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Biological Activity of Human Immunodeficiency Virus Type 1 Vif Requires Membrane Targeting by C-Terminal Basic Domains

JOAO GONCALVES,[1,2] BIN SHI,[1,2] XIAOYU YANG,[1,2] AND DANA GABUZDA[1,3]*

Division of Human Retrovirology, Dana-Farber Cancer Institute,[1] and Departments of Pathology[2] and Neurology,[3] Harvard Medical School, Boston, Massachusetts 02115

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Human immunodeficiency virus type 1 (HIV-1) encodes a Vif protein which is important for virus replication and infectivity. Vif is a cytoplasmic protein which exists in both membrane-associated and soluble forms. The membrane-associated form is an extrinsic membrane protein which is tightly associated with the cytoplasmic side of membranes. We have analyzed the mechanism of membrane targeting of Vif and its role in HIV-1 replication. Mutagenesis studies demonstrate that C-terminal basic domains are required for membrane association. Vif mutations which disrupt membrane association also inhibit HIV-1 replication, indicating that membrane localization of Vif is likely to be required for its biological activity in vivo. Membrane binding of Vif is almost completely abolished by trypsin treatment of membranes. These results demonstrate that membrane localization of Vif requires C-terminal basic domains and interaction with a membrane-associated protein(s). This interaction may serve to direct Vif to a specific cellular site, since immunofluorescence staining and plasma membrane fractionation studies show that Vif is localized predominantly to an internal cytoplasmic compartment rather than to the plasma membrane. The mechanism of membrane targeting of Vif is different in some respects from that of other extrinsic membrane proteins, such as Ras, Src, and MARCKS, which utilize a basic domain together with a lipid modification for membrane targeting. Membrane targeting of Vif is likely to play an important role in HIV-1 replication and thus may be a therapeutic target.

The human immunodeficiency virus type 1 (HIV-1) genome encodes six nonstructural proteins with regulatory functions (Tat, Rev, Nef, Vif, Vpu, and Vpr) in addition to the Gag, Pol, and Env proteins common to all retroviruses. The open reading frame which encodes Vif is conserved in HIV-1 strains and in most of the other lentiviruses, including HIV-2, simian immunodeficiency virus, visna virus, and feline immunodeficiency virus, indicating that its biological function during natural infections is likely to be important (21, 22, 34, 42). Vif is essential for establishing productive HIV-1 infection in peripheral blood T lymphocytes and monocytes/macrophages, the major target cells for HIV-1 infection in vivo (3, 6, 20, 41). Previous studies have shown that Vif significantly enhances the infectivity of HIV-1 virus particles (4, 38). Vif is required at the time of virus production (1, 5, 29, 41), but its biological function and mechanism of action are unknown. The action of Vif is essential for the completion of proviral DNA synthesis after virus entry, most likely indirectly as a result of its effect during virus production (35, 41).

The requirement for Vif is cell type dependent. Peripheral blood mononuclear cells and some cell lines such as H9 require Vif to produce fully infectious virus particles, whereas other cell lines such as COS-1 and SupT1 do not (1, 5, 29, 41). The observation that some but not all cell lines require Vif for the production of infectious virus suggests that Vif may compensate for cellular factors required for the production of infectious particles that are present in some cell lines but absent in others. Alternatively, Vif may neutralize a cell-specific inhibitory factor which interferes with proper HIV-1 particle assembly. However, computer database searches have not revealed any significant homologies with any known cellular proteins (9, 22, 42). A striking feature of the 192-amino-acid Vif sequence is the high content of basic residues and predicted isoelectric point of 10.7 (22). The basic residues are clustered predominantly in the N- and the C-terminal regions.

Vif is present in HIV-1-infected cells and is associated with the mature virus particle only at very low levels (2, 9), suggesting that Vif may act via cellular pathways. The cellular pathways which may be important for Vif function have not been identified. We and others previously demonstrated that Vif is a cytoