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Human Immunodeficiency Virus Type 1 Vif Protein Binds to the Pr55\(^{\text{Gag}}\) 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\(^{\text{Gag}}\) precursor in vitro as well as in infected cells. No interaction is observed between Vif and the mature capsid protein. The Pr55\(^{\text{Gag}}\)-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\(^{\text{Gag}}\) 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\(^{\text{Gag}}\) 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 HIV-1. 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–pheno-type 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\(^{\text{Gag}}\)-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-1NDK 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 src 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 pNL4.3, using primers pNL4.3 for amplification of full-length DNA plasmid sequence (nt 5323 to 5344), and primers VifNeo and Vif/Kpn, corresponding to the 3' 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 NdeI and KpnI and purified by electrophoresis on a 1% agarose gel. The DNA was recovered using Qiagen extraction columns and resuspended in deionized water. Primer sequences are available upon request.

**Generation of mutant A.** Mutations were introduced into plasmid pNDK by oligonucleotide-directed mutagenesis. Two PCR amplifications were performed with Pho polymerase, one using VifNeo 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 performing primers VifNeo and Vif/Kpn. The resulting PCR product was then subcloned into the pCMV-15Ac 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.** A strategy described above was used to construct plasmids pGST-C and pGST-E using primer pairs B1c and B2c for B1, B2 and Mut3 and Mut4, respectively. The PCR products were subcloned into either pGluAc-15T 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 (94-100) in pGRPR.

**Introduction of mutants A, B, and C into the full-length HIV-1 molecular clone (pNDK/Mut-A, B, and C).** Plasmids Bac/vif-mutA, Bac/vif-mutB, and Bac/vif-mutC 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. PKND* is similar to pNDK except that it lacks the Ndel site in the pUC plasmid sequence. The pNDKvifΔ mutant was constructed as follows. Two mutated overlapping mutant PCR fragments were generated by using primer pairs Vifb and Δc2-E2, using primers described above. The resulting mutant 65-bp fragment was reamplified with primers Vifb and Vifb-E2 before being digested with BamHI and replaced in pNDK at the corresponding site.

**Construction of GEX 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 contain 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 using Qiagen extraction columns and resuspended in deionized water.

(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. Meredie, Melbourne, Australia). The DNA was cleaved with BamHI and EcoRI and reamplified using primers described above. The resulting 135-bp fragment was reamplified with primers Vifb and ¥2 before being digested with BamHI and replaced in pNDK at the corresponding site.

(iii) Introduction of point mutations in pGST-Gag. The stop codon mutations introduced into pGST-Gag open reading frame were constructed by replacing primers Mut1 and Mut2 with Mut3 and Mut4, respectively. The PCR products were subcloned into pGluAc-15T 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 (94-100) in pGRPR.

**Expression and purification of GST fusion proteins.** Escherichia coli Top10 strain; a kind gift of G. Sutter) (43) per cell to express T7 polymerase and then 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, 150 mM NaCl, 100 mM Tris-HCl, pH 7.4, 0.1% Tween 20 or 0.2% Triton X-100) supplemented with protease inhibitors (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 removed by centrifugation at 10,000 rpm for 20 min, and the supernatant was adjusted to 10% glycerol and stored at −70°C. The bacterial lysate was incubated with 200 μg 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, the GSH-agarose beads were quantified by electrophoresing an aliquot on a sodium dodecyl sulfate (SDS)-12% 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/HindII as the 3' primer for amplification of pNDK or Vif-expressing plasmid pCDNA1 (21), respectively. The open reading frame was amplified from pNL4.3 by PCR using T3Gag and P6 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 μCi/μmol; Amersham), resolved on 10% SDS–12% polyacrylamide gels, and quantitated by autoradiography and phosphorimager analysis.

**Preparation of cell lysates for analysis of viral protein interactions.** Cells were washed once in PBS and lysed in 1 mg of GST buffer (20 ml for 10 × 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 (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.

**Transfections.** Transfections were performed by the Lipofectamine method (24) as recommended by the manufacturer (GIBCO). Briefly, HeLa cells were plated at 3.7 × 105 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 μl of plasmid DNA before addition of complete culture medium.

