anti-HA.11 (Covance) or mouse anti-α-tubulin (Covance) primary antibodies followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies. Membranes were developed using HyGLO chemiluminescent HRP detection reagent (Denville Scientific) and exposed to film. Blots were stripped using 0.2 M glycine, 1.0% SDS, 1.0% Tween-20, pH 2.2 between sequential probing with primary antibodies.

**Single-cycle infectivity assays** – 250,000 293T cells were plated in 2 mL DMEM in 6-well plates. One day later, Trans-IT transfection reagent (Mirus Bio) was used to cotransfect these cells.
with 1.6 µg Vif-deficient HIV-1\textsubscript{LIMB}, 100 ng of a codon-optimized HIV-1\textsubscript{LIMB} Vif-HA expression construct (or 50-200 ng supplemented to 200 ng total with a vector control in Fig. 2C) and 200 ng of a given APOBEC3-V5 construct. Approximately two days later, virus-containing supernatants were filtered through 0.45 µm PVDF filters (Millipore) and 75 or 150 µL were used to infect 25,000 CEM-GFP reporter cells plated at a final total volume of 250 µL. At this time, 293T cells were resuspended with 1 mL phosphate buffered saline (PBS) and 500 µL were spun down and resuspended in 250 µL lysis buffer [25 mM HEPES pH 7.4, 150 mM NaCl, 1 mM MgCl\textsubscript{2}, 50 µM ZnCl\textsubscript{2}, 10% glycerol and 1% Triton X-100 supplemented with 50 µM MG132 (American Peptide) and complete protease inhibitor (Roche)] for analysis of APOBEC3 intracellular stability. Three days post-harvest, CEM-GFP reporter cells were fixed with 4% paraformaldehyde and analyzed by flow cytometry on a Beckman-Coulter Quanta MPL or a Becton-Dickinson LSR II to determine infectivity as measured by the percentage of GFP-positive cells. Relative infectivity was calculated by normalizing the infectivity of viruses produced in the presence of each APOBEC3 protein +/- Vif to the infectivity of viruses produced in the presence of an APOBEC3 vector control +/- Vif in each experiment. Data shown represent the mean and SEM of the number of independent transfection-infection series indicated in the figure legend.

**Analysis of intracellular APOBEC3 stability** – Cotransfected cells were lysed as described above, and aliquots were mixed with a 5x or 7.5x version of the sample buffer described to a final concentration of 2x and boiled for 10 minutes. Proteins were then separated by 10% SDS-PAGE and transferred to a PVDF membrane. Membranes were probed as described above and stripped using 62.5 mM Tris pH 6.8, 2% SDS and 100 mM β-mercaptoethanol at 50°C prior to sequential blocking and reprobing.

**Analysis of intracellular APOBEC3 expression** – APOBEC3-expressing and vector control derivatives of SupT11 as well as the model nonpermissive cell lines CEM and H9 were grown to confluence in 10 cm dishes. 5x10\textsuperscript{6} cells were then lysed in 250 µL and analyzed for huA3F or huA3G expression using antibodies #1474 or #10201 from Drs. Michael Malim or Jaisri Lingappa, respectively, obtained through the AIDS Research and Reference Reagent Program.

**Virus titration and spreading infections** – Viruses were produced by plating 3.5x10\textsuperscript{6} 293T cells in 10 cm dishes and one day later transfecting those cells with 5-10 µg of a given proviral plasmid using Trans-IT transfection reagent (Mirus Bio). Approximately two days post-transfection, supernatants were harvested, and different volumes were used to infect 150,000 CEM-GFP reporter cells at a constant total volume of 1 mL in 24-well plates. Three days later, these CEM-GFP cells were fixed in 4% paraformaldehyde, and the total percentage of GFP-positive cells was quantified by flow cytometry as before. Linear regression was then employed to determine the volume of a given viral stock required to initiate infection at a CEM-GFP MOI of 0.01.

