

Human Immunodeficiency Virus Type 1 Vif Protein Binds to the Pr55^{Gag} Precursor

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The Vif protein of human immunodeficiency virus type 1 is required for productive replication in peripheral blood lymphocytes. Previous reports suggest that *vif*-deleted viruses are limited in replication because of a defect in the late steps of the virus life cycle. One of the remaining questions is to determine whether the functional role of Vif involves a specific interaction with virus core proteins. In this study, we demonstrate a direct interaction between Vif and the Pr55^{Gag} precursor in vitro as well as in infected cells. No interaction is observed between Vif and the mature capsid protein. The Pr55^{Gag}-Vif interaction is detected (i) in the glutathione *S*-transferase system, with in vitro-translated proteins demonstrating a critical role of the NC p7 domain of the Gag precursor; (ii) with proteins expressed in infected cells; and (iii) by coimmunoprecipitation experiments. Deletion of the C-terminal 22 amino acids of Vif abolishes its interaction with the Pr55^{Gag} precursor. Furthermore, point mutations in the C-terminal domain of Vif which have been previously shown to abolish virus infectivity and binding to cell membranes dramatically decrease the Gag-Vif interaction. These results suggest that the interaction between Vif and the Pr55^{Gag} precursor is a critical determinant of Vif function.

In addition to the structural *gag*, *pol*, and *env* genes, the human immunodeficiency virus type 1 (HIV-1) genome contains several regulatory genes: *tat*, *rev*, *vpr*, *vif*, *nef*, and *vpu* (47). The Vif protein of HIV-1 has thus far been considered an accessory factor. Vif is encoded by all lentivirus genomes except that of equine infectious anemia virus (31). Furthermore, in vivo experiments in several models have shown a total absence of infection in animals inoculated with *vif*-deleted (Vif⁻) viruses (13, 23), demonstrating a crucial role for *vif* in establishing infection in vivo. The requirement for *vif* is cell type dependent (14, 17, 35, 41, 46). Although most CD4⁺ continuous cell lines are permissive for Vif⁻ mutants, others, including H9 cells as well as peripheral blood mononuclear cells and monocytes/macrophages, do not allow the replication of these mutants (12, 14, 18). When Vif virus is produced in restrictive cells, the newly synthesized Vif⁻ proviral DNA appears to be unstable in the target cell, suggesting that it might be not efficiently reverse transcribed due to a replication block that occurs before or during reverse transcription (5, 12, 20, 41, 46) or might be abnormally susceptible to degradation (39). Although the function of Vif is still unknown, Vif has been found to act late in the virus life cycle of the producer cell and is required for the assembly of fully infectious virions (4, 5, 12, 37). Recent reports indicate that Vif⁻ particles exhibit a normal protein content (7, 16), although some previous studies reported an aberrant composition of Gag proteins (5, 37) as well as a decrease in the incorporation of Env in virus particles (35). However, Vif⁻ particles are morphologically abnormal when examined by transmission electron microscopy, displaying an abnormally condensed nucleoid (5, 7, 25).

The HIV-1 *vif* gene encodes a 23-kDa protein of 192 amino acids which is present in infected cells (27). Vif is located in the cytoplasm both in a soluble cytosolic form and in a membrane-associated form that is associated with the cytoplasmic side of cell membranes (19). The C-terminal basic domain of Vif is required for membrane association and for the Vif⁺ phenotype in vitro (21). Vif is highly phosphorylated on serine and threonine residues (49). Vif has been found in virions associated with the core structures of HIV-1, HIV-2, and simian immunodeficiency virus (26, 29). However, the relative concentration of Vif is much lower in virions than in infected cells, in contrast to the other major virion components (8). Therefore, the specificity of Vif incorporation into virions remains an open question.

The aim of this study was to investigate whether HIV-1 Vif directly interacts with viral components of the mature core or with the immature Gag precursor during the assembly process. We demonstrate a strong direct interaction between Vif and the Gag precursor, using an in vitro glutathione *S*-transferase (GST) system. We show that the C-terminus basic domain of Vif and two domains of Gag are required for this interaction. The interaction between Vif and Gag is also demonstrated in HIV-1-infected cells. Vif⁻ mutants which show an absence of Pr55^{Gag}-Vif interaction cannot replicate in restrictive cells, suggesting that this interaction plays a biological role in HIV-1 replication in vivo.

MATERIALS AND METHODS

Cells. HeLa cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), antibiotics (penicillin and streptomycin), and 2 mM glutamine. SupT1 and H9 cells were grown in RPMI 1640 medium supplemented with FCS, antibiotics, and 2 mM glutamine. H9 cells chronically infected with wild-type virus have been previously described (7).

Molecular clones. The HIV-1_{NDK} molecular clone used in all our studies has been described elsewhere (42). This clone has an additional stop codon replacing

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amino acid 65 of the *vpr* gene (7). The B1, B2, B3, and B4 point mutations in *vif* were constructed in the full-length HIV-1 HXB2 molecular clone (15) and have been described elsewhere (21). The Vif A, B, and C mutants were constructed as follows. The *vif* gene was amplified by PCR from the HIV-1 molecular clone pNDK (4, 42), using the *Pfu* proofreading thermostable DNA polymerase (Stratagene) and primers Vif/Nco and Vif/Kpn, corresponding to the 5' and 3' ends of the full-length *vif* gene (see below). Amplification was performed on a Hybaid thermocycler for 30 cycles of 30 s at 94°C, 1 min at 60°C, and 1.5 min at 72°C. The amplified product was cleaved with *NcoI* and *KpnI* and purified by electrophoresis on a 1.5% agarose gel. The DNA was recovered by using Qiaex beads (Qiagen) and ligated to the baculovirus expression vector pGmAc-115T (9) digested with *NcoI* and *KpnI* to generate the pBac/*vif* recombinant baculovirus expression plasmid. DNA sequencing was performed to verify the absence of nucleotide misincorporations during the PCR amplification step.

Generation of mutant A. Mutations were introduced into plasmid pNDK by oligonucleotide-directed mutagenesis. Two PCR amplifications were performed with *Pfu* polymerase, one using Vif/Nco and Mut1 as sense and antisense primers and the second using Mut2 and Vif/Kpn as sense and antisense primers. PCR products were gel purified as described above and were then coamplified by using primers Vif/Nco and Vif/Kpn. The resulting PCR product was then subcloned into the pGmAc-115T vector as described above. The resulting construct, Bac/*vif*-mutA, was sequenced to confirm the changes in amino acids 76 to 79 (EREW to DINQ).

Generation of mutants B and C. The strategy described above was used to construct mutants B and C by replacing primers Mut1 and Mut2 with Mut3 and Mut4, respectively. The PCR products were subcloned into either pGmAc-115T vector, generating Bac/*vif*-mutB, or into Bac/*vif*-mutA, generating Bac/*vif*-mutC. The resulting constructs contain changes in a domain which spans amino acids 89 to 94 (WRKRRY to FEKRRF).

Introduction of mutants A, B, and C into the full-length HIV-1_{NDK} molecular clone (pNDK/Mut-A, -B, and -C). Plasmids Bac/*vif*-mutA, -B, and -C were cleaved with *NdeI* to generate a 240-bp fragment which was ligated to the 14-kb fragment obtained after digestion of pNDK* by *NdeI*. pNDK* is similar to pNDK except that it lacks the *NdeI* site in the pUC plasmid sequence. The pNDK/*vif*ΔC mutant was constructed as follows. Two mutated overlapping mutant PCR fragments were generated by using primer pairs VifB-Δc1 and Δc2-E2, using the methods described above. The resulting mutant 365-bp fragment was reamplified with primers VifB and E2 before being digested with *BamHI* and replaced in pNDK at the corresponding sites.

Construction of pGEX vectors. (i) Construction of pGST-Gag. Gag fragments were amplified by PCR from the HIV-1 NL4.3 plasmid (1) as described above. Vif fragments were amplified by PCR from plasmid pNDK (42). Sense primers containing an *EcoRI* site and antisense primers containing a *NotI* site were used (see below). Amplification was performed on a Hybaid thermocycler for 30 cycles of 30 s at 94°C, 1 min at 60°C, and 1.5 min at 72°C. The amplified product was cleaved with *NotI* and *EcoRI* and purified by electrophoresis on a 1% agarose gel. The DNA was recovered by using Qiaex beads (Qiagen) and ligated to the pGEX-5X-2 expression vector (Pharmacia) digested with *NotI* and *EcoRI* to generate pGST-Gag, which contains a GST-Gag open reading frame.

(ii) Construction of pGST-Vif. The full-length *vif* gene of pNL4.3 was amplified by PCR using primers Vif/Bam and Vif/as (a generous gift from A. Azad and I. McCreadie, Melbourne, Australia). The DNA was cleaved with *BamHI* and *EcoRI*, and the 700-bp fragment was subcloned into pGEX-2T expression vector (Pharmacia) digested with *BamHI* and *EcoRI*, as described above, to generate pGST-Vif, which contains a GST-Vif fusion open reading frame.

(iii) Introduction of point mutations in pGST-Gag. The stop codon mutations introduced into the Gag open reading frame in the pGmAc baculovirus expression plasmid have been described elsewhere (10). These constructs were subcloned into plasmid pGEX-5X-2 in frame with GST by switching fragments located either between the *EcoRI* site at the 5' end of the *gag* gene and the *SpeI* site corresponding to nucleotide (nt) 1507 in pNL4.3 or between the *SpeI* and *NotI* sites at the 3' end of the *gag* gene. The mutant which contains a stop codon at codon 211 was created by blunting the unique *SpeI* site.

Construction of Pos7-Gag. Pos7 DNA was kindly donated by Bernard Moss (48). Gag sequences were amplified by PCR as described above, using oligonucleotides Gag/Nco and Gag/Xho. The resulting fragment was cleaved by *NcoI* and *XhoI* and ligated to Pos7 DNA digested with the same enzymes.

Oligonucleotides. The oligonucleotides used were as follows: Vif/Bam, 5'CC TGGGGATCCATGGAACAGATGGC3' (nt 5041 to 5056); Vif/as, 5'ATT CTGCTATGTTGACACCC3' (nt 5800 to 5781); Vif/Nco, 5'CCCATGCCATG GAAAACAGATGGCAGGTGATGATTGTG3' (nt 5032 to 5061); Vif/HXB2, 5'CTAGTGTCCATTTCATTGTGTGGC3' (nt 5619 to 5597); Vif/Kpn, 5'GGG GGTACTCTTAAAGTCCCTCTAAAAGCTCTAATG3' (nt 5628 to 5608); VifB, 5'GTAGACCTGGCCTGGCAGCC3' (nt 5323 to 5344); E2, 5'CTGC TATGTGACACCAATCTG3' (nt 5788 to 5765); P17/*EcoRI*, 5'AAAGGA ATTCTATGGGTGCGAGCGCTGGTA3' (nt 790 to 810); P17/*NotI*, 5'A AAAAAAGCGCCGCTAGTAATTTGGCTGACCTGGCTG3' (nt 1185 to 1164); P6/*NotI*, 5'ATCAGCGCCGCTATTGTGACGAGGGGTC3' (nt 2292 to 2275); P24/*EcoRI*, 5'AAAGGAATCTTCCTATAGTCAGAACCTC CAGG3' (nt 1186 to 1207); P24/*NotI*, 5'AAAAAAGCGCCGCTGCTACAAA CTCTGTCTTATGGCCGG3' (nt 1878 to 1856); P7/*EcoRI*, 5'AAAGGAAT TCTTATACAGAAAGCAATTTAGGAACC3' (nt 1921 to 1945); P7/*NotI*,

5'AAAAAAGCGCCGCTTAAATTAGCTGTCTCTCAGTACAATC3' (nt 2073 to 2059); P6/*EcoRI*, 5'CCGCGAATTCTACTTCAGAGCAGACCAGA C3' (nt 2134 to 2151); Gag/*Nco*, 5'CCCATGCCATGGGTGCGAGCGCTCA GG3' (nt 790 to 808); Gag/*Xho*, 5'CCCCCTCGAGCTAGTGTCCATTCA TTGTATGGCC3' (nt 2296 to 2273); Mut1, 5'ATTCTGGTGGATATCTCCT GTATGCAGACCCCAATTTG3' (nt 5271 to 5233); Mut2, 5'ACAGGAGATA TCAACCAGAATCTGGGTCAGGGAGTCTCC3' (nt 5248 to 5290); Mut3, 5'AAATCTCCTTTTTTCGAATTCATGGAGACTCCCTGACCC3' (nt 5313 to 5284); Mut4, 5'ATAGAATTCGAAAAAGGAGATTTAGCACACAAGT AGACCC3' (nt 5290 to 5330); Δc1, 5'CCATCTATCTCATGATAGCTTCTCA AC3' (nt 5553 to 5527); Δc2, 5'GTTAGGAAGCTATCATGAGATAGATGG3' (nt 5527 to 5553); T3/Vif, 5'CGAATTACCCTACTAAAGAAAGAAGGAT GAAAAACAGATGGCAGGTGATG3'; and T3/Gag, 5'CGAATTCACCTCA CTAAGAAAGAAGGCCGCGCCATGGTGGTGCGAGAGCGTCGGTA3'.

Expression and purification of GST fusion proteins. *Escherichia coli* Top10 cells (Invitrogen) transformed with fusion protein expression plasmids were grown at 37°C to an optical density at 590 nm of 0.5 to 0.6. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 30°C. The bacteria were then centrifuged at 5,000 × g for 15 min, and the pellet was frozen at -70°C, thawed in 1/10 volume of phosphate-buffered saline (PBS; 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) with protease inhibitor cocktail (aprotinin [1 μg/ml], leupeptin [1 μg/ml], pepstatin [2 μg/ml], and antipain [1 μg/ml]), and gently mixed for 10 min at 4°C. Bacteria were then lysed by sonication on ice, and the lysate was incubated for 30 min at 4°C in the presence of 1% Triton X-100 with shaking. Insoluble material was pelleted for 20 min at 30,000 × g, and the supernatant was adjusted to 10% glycerol and stored at -70°C. The bacterial lysate was incubated with 200 μl of 50% (vol/vol) glutathione (GSH)-agarose beads (Sigma) added per ml of lysate at 4°C for 30 min. After three washes in 1 M NaCl and two washes in PBS, the GST-protein fusions immobilized on GSH-agarose beads were quantified by electrophoresing an aliquot on a sodium dodecyl sulfate (SDS)-12 or 15% polyacrylamide gel, and the beads were stored at 4°C for further analysis.

In vitro transcription and translation. For in vitro transcription, the *vif* open reading frame was amplified from plasmid pNDK or pCDNA1Vif (21) as described above, using the 5' primer T3Vif, which contains the T3 RNA polymerase recognition site upstream of the Vif ATG initiation codon, and Vif/Kpn or Vif/HXB2 as the 3' primer for amplification of pNDK or Vif-expressing plasmid pCDNA1 (21), respectively. The *gag* open reading frame was amplified from pNL4.3 by PCR using T3Gag and P6/Not as 5' and 3' primers, respectively. The PCR product was then treated with proteinase K (40 μg/ml) for 30 min at 37°C, subjected to phenol extraction and ethanol precipitation, and resuspended in diethyl pyrocarbonate-treated water. DNA template (1 μg) was then subjected to in vitro transcription-translation using the TNT coupled wheat germ extract system (Promega) as recommended by the manufacturer. Proteins were translated in the presence of [³⁵S]methionine (>1,000 Ci/mmol; Amersham), resolved on SDS-12% polyacrylamide gels, and quantitated by autoradiography and phosphorimager analysis.

Preparation of cell lysates for analysis of viral protein interactions. Cells were washed twice in PBS and lysed in TBST buffer (20 μl for 5 × 10⁶ cells) containing 50 mM Tris-HCl (pH 7.6), 0.2% Tween 20 or 0.2% Triton X-100, and different concentrations of NaCl (150 to 400 mM) supplemented with the protease inhibitor cocktail described above. Nucleic acids and insoluble materials were removed by centrifugation at 15,000 × g for 15 min at 4°C.

In vitro protein-protein interactions. Binding reactions were performed for 2 h at 4°C in TBST binding buffer containing 50 mM Tris-HCl (pH 7.6), 0.2% Tween 20 or 0.2% Triton X-100, and different concentrations of NaCl (0 to 1 M) in the presence of bovine serum albumin (200 μg/ml) in a total volume of 300 μl. GSH-agarose beads were mixed with either 20 μl of cytoplasmic extract, 250 μl of total cell extract previously dialyzed against TBST, or 5 μl of in vitro-translated ³⁵S-labeled proteins and then extensively washed in binding buffer. Samples were resuspended in 2× sample buffer (0.2% SDS, 50 mM NaCl, 60 mM Tris [pH 6.8], 0.5 mM EDTA, 700 mM β-mercaptoethanol). Bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting or by autoradiography.

Transfections. Transfections were performed by the Lipofectamine method (24) as recommended by the manufacturer (GIBCO). Briefly, HeLa cells were plated at 3.7 × 10⁶ cells/75-cm² flask and grown overnight in DMEM supplemented with 10% FCS. Cells were washed with OptiMEM medium (GIBCO) and incubated for 6 h with 75 μl of Lipofectamine and 10 μg of plasmid DNA before addition of complete culture medium.

T7-MVA vaccinia virus expression system. HeLa cells (5 × 10⁶) were first infected for 30 min with 3 PFU of recombinant vaccinia virus (T7-MVA, Ankara strain; a kind gift of G. Sutter) (43) per cell to express T7 polymerase and then transfected with 9 μg of Pos7 or Pos7-Gag, using the transfection conditions described above. Cells were harvested 20 h after transfection.

Antibodies. Two anti-p24 monoclonal antibodies, P25-A and P25-B (a kind gift from J. C. Mazié, Hybridolab, Institut Pasteur), were used. P25-B recognizes a p24 epitope which includes residues 209 to 218; P25-A recognizes a different p24 epitope which has been not yet characterized. With these antibodies, Western blot analysis of wild-type HIV-1-infected cell extracts allows the detection of p24 and of Pr55^{Gag} precursor; the p40 intermediate Gag cleavage product is also recognized to a lesser extent. The anti-P7 antibody, a generous gift from Valérie

Tanchou (INSERM, Lyon, France), recognizes a conformational NC p7 epitope, which is also present in the Pr55^{Gag} precursor (44). A previously described (19) rabbit anti-Vif polyclonal serum was used in all Western blot analyses.

Western blot analysis. Following SDS-PAGE, proteins were electrotransferred to polyvinylidene difluoride membranes (Amersham). Blots were incubated with either rabbit anti-Vif antibodies diluted 1:2,000 or mouse anti-Gag antibodies diluted 1:10,000, followed by horseradish peroxidase-linked donkey anti-rabbit or anti-mouse immunoglobulin (Amersham), respectively, using the conditions recommended by the manufacturer. Antibody binding was detected by ECL Western blotting detection reagents (Amersham).