**T7-MVA vaccinia virus expression system.** HeLa cells (5 × 105) were first infected for 30 min with 3 μl 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 M. Maziz, Hybridon, Takara Shuzo, Kyoto, Japan), were used. P25-B recognizes a 24-kDa epitope which contains residues 209 to 218; P25-A recognizes a different 24-kDa epitope which has not been yet characterized. With these antibodies, Western blot analysis of wild-type HIV-1-infected cell extracts allows the detection of p24 and its cleavage product, p17, at this stage of infection and assays of cell-free virus production and receptor usage in the [3H]labeled sodium dodecyl sulfate analysis system (34) to be recognized to a lesser extent. The anti-p7 antibody, a generous gift from Valerie
Tanchou (INSERM, Lyon, France), recognizes a conformational NC p7 epitope, which is also present in the Pr55\(^{env}\) 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 \(\times g\) 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 \(\mu\)g of antibody in a volume of 500 \(\mu\)l. 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 infection of molecular clone pNDK or pHXB2 in HeLa cells or from acutely infected SupT1 cells. 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\(^{env}\) precursor in fusion with GST and has a 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, \(^{35}\)S-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 \(^{35}\)S-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 preprotein. No binding was observed between Gag and GST alone (Fig. 1b, lane 2) or between GST-Vif and in vitro-translated \(^{35}\)S-labeled luciferase (data not shown). Together, these results demonstrate a direct interaction between Gag and Vif in vitro in a GST fusion-based assay.

**Domains of Gag involved in binding to Vif.** To map the regions of the Pr55\(^{env}\) precursor required for its in vitro association with Vif, plasmids encoding the mature processed Gag precursors 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-MA 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-p55\(^{132}\)). This loss of binding to Vif was greater than that observed with GST-p55\(^{143}\), which contains MA and the p24 N terminus (Fig. 2c; compare lanes GST-p55\(^{132}\) and GST-p55\(^{143}\)). 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 pNDKVifAC, 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...
(VifΔC) 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 ΔVif 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 demonstrate that basic residues in the C-terminus domain of Vif are required for binding of Vif to GST-Gag in vitro.

**Pr55Gag-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 Pr55Gag 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 Pr55Gag 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 Pr55Gag under high-stringency conditions such as 400 mM NaCl (Fig. 4a). The same blots were then reprobed 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 Pr55Gag and Vif resulted from an indirect interaction due to the presence of other viral proteins, we expressed Pr55Gag 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 Pr55Gag precursor, while Pr55Gag was not detected after incubation with GST alone.
To exclude the possibility that GST-Vif could bind the Pr55Gag 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 Pr55Gag 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 Pr55Gag 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 molecular 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 performed with infected H9 cell lysates. Thus, these results suggest that Vif is associated with the immature Pr55Gag precursor in infected cells.

FIG. 3. Binding of Vif proteins with GST-Pr55Gag. Equivalent amounts of GST and GST-Pr55Gag 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. The input lane was loaded with one-fifth of the amount of 35S-labeled proteins used in the binding reactions.

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 x 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 x 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.

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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\textsuperscript{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\textsuperscript{−} phenotype. Notably, this mutant showed the most significant loss of binding to Pr55\textsuperscript{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\textsuperscript{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\textsuperscript{Gag} correlate with a Vif\textsuperscript{−} 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\textsuperscript{Gag} with the same efficiency as the wild-type protein (Fig. 3c), was not able to replicate in H9 cells, consistent with a Vif\textsuperscript{−} 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\textsuperscript{−} phenotype.

**DISCUSSION**

In this study, we demonstrate a direct interaction between the HIV-1 Pr55\textsuperscript{Gag} precursor and the Vif protein. This was demonstrated both in vitro, using GST systems and in vitro-
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 Pr55Gag and Vif. The strength of this interaction suggests that the Pr55Gag-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 TFIIH transcription factor (2). In addition, Nef has been shown to bind p56Lck (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 other 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 Pr55Gag remains unknown. It is tempting to speculate that Pr55Gag 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 pNDE/Mut-C or to a lesser extent pNDE/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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