Spreading infections were initiated by infecting 150,000 cells of a given cell line at an MOI of 0.01 in a total volume of 1 mL in 24-well plates. Cultures were subsequently split and fed as necessary to prevent cell overgrowth. Viral spread was monitored by periodically harvesting 150 µL of supernatant from each culture and using it to infect 25,000 CEM-GFP cells at a final volume of 250 µL in 96-well plates. Three days post-infection, these CEM-GFP were fixed in 4% paraformaldehyde, and the percentage of GFP-positive cells was analyzed by flow cytometry on a Becton-Dickinson LSR II.

**Homology Modeling of the huA3F C-terminal Deaminase Domain** – The A3F\textsubscript{186-373} model was generated using YASARA (22) based on the crystal structure of A3G\textsubscript{191-384} 2K3A [PDB ID 3IR2, (23)]. Alignment with the A3G\textsubscript{191-384} sequence (Supplementary Fig. 1) was iteratively optimized using related SwissProt and TrEMBL sequences, the predicted secondary structure and the structural information of the template. Knowledge-based and electrostatic interactions in unrestrained molecular dynamics with explicit solvent molecules were used to refine amino acid side chain geometry. Insertions were accounted for by a search of the Protein Data Bank for loop ends superimposable with model anchor points. Further optimization was achieved by placement of loops into their lowest energy conformations.
Coimmunoprecipitation – Coimmunoprecipitation of V5-tagged huA3F with HA-tagged HIV-1 Vif or with HA-tagged Vif with mutation of the conserved BC Box residues SLQ>AAA that ablate Vif-mediated degradation was carried out by lysing cotransfected 293T cells with RIPA buffer [50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with complete protease inhibitor (Roche)]. Lysates were then incubated with 2.5 μL of mouse anti-HA.11 at 4°C followed by the addition of 40 μL Dynabeads Protein G (Invitrogen). Immunoprecipitated complexes were isolated by magnetic separation, washed four times with PBS and eluted by addition of 30 μL of 5x sample buffer as above. SDS-PAGE and Western blotting were then carried out as before. Western blots were quantified by analysis with ImageJ software (24). Binding quantification represents the intensity of V5 bands immunoprecipitated divided by the intensity of the corresponding lysate bands and normalized to the ratio found for huA3F in a given experiment.

RESULTS

HIV-1 Vif recognizes the huA3F C-terminal deaminase domain. Two previous reports have indicated that HIV-1 Vif binds the CTD of huA3F (9,12). To confirm and extend these results, we took two approaches. First, we created chimeras between huA3F and rhA3F, which share 87% identity and 92% overall similarity at the protein level (Fig. 1A). This strategy takes advantage of the fact that rhA3F is resistant to HIV-1 Vif while huA3F is sensitive to HIV-1 Vif (13,14). Therefore, by comparing the sensitivity of chimeric proteins to HIV-1 Vif-mediated degradation, one can broadly infer whether a given chimera contains a site functionally recognized by HIV-1 Vif (12). A schematic of the substitutions made is shown in Fig. 2A. To test the Vif susceptibility of these mutants, we carried out single-cycle infectivity assays. As shown in Fig. 2B, infectivity restoration upon the cotransfection of HIV-1IIIB Vif with most huA3F mutants containing 1-2 rhA3F residues at sites where these two differ within their CTDs. A schematic of the substitutions made is shown in Fig. 2A. To test the Vif susceptibility of these mutants, we carried out single-cycle infectivity assays. As shown in Fig. 2B, infectivity restoration upon the cotransfection of HIV-1IIIB Vif with most huA3F mutants was similar to that seen with wild-type huA3F. For the substitution QE323-324EK, however, Vif sensitivity was ablated (Fig. 2B). These infectivity data correlated with producer cell huA3F levels, making this mutant phenotypically analogous to the control huA3G D128K.

Interestingly, neither of these substitutions is contained within the Vif binding region proposed by Russell et al. (9), residues 283-300, while the substitutions that do fall within this region (NLT298-300KLA) have no apparent phenotype [Fig. 2A-B and Discussion]. Furthermore, huA3F D313H, which corresponds to the change D130K in the evolutionarily related NTD of huA3G, has no apparent phenotype [Fig. 2A-B, Discussion and (25)].