Coimmunoprecipitation assay. Cells were lysed at 4°C in a buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.2% Triton X-100, and the protease inhibitor cocktail described above for 15 min with shaking. After centrifugation at 15,000 × *g* for 15 min, the supernatant was removed and precleared on protein G-Sepharose coupled with normal mouse serum at 4°C for 2 h. The supernatant was incubated overnight at 4°C with 1 μg of antibody in a volume of 500 μl. Protein G-Sepharose beads (Pharmacia) were added for 2 h and were extensively washed with lysis buffer. Bound proteins were eluted by boiling in 2× sample buffer, resolved by electrophoresis on an SDS-12% polyacrylamide gel, and analyzed by Western blotting.

Infection and virus propagation. Virus stocks were obtained 48 h after transfection of molecular clone pNDK or pHXB2 in HeLa cells or from acutely infected SupT1 cells. Virus was quantified in cell-free supernatants by measuring reverse transcriptase (RT) activity as previously described (34). Cells were infected by incubating 2 × 10⁶ cells in 1 ml of virus supernatant at 37°C with gentle shaking. After centrifugation at 800 × *g* for 5 min, cells were resuspended at 5 × 10⁵/ml in culture medium. Virus replication was assayed twice a week by determining the RT activity in the cell-free supernatant.

RESULTS

GST-Gag binds to in vitro-translated Vif protein, and GST-Vif binds to in vitro-translated Gag. Vif is associated with virus core structures (13). Based on this observation, we hypothesized that a direct interaction may occur between Vif and Gag. To test this possibility, we constructed a vector which encodes a GST-Gag fusion protein in *E. coli*. This protein consists of the full-length Pr55^{Gag} precursor in fusion with GST and has an apparent molecular mass of 80 kDa, as demonstrated by electrophoresis on an SDS-gel and Coomassie blue staining (data not shown). The GST-Gag fusion protein is recognized by Western blotting using specific anti-p24 monoclonal antibodies (data not shown). Radiolabeled in vitro-translated Vif protein was incubated with the GST-Gag fusion protein or with GST alone bound to GSH-Sepharose, washed, eluted, and separated by SDS-PAGE. NaCl concentrations ranging from 0.1 to 1 M were used. Bound radiolabeled Vif protein was detected by autoradiography. As shown in Fig. 1a, ³⁵S-labeled Vif binds to GST-Gag but not to GST. This interaction is not affected by adding NaCl up to 0.4 M during the binding and washing steps, suggesting a high-affinity GST-Gag/Vif interaction. In vitro-translated ³⁵S-labeled luciferase proteins failed to bind to GST-Gag, suggesting that the GST-Gag/Vif interaction is specific. Furthermore, no interaction was observed between in vitro-translated Vif and GST fused with the caprine arthritis-encephalitis virus dUTPase (45) (data not shown).

To determine whether the GST-Gag/Vif interaction was due to a particular conformation of the GST-Gag fusion protein, the converse experiment was performed. A GST-Vif protein was expressed in *E. coli*, and its recognition by a specific Vif polyclonal antibody was confirmed by Western blotting (data not shown). Radiolabeled in vitro-translated Gag protein was incubated with the GST-Vif fusion protein or with GST alone bound to glutathione-Sepharose, washed, eluted, and separated by SDS-PAGE. The results shown in Fig. 1b indicate that GST-Vif binds specifically to the in vitro-translated Gag precursor. No binding was observed between Gag and GST alone (Fig. 1b, lane 2) or between GST-Vif and in vitro-translated ³⁵S-labeled luciferase (data not shown). Together, these results demonstrate a direct interaction between Gag and Vif in vitro in a GST fusion-based assay.

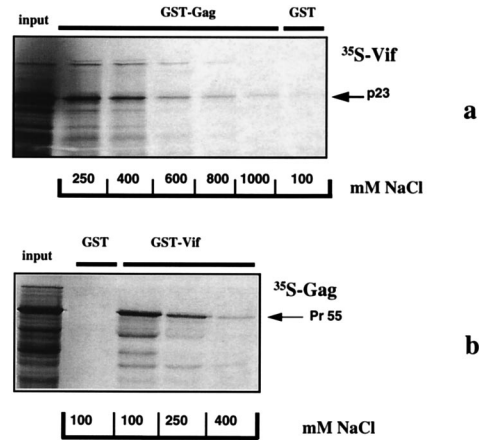


FIG. 1. Binding of Vif with GST-Pr55^{Gag} (a) and binding of Gag with GST-Vif (b). Equivalent amounts of GST or GST-Gag (a) or GST-Vif (b) affinity purified on GSH-agarose beads were incubated with 5 μl of ³⁵S-labeled in vitro-translated Vif (a) or Gag (b) in TBST binding buffer containing different concentrations of NaCl. After three washes performed under the same NaCl conditions, samples were analyzed by SDS-PAGE (12% gel) and autoradiography. Vif and Gag were transcribed and translated in vitro as described in Materials and Methods. The input lane was loaded with one-fifth of the amount of ³⁵S-labeled proteins used in the binding reactions.

Domains of Gag involved in binding to Vif. To map the regions of the Pr55^{Gag} precursor required for its in vitro association with Vif, plasmids encoding the mature processed Gag proteins were constructed. In vitro-translated Vif protein was then assayed for binding to equal amounts of GST-MA, GST-CA, GST-NC, or GST-LI. The results shown in Fig. 2a demonstrate that GST-CA and GST-LI bound Vif inefficiently. However, the GST-NC protein bound efficiently to Vif. To further confirm these results, stop codons were introduced at various positions in the GST-Gag protein (Fig. 2b). As shown in Fig. 2c, no difference in the ability to bind Vif was observed when a stop codon was introduced at amino acid 437 of the Gag precursor, resulting in deletion of the LI p6 domain. In contrast, a striking decrease in binding to Vif was observed when the truncated Gag precursor expressed only the MA and CA domains (stop codon at position 374), providing further evidence for the role of the NC p7 domain of Gag in binding to Vif. GST-MA-CA fusion proteins that contain the junction domain between MA and CA showed a less significant reduction in the capacity to bind to Vif, suggesting that this domain plays a role in the Gag-Vif interaction. The GST-MA fusion protein, which contains a stop codon at amino acid 132, showed a nearly complete loss of the ability to bind to Vif (Fig. 2c, lane GST-p55¹³²). This loss of binding to Vif was greater than that observed with GST-p55¹⁴³, which contains MA and the p24 N terminus (Fig. 2c; compare lanes GST-p55¹³² and GST-p55¹⁴³). Taken together, these results indicate that at least two domains are involved in the Gag-Vif interaction: the NC p7 domain and a region located between MA and the N-terminal region of CA.