To confirm the intrinsic Vif-resistance of huA3F QE323-324EK and eliminate the possibility that the C-terminal V5 tag initially used might affect our observations, we carried out single-cycle titration experiments using increasing levels of Vif cotransfected with a constant amount of untagged huA3F, huA3F QE323-324EK, huA3G or huA3G DPD128-130KPK. As shown in Fig. 2C, both huA3F QE323-324EK and huA3G DPD128-130KPK retained similar restriction regardless of Vif levels. The Vif-resistance of both constructs was further confirmed by the intracellular stability of each in comparison with its wildtype control in the presence of Vif.
Because the single-cycle infectivity assays described to this point are vulnerable to potential overexpression artifacts [e.g. (26-28)], we also sought to assess the Vif-resistance of the huA3F QE323-324EK construct in a more physiologic setting. To that end, we created derivatives of a previously-described APOBEC3-deficient T cell line, SupT11, stably transfected with untagged huA3F QE323-324EK or huA3G DPD128-130KPK to go with our previously described derivatives expressing wildtype huA3F or huA3G (15,29). The expression levels of huA3F and huA3G in each cell line used are shown in Fig. 2D. We then initiated spreading infections an at MOI of 0.01 on these cell lines using Vif-deficient or wildtype IIIB or LAI-GFP viruses. All cell lines with the exception of the vector controls restricted the spread of Vif-deficient HIV-1IIIB and HIV-1 LAI-GFP (data not shown). In contrast, both wildtype HIV-1IIIB and HIV-1 LAI-GFP, which differ at 20/192 Vif amino acids, spread efficiently on cell lines expressing wildtype huA3F or huA3G (Fig. 2E and data not shown). Despite this, similar levels of huA3F QE323-324EK or huA3G DPD128-130KPK restricted the spread of even these Vif-proficient viruses (Fig. 2E and data not shown). We thus conclude that huA3F QE323-324EK, like huA3G DPD128-130KPK, is fully resistant to HIV-1 Vif and fully capable of inhibiting virus replication.

Reciprocal amino acid substitutions rhA3F EK323-324QE do not sensitize rhA3F to HIV-1 Vif. To ask whether the reciprocal amino acid substitutions in rhA3F might render it susceptible to HIV-1 Vif in a manner analogous to the sensitization of agmA3G by the humanizing mutation K128D (4-7), we carried out single-cycle infectivity and expression analyses as above using rhA3F and rhA3F EK323-324QE. Under these conditions, rhA3F EK323-324QE showed no significant recovery in infectivity in the presence of HIV-1 Vif over wildtype rhA3F (Fig. 3). Thus, residues 323-324 are not exclusively responsible for the differential Vif sensitivity of rhA3F and huA3F. This observation is consistent with the emerging view that a larger surface on APOBEC3 proteins is recognized by Vif (See Discussion).

It is important to note that such separation of function experiments are not possible using SIVmac239 Vif, as this Vif neutralizes both huA3F and rhA3F (14). This parallels the results of several of the original papers characterizing huA3G D128K, which show that SIVmac239 Vif is able to neutralize both huA3G and rhA3G [e.g. (6,7)]. It is also consistent with our own studies suggesting that the Vifs of various species’ lentiviruses are optimized for recognition of their own host species’ APOBEC3Z3 proteins but often retain considerable activity against the APOBEC3Z3 proteins of other species (18).

Mutation of huA3F E324 alone alters functional susceptibility to HIV-1 Vif in the absence of a quantitative reduction in physical binding. To further characterize the changes at residues 323-324, we created huA3F mutants with single cognate rhesus substitutions at each position as well as single and double alanine mutations at these positions and assessed their restriction activities and Vif susceptibilities as before. This analysis revealed that any huA3F variant lacking glutamate at position 324 is resistant to HIV-1 Vif regardless of the identity of residue 323, which correlates with intracellular A3F levels (Fig. 4A). We therefore conclude that residue 324 is a single amino acid determinant of huA3F HIV-1 Vif susceptibility.