The C-terminus domain of Vif is required for the Gag-Vif interaction. The C-terminus domain of Vif has been shown to be important for membrane localization (21). We therefore examined whether this domain is also involved in a specific interaction with Gag during virus assembly. To test this hypothesis, in vitro-translated Vif was expressed from plasmid pNDKVifΔC, in which a stop codon was introduced at amino acid 171, resulting in deletion of the last 22 amino acids (Fig. 3a). Equal amounts of in vitro-translated wild-type and mutant

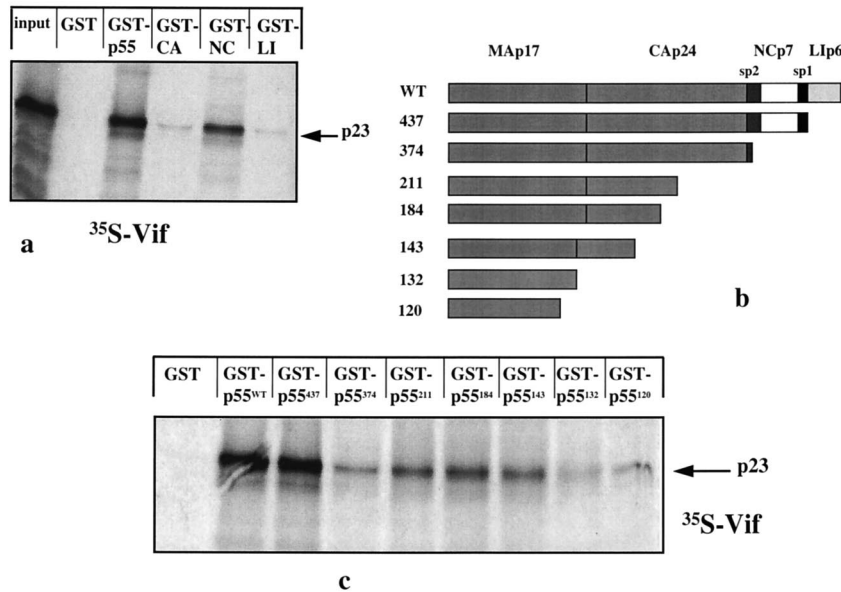


FIG. 2. Mapping of the Gag regions involved in the Gag-Vif interaction. (a) Binding of Vif with Gag proteins fused with GST. (c) Binding of Vif with GST-Gag fusion proteins in which stop codons have been introduced at the positions shown in panel b. Equivalent amounts of GST, GST-Pr55^{Gag}, GST-CA p24, GST-NC p7, GST-LI p6, and truncated GST-Gag affinity purified on GSH-agarose beads were incubated with 5 μ l of ³⁵S-labeled in vitro-translated Vif protein in TBST binding buffer containing 400 mM NaCl. After three washes in 400 mM NaCl, samples were analyzed by SDS-PAGE (12% gel) and autoradiography. Vif was transcribed and translated in vitro as described in Materials and Methods. The input lane was loaded with one-fifth of the amount of ³⁵S-labeled proteins used in the binding reactions. WT, wild type.

(Vif Δ C) Vif proteins were tested for the ability to bind to GST-Gag. As shown in Fig. 3a, full-length Vif bound to GST-Gag but not to GST. In contrast, the binding of GST-Gag to the mutant Vif protein with the C-terminus truncation was significantly reduced. Signal quantification obtained with a phosphorimager showed that the amounts of bound full-length Vif and Δ CVif were 75 and 21%, respectively, of the input level.

We then investigated whether the basic amino acids within the C-terminus domain of Vif are important for the Gag-Vif interaction. To test this hypothesis, we used previously described point mutants in which alanines were substituted for C-terminal basic residues (B1 to B7 mutants) (21). The Vif mutant sequences are shown in Fig. 3b. Vif proteins were translated in vitro from pCDNA1 plasmids expressing wild-type Vif or Vif bearing the B1 to B7 mutations. Equal amounts of ³⁵S-labeled mutant Vif proteins quantitated with a phosphorimager were then compared to the ³⁵S-labeled wild-type Vif for the ability to bind GST-Gag. None of the Vif proteins were able to bind to GST alone (Fig. 3b). All of the B1 to B7 mutants demonstrated a reduced binding to GST-Gag compared to the wild-type Vif protein (Fig. 3b). The most significant reductions were demonstrated for the B4 and B7 proteins, suggesting that the basic residues located between Vif amino acids 157 to 179 of the Vif protein play a critical role in the interaction of Vif with the Gag precursor. Similar results were obtained in three independent experiments, using equal amounts of ³⁵S-labeled Vif proteins as determined by phosphorimager analysis. To control for the specificity of the results obtained with the C-terminus mutants, three additional mutants were constructed in the N terminus of Vif, modifying the residues located at positions 76 to 79 and/or 89 to 94 (Mut-A, Mut-B, and Mut-C [Fig. 3c]). No significant loss of binding to GST-Gag was observed when the wild-type Vif protein was compared to mutant Vif proteins containing N-terminus amino acid substitutions (Fig. 3c). Together, these results demon-

strate that basic residues in the C-terminus domain of Vif are required for binding of Vif to GST-Gag in vitro.

Pr55^{Gag}-Vif interaction in HIV-1-infected cells. We then investigated whether the Gag precursor expressed in infected H9 cells was able to bind to the GST-Vif fusion protein. H9 cells were used, since they represent the most restrictive cell line for the propagation of Vif⁻ viruses (7, 17, 46). GST or GST-Vif immobilized on GSH-agarose beads was first incubated with infected H9 cytoplasmic lysates to allow binding of Gag proteins, extensively washed, eluted from the beads, and then analyzed by Western blotting using a mixture of two specific monoclonal anti-p24 antibodies (P25-A and -B). As shown in Fig. 4a, these antibodies are able to recognize the p24 mature capsid protein, the immature full-length Pr55^{Gag} precursor, and an intermediate Gag cleavage product of 40 kDa (positive control lane). No Gag protein was detected in HIV-1-infected or uninfected cell lysates after binding on GST beads alone. In contrast, incubation of GST-Vif with cell lysates extracted from infected H9 cells specifically retained the Pr55^{Gag} precursor but not the mature CA protein. No Gag protein was detected after incubation of GST-Vif with cytoplasmic lysates from uninfected H9 cells. The results were confirmed by using different NaCl binding conditions. GST-Vif bound specifically to Pr55^{Gag} under high-stringency conditions such as 400 mM NaCl (Fig. 4a). The same blots were then probed with an anti-p17 antibody. MA was not detected (data not shown), indicating that MA in infected cells does not bind to GST-Vif. To eliminate the possibility that the binding between Pr55^{Gag} and Vif resulted from an indirect interaction due to the presence of other viral proteins, we expressed Pr55^{Gag} in the T7-MVA expression system (43, 48). Cytoplasmic lysates were prepared from HeLa cells infected with strain T7-MVA and transfected with the Pos7 expression vector with or without the Gag precursor. As shown in Fig. 4b, GST-Vif beads specifically bound to Pr55^{Gag} precursor, while Pr55^{Gag} was not detected after incubation with GST alone.