To determine whether mutation of E324 alters the quantitative binding of Vif to huA3F, we cotransfected HA-tagged Vif with V5-tagged huA3F and variants mutated at residues 323 and 324. We then immunoprecipitated HA-tagged Vif from these lysates and blotted for associated V5-tagged huA3F. Despite the resistance of huA3F E324 variants to HIV-1 Vif-mediated degradation, however, we found that mutation of this residue does not reduce coimmunoprecipitation with HIV-1 Vif relative to wildtype (Fig. 4B-C). These results were confirmed by reciprocal immunoprecipitation of V5-tagged huA3F or huA3F E324K and blotting for cotransfected, untagged Vif in both RIPA and NP40 lysis buffers (data not shown).

Determinants of HIV-1 Vif recognition localize to the α4 helix of a susceptible deaminase domain. To visualize E324 in its three-dimensional context, we created a model of the CTD of huA3F based on a recent crystal structure of the huA3G CTD [PDB ID 3IR2, (23)]. As shown in Fig. 5A-B, E324 is located on the surface of the α4 helix. Importantly, this region is also adjacent to the linearly separate stretch of amino acids previously implicated in the huA3F
interaction with HIV-1 Vif, residues 283-300 (9). We also noted, however, that several additional negatively charged residues occurred on or near the surface of this helix: D311, D313 and E316. On aligning these residues to those found in other Vif-susceptible deaminase domains in Fig. 5C, we noted that they each align to a previously described APOBEC3 determinant of Vif susceptibility (4-10,30). D311 and D313 in huA3F correspond to D128 and D130 in the NTD of huA3G, while huA3F E316 corresponds to E/D121 in human APOBEC3H (huA3H). Thus, all known APOBEC3 determinants of Vif susceptibility cluster along the surface of the α4 helix, and all are negatively charged.

**DISCUSSION**

The studies described here are the first to identify a single amino acid determinant of the susceptibility of huA3F to HIV-1 Vif. This represents an important advance in our understanding of the HIV-1 Vif-huA3F interaction, the relevance of which is strongly supported by a large body of work demonstrating the potency of huA3F-mediated restriction of HIV-1 [e.g. (31-34)]. Our own long-term viral evolution studies have also suggested that functional neutralization of huA3F by HIV-1 Vif is required for the virus to propagate in the presence of huA3F (15). Thus, shielding the α4 region of huA3F described here from HIV-1 Vif may represent a viable strategy for the development of novel pharmacotherapies for HIV-1 infection (3,35).

Our work confirms prior reports that broadly localized Vif interaction to the CTD of huA3F [(9,12), Fig. 1]. An additional recent report on the existence of a Vif-susceptible splice variant of huA3F composed largely of the CTD is also consistent with these data (36).

Our identification of a single amino acid determinant of HIV-1 Vif susceptibility in huA3F echoes several prior reports localizing Vif susceptibility in huA3G and huA3H in that the residue identified is a single negative charge localized to the surface of an APOBEC3 protein [(4-7), Figs. 4-5]. E324 differs from these reports in one key respect, however, as a charge substitution was involved in all prior reports. For example, huA3G D128A has no phenotype, while D128K is Vif-resistant [e.g. (5,6,8)]. The fact that both alanine and lysine substitutions at huA3F E324 ablate Vif susceptibility (Fig. 4A) suggests that E324 is required either for overall stability of the broader Vif binding surface or for direct functional interaction with HIV-1 Vif. Changes to this residue do not, however, affect the ability of huA3F to restrict HIV-1; in fact, none of the changes described in these studies affected restriction activity (e.g. Figs. 2-4).

It is notable that E324 does not fall within the region previously found by Russell et al. to be critical for HIV-1 Vif recognition of huA3F (9). These residues, 283-300, encompass most of the α3 helix, which is structurally adjacent to α4 and E324 and appears to form a common surface [Fig. 5A-B and (9)]. It is therefore possible that E324 may cooperate with residues in α3 and/or α4 to create a stable surface recognized by HIV-1 Vif, in which case mutational alteration of any critical component of this putative Vif interaction node may affect Vif sensitivity. Alternatively, Russell et al. used chimeras between huA3F and huA3G to map the huA3F Vif interacting region (9). The C-terminal deaminase domains of huA3F and huA3G, however, are evolutionarily divergent Z2 and Z1 types, respectively (25). This means that chimeras in this domain will contain a relatively large number of amino acid substitutions versus wildtype. For example, only half of residues 283-300 are biochemically similar or identical between huA3F and huA3G. Thus, we think it likely that the structure of huA3F-huA3G chimeras in this region will be altered relative to huA3F, which may affect interaction with Vif.

In addition to suggesting the structural unity of our findings with those of Russell et al., our model of the huA3F CTD allowed us to make an important observation about the nature of the region surrounding E324. Namely, both the α4 and the neighboring α3 helices have a number of negatively charged surface residues (Fig. 5A-B). This led us to align these surface residues with other Vif-susceptible APOBEC3 deaminase domains such as the huA3G NTD and huA3H, which showed that each negativelycharged surface residue in the α4 helix of huA3F corresponds to a known negatively charged determinant of Vif susceptibility in another
APOBEC3 protein (Fig. 5). Thus, while determining the identity of all the amino acid residues with which Vif interacts (i.e. the broader Vif binding surface in APOBEC3 proteins) will require a great deal of future genetic and structural study, it is intriguing that all known Vif susceptibility determinants map to the same structural motif. This implies a degree of structural conservation among Vif-APOBEC3 interaction surfaces that would not be apparent from a simple linear comparison of these single amino acid determinants.

While the lack of functional interaction between Vif and huA3F E324 variants is clear, this appears to be due to a qualitative change in the nature of the Vif-huA3F interaction in E324 mutants since coimmunoprecipitation of huA3F E324 mutants with HIV-1 Vif is unimpaired. Our data therefore support the potential for both qualitative and quantitative changes in huA3F binding to Vif that may alter susceptibility. In the absence of structural data, however, we are unable to explain the nature of the qualitative defect found in huA3F E324 mutants. It is possible that the qualitative defect in E324 mutants may involve a conformational change that prevents productive interaction with HIV-1 Vif. Alternatively, mutation of E324 may impair the recruitment of other components of the E3 ligase complex by HIV-1 Vif en route to degradation. It is also conceivable that E324 mutants may have a functionally relevant, altered affinity for HIV-1 Vif that is not readily apparent by coimmunoprecipitation.

In summary, we have described here a single amino acid determinant of huA3F susceptibility to HIV-1 Vif. This advance in our understanding of the Vif-huA3F interaction echoes the single amino acid determinants previously identified in other APOBEC3 proteins, as all are negatively charged residues that may interact directly with the highly basic Vif protein. Importantly, the observation that all of these single amino acid determinants cluster along the α4 helix raises the exciting possibility that certain features of the Vif-APOBEC3 interaction may be structurally conserved, which would facilitate the design of hypothetical single molecules which may simultaneously block the functional interaction of Vif with multiple APOBEC3 proteins. Indeed, while its exact mechanism of action remains unknown, the compound RN-18 provides proof of concept for just such a scenario (37).

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Susceptibility of huA3F to HIV-1 Vif maps to the huA3F C-terminal deaminase domain.  
A: A schematic depiction of the chimeras used in Fig. 1B.  
B: A cotransfection experiment demonstrating the instability in the presence of HIV-1 Vif of chimeras between rhA3F and huA3F that contain the huA3F CTD.  
C-D: Cotransfection experiments demonstrating that the CTDs of huA3F and rhA3F are destabilized by HIV-1 Vif or SIV Vif, respectively, while the corresponding NTDs remains highly expressed.