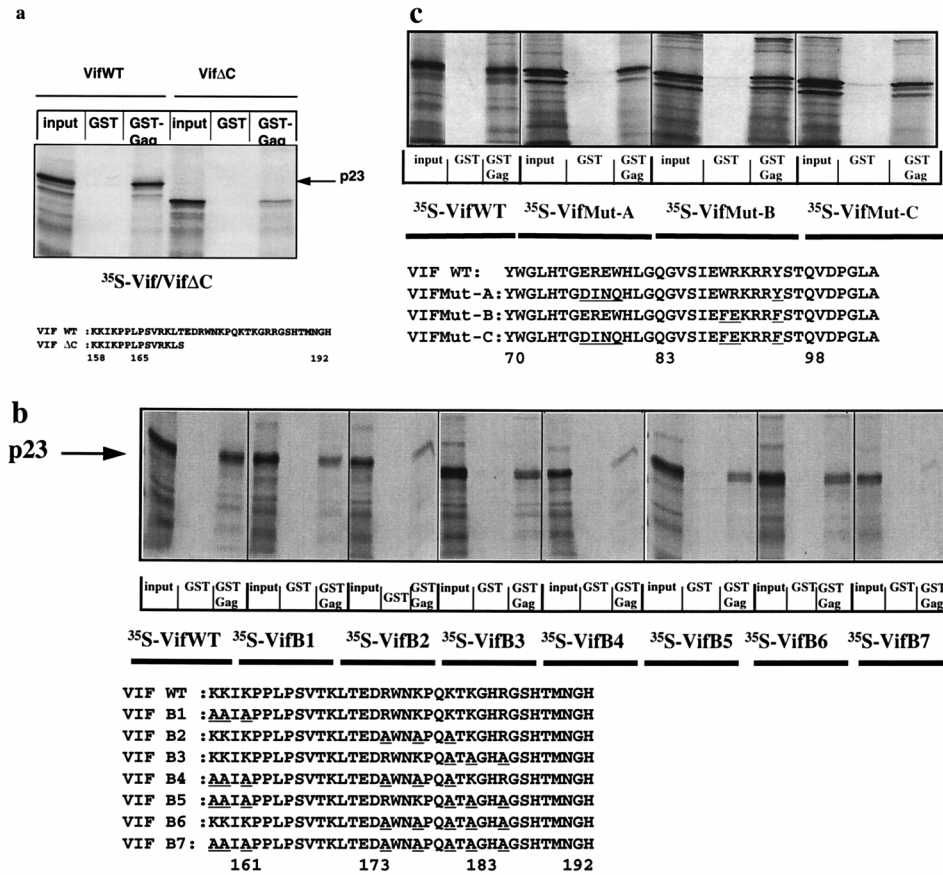


FIG. 3. Binding of Vif proteins with GST-Pr55^{Gag}. Equivalent amounts of GST and GST-Pr55^{Gag} affinity purified on GSH-agarose beads were incubated with 5 μl of ³⁵S-labeled in vitro-translated wild-type (WT) or mutant Vif proteins in TBST binding buffer containing 400 mM NaCl. The mutant Vif sequences are shown in each panel. After three washes in 400 mM NaCl, samples were analyzed by SDS-PAGE (12% gel) and autoradiography. Vif was transcribed and translated as described in Materials and Methods from pNDK and pNDKΔC (a), pCDNA1-Vif and pCDNA1B1-B7 (21) (b), and pNDK, pNDK/Mut-A, pNDK/Mut-B, and pNDK/Mut-C (c). The input lane was loaded with one-fifth of the amount of ³⁵S-labeled proteins used in the binding reactions.

To exclude the possibility that GST-Vif could bind the Pr55^{Gag} precursor in infected cells because of a particular conformation of the GST-Vif fusion protein, the converse experiment was performed. A very faint but reproducible signal corresponding to the Vif protein was recognized by the anti-Vif antibodies in HIV-1-infected H9 cell lysate bound to GST-Gag (data not shown). The weakness of the signal was probably due to the association of Vif with membranes, since Vif bound to GST-Gag beads could be detected only when cell extracts were prepared with detergents such as Triton X-100 and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), which solubilize Vif from the membrane compartment. No binding was observed when cell extracts were prepared by using buffers containing Nonidet P-40 or Tween 20. Together, these results indicate that a specific interaction between the Gag precursor and Vif can be demonstrated in the context of infected cell lysates.

To demonstrate that Vif is associated with the immature Pr55^{Gag} precursor in infected cells, coimmunoprecipitation experiments were performed with infected H9 cells. Cell extracts were prepared from either uninfected or infected H9 cells and subjected to immunoprecipitation with an anti-NC p7 monoclonal antibody which immunoprecipitates the Pr55^{Gag} precursor (44). Proteins present in the immunocomplexes were then resolved by denaturing SDS-PAGE and analyzed by Western blotting using an anti-Vif polyclonal serum. As indicated in Fig. 5, anti-Vif antibodies detected a protein with the molec-

ular mass of Vif in HIV infected cells (lane 4), but not in mock-infected cells (lane 3), when lysates were immunoprecipitated with the monoclonal anti-Gag antibody. This 23-kDa protein was undetectable when the immunoprecipitation was

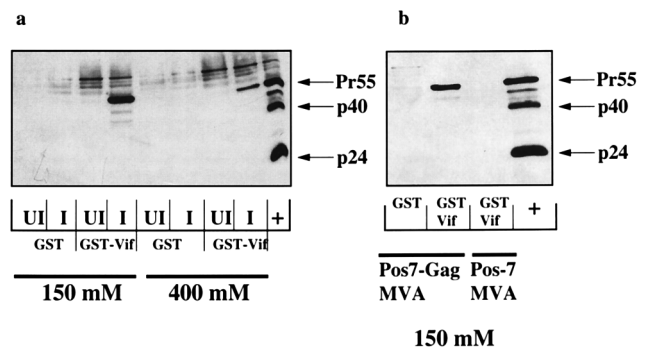


FIG. 4. Binding of GST-Vif to Gag expressed in infected cells (a) or in HeLa cells infected with the T7-MVA recombinant vaccinia virus and transfected with Pos7-Gag (b). Equivalent amounts of GST or GST-Vif affinity purified on GSH-agarose beads were incubated with cytoplasmic lysates from 5×10^6 infected (I) or uninfected (UI) H9 cells (a) in binding buffer containing 50 mM Tris (pH 7.6), 0.2% Tween 20, and 150 or 400 mM NaCl as indicated, or with lysates from 4×10^6 HeLa cells transfected with Pos7 or Pos7-Gag and infected with T7-MVA (b). After three washes in binding buffer, samples were analyzed by Western blotting using monoclonal antibodies P24-A and P24-B each diluted 1/10,000.

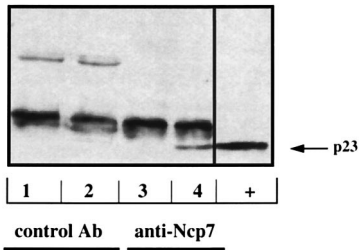


FIG. 5. Coimmunoprecipitation of Vif and Pr55^{Gag} in H9 cells. Cell extracts were prepared from 10⁷ uninfected H9 cells (lanes 1 and 3) or chronically HIV_{NDK} infected H9 cells (lanes 2 and 4) and immunoprecipitated with an anti-Nc p7 monoclonal antibody (lanes 3 and 4) or with a nonspecific control immunoglobulin G2b antibody (Ab) (lanes 1 and 2). Immunoprecipitates were resolved on an SDS-12% polyacrylamide gel and analyzed by Western blotting with a polyclonal rabbit antiserum raised against recombinant Vif protein. The positive control (+) is a cytoplasmic lysate prepared from HIV-1-infected H9 cells.

performed with an irrelevant antibody of the same species and isotype (lanes 1 and 2). These results provide evidence for a specific interaction between Vif and the Gag precursor in HIV-1-infected H9 cells.

The Gag-Vif interaction is important for the biological function of Vif. We then investigated whether Vif mutations which affect its binding to Pr55^{Gag} affect the Vif phenotype. Full-length molecular clones in which C-terminal basic amino acids were substituted with alanines (B1, B2, B3, and B4 [Fig. 3b]) were transfected into HeLa cells, and virus stocks were used to infect the permissive SupT1 and restrictive H9 cell lines. As expected, the results demonstrated (Fig. 6a) that all of these mutants were able to replicate to levels similar to that of the wild type in SupT1 cells, consistent with previous studies (21).

The analysis of cell extracts from infected SupT1 cells by Western blotting demonstrated expression of the mutant Vif proteins at levels similar to that of the wild type, indicating that the mutant Vif proteins are stably expressed (data not shown). In contrast, when H9 cells were used as target cells, no productive infection was seen for the B4 mutant (Fig. 6b), indicating that this mutant has a Vif⁻ phenotype. Notably, this mutant showed the most significant loss of binding to Pr55^{Gag} (Fig. 3b). A significant delay in virus replication in H9 cells was shown for the B1 and B2 mutants, while only a minor delay was observed for the B3 mutant. The B3 mutant showed a less significant loss of binding to Pr55^{Gag} compared to the B1, B2, and B4 mutants (Fig. 3b). Thus, mutations in the Vif C terminus which do not significantly affect binding to Pr55^{Gag} correlate with a Vif⁺ phenotype. The replication of molecular clones containing mutations in the N terminus of Vif (pNDK/Mut-A, and pNDK/Mut-C) was also studied in SupT1 and H9 cells. The replication of these viruses in SupT1 cells was not affected by the mutations (Fig. 6c). Interestingly, the pNDK/Mut-C virus, for which Vif was fully able to bind to Pr55^{Gag} with the same efficiency as the wild-type protein (Fig. 3c), was not able to replicate in H9 cells, consistent with a Vif⁻ phenotype (Fig. 6d). The replication of the pNDK/Mut-A virus was delayed in H9 cells, consistent with an intermediate phenotype. Replication of the pNDK/Mut-B virus was not studied since the corresponding Vif protein was unstable in SupT1 cells. Together, these data suggest that the Gag-Vif interaction is necessary but not sufficient for the Vif⁺ phenotype.

DISCUSSION

In this study, we demonstrate a direct interaction between the HIV-1 Pr55^{Gag} precursor and the Vif protein. This was demonstrated both in vitro, using GST systems and in vitro-