FIGURE 2. Substitution of rhA3F residues at positions 323-324 of huA3F results in phenotypic Vif resistance.  
A: A schematic depiction of the CTDs of huA3F and rhA3F with relevant amino acids shown.  
B: Single-cycle infectivity data quantifying the effects of HIV-1 Vif on the rescue of HIV-1 infectivity. All mutants remained competent for restriction. Relative infectivity represents the mean and SEM of four independent experiments. Western blots demonstrating intracellular APOBEC3 and Vif levels corresponding to one of these experiments are shown below.  
C: Single-cycle infectivity data demonstrating the continued restriction of HIV-1 by untagged huA3F QE323-324EK and huA3G DPD128-130KPK in the presence of increasing amounts of Vif. Relative infectivity represents the mean and SEM of two independent experiments done in duplicate. The Western blots demonstrating the stability of Vif-resistant huA3F and huA3G variants in the presence of Vif correspond to one of these infectivity experiments.  
D: Western blots showing the expression levels of huA3F, huA3G and their Vif-resistant variants in the SupT11 cell lines used in Fig. 2E.  
E: Representative spreading infection curves demonstrating that wildtype HIV-1IIIB is restricted by Vif-resistant variants huA3F QE323-324EK and huA3G DPD128-130KPK but not the corresponding wildtype APOBEC3 proteins. Open symbols indicate vector control or huA3F- or huA3G-expressing cell lines. Closed symbols indicate cell lines expressing huA3F QE323-324EK or huA3G DPD128-130KPK. The x-axis is offset from zero to permit visualization of curves yielding no detectable spread.

FIGURE 3: Substitution of human residues at positions 323-324 of rhA3F does not sensitize rhA3F to HIV-1 Vif. Single-cycle infectivity experiments demonstrating that substituting the human residues at positions 323-324 of rhA3F does not sensitize this restriction factor to permit infectivity recovery in the presence of Vif. Data represent the mean and SEM of three independent experiments. Western blots corresponding to one of the single-cycle experiments shown demonstrate the correlation between intracellular stability of APOBEC3 variants and functional recovery in infectivity.

FIGURE 4. The identity of residue 324 is a primary determinant of the degradation sensitivity of huA3F to HIV-1 Vif. A: Single-cycle infectivity data quantifying the restriction and Vif-sensitivity
phenotypes of single and double human-to-rhesus and human-to-alanine mutations at positions 323 and/or 324 of huA3F, where only mutations at position 324 ablate Vif-responsiveness. Data represent the mean and SEM of three independent experiments. Western blots corresponding to one of these experiments demonstrating that mutations at position 324 of huA3F result in resistance to Vif-mediated degradation are shown below. B: A representative experiment demonstrating the lack of effect of mutations at huA3F positions 323-324 on coimmunoprecipitation with HA-tagged Vif. The bands in each row are taken unaltered from different parts of the same blot. C: Quantification of the results from a total of five independent experiments including the one shown in Fig. 4B. Relative binding represents the ratio of IP V5 signal to cellular V5 signal normalized to the ratio observed for A3F in a given experiment (set to 1).

FIGURE 5: A model structure of the C-terminal deaminase domain of huA3F. A: A ribbon diagram depicting the CTD of huA3F. The region encompassing the huA3F equivalents of all known single amino acid determinants of Vif-sensitivity is shown in blue, with D311, D313 and E316 shown in orange and E324 in red. The region previously implicated in huA3F interaction with Vif (residues 283-300) is colored purple. B: The predicted surface of the huA3F CTD. C: An alignment of residues in the α4 helix encompassing known determinants of Vif susceptibility.
Albin et al., Fig. 3

Relative Infectivity

HIV Vif

- +

rhA3F WT
rhA3F EK-QE
huA3F WT
hsA3F QE-EK

Vif-HA
A3-V5
Tub
A)

B)

C)

Albin et al., Fig. 4
A

huA3F 311–324 DTDYQEGLRSLSQ--E
huA3G 128–143 DPDYQEALRSLCQKR
huA3H 116–128 CKPQQDGLRLLCG---

Albin et al., Fig. 5
A single amino acid in human APOBEC3F alters susceptibility to HIV-1 Vif
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