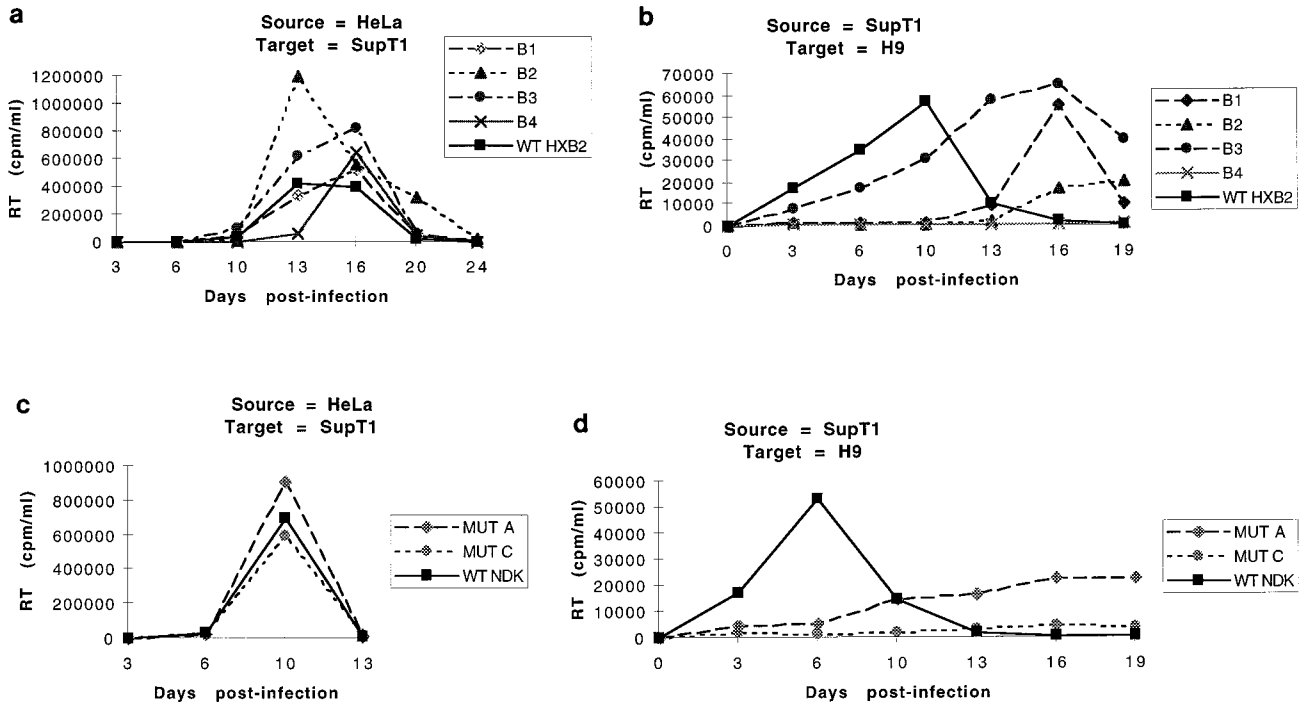


FIG. 6. Replication kinetics of wild-type and mutant HIV-1 derived from full-length molecular clones HXB2 (a and b) and HIV-1_{NDK} (c and d) in SupT1 (a and c) and H9 (b and d) cells. Cells were infected with 30,000 cpm of RT equivalents of virus harvested from the indicated producer cell, and RT activity was determined twice a week in cell-free culture supernatants.

translated proteins, and in vivo in the context of infected cells, as well as in transfected cells coexpressing Gag and Vif. Interactions between Gag and other retroviral proteins, including an interaction between Vpr and MA p17 (36) and between Vpr and NC p7 (28), which are all part of the virus particle, have been previously described. Similarly, an interaction was demonstrated between the TM-gp41 and MA-p17 proteins (11). Using a GST system, we demonstrated that the Gag-Vif interaction is maintained in the presence of 400 mM NaCl, indicating a strong affinity between Pr55^{Gag} and Vif. The strength of this interaction suggests that the Pr55^{Gag}-Vif interaction may have a structural role rather than a regulatory function. Other reports have demonstrated specific interactions between HIV regulatory proteins and cellular targets, using similar GST-based assays. HIV-1 Vpr has been shown to interact with the uracil DNA glycosylase repair enzyme (6) and with the TFIIB transcription factor (2). In addition, Nef has been shown to bind p56^{Lck} (22) and to β -COP (3), using GST systems. For these interactions involving a cellular protein and a virus component, binding was detected in the presence of NaCl concentrations lower than 50 mM and was usually lost when higher concentrations of NaCl were used. Whether a Gag-Vif interaction occurs in other lentivirus systems merits investigation, as does the question of whether a heterologous interaction can occur between Gag and Vif proteins of different lentiviruses. Vif proteins of primate lentiviruses can complement only primate Vif⁻ viruses (40). Whether there is a correlation between heterologous Gag-Vif interactions and the capacity for Vif to transcomplement Vif⁻ mutants remains to be determined.

The C-terminus basic domain of Vif was shown to be important for the interaction of Vif with Gag. Furthermore, basic residues in this region which are essential for Vif function and for Vif localization to membranes (21) were required for the binding of Vif to the Gag precursor. These findings are consistent with a role of Vif during the virus assembly process. Our data are also consistent with a recent report (38) in which Vif and Gag were shown to colocalize at the plasma membrane in infected cells in confocal microscopy studies. Vif might associate with the Gag precursor during viral protein synthesis in infected cells and thereby be directed to cellular membranes through interaction with the myristoylated Gag precursor. The disruption of Gag-Vif complexes might occur due to a subsequent interaction of Vif with cellular membranes, a possibility which would be consistent with the low levels of Vif in virus particles (8). How Vif is released from its interaction with Pr55^{Gag} remains unknown. It is tempting to speculate that Pr55^{Gag} might be assembled into the virus particle, while Vif might tightly interact with a component of the plasma membrane, since the same basic amino acids of Vif that are important for the Gag-Vif interaction are also involved in its membrane localization. Another possibility is that Vif phosphorylation on previously described serine and threonine residues (49), particularly the highly conserved Ser¹⁴⁴, may modulate Vif binding to Gag or a putative interaction with a membrane component, subsequently retaining Vif inside the plasma membrane.

Our study indicates that at least two domains of the Gag precursor are involved in binding to Vif. One domain is located in the region of the MA p17/CA p24 junction. The second domain corresponds to the NC p7 region. Our in vivo results were obtained in infected cells, but not in the context of mature virion components. The possibility that the mature NC p7 or the MA p17/CA p24 junction associates specifically with the low levels of Vif within the virus particle merits further study. Interestingly, some viruses with mutations in the NC p7 domain (32) or in the matrix region (33) exhibit morphological

defects that are similar to those observed for Vif⁻ viruses. Thus, it is tempting to speculate that the Gag-Vif interaction may correctly localize NC p7 on the viral RNA during assembly in order to optimize the reverse transcription step of the next cycle of infection. This possibility would be consistent with defects in initiation (12) or completion of reverse transcription in the target cell (20, 41, 46), or in stabilization of the reverse transcription complex (39), that are observed in Vif⁻-infected cells.

Our data demonstrate that the Vif-Gag interaction is a critical determinant of Vif function, since Vif mutants which have lost the ability to bind to Gag exhibit a Vif⁻ phenotype. These mutations are localized in the C-terminus basic domain of Vif. Other Vif mutants that are altered in the N-terminal domain, such as pNDK/Mut-C or to a lesser extent pNDK/Mut-A, appear to bind normally to Gag but nevertheless behave phenotypically like a Vif⁻ virus. This observation suggests that other mechanisms, in addition to the Gag-Vif interaction, are involved in Vif function. Cellular proteins may play an important role in the mechanism of Vif action, since the Vif phenotype is highly dependent on the producer cell. Vif has been shown to colocalize with vimentin (26), but a physical association between these two molecules has not yet been demonstrated. The Gag precursor has been found to be associated with cyclophilin A (30). However, the functional role of a possible complex between cyclophilin, Gag, and Vif is unlikely since cyclophilin A is expressed in a wide variety of cells which are either permissive or restrictive for the replication of vif mutants. The recent finding that 90% of Vif is associated with cellular membranes (38) suggests that the identification of cellular proteins that interact with Vif is important for understanding the mechanism of Vif action and represents a major challenge for basic research. Further analysis of the virus assembly complex in which the Gag-Vif interaction represents one of the early steps may help in the design of new therapeutic strategies to inhibit HIV replication.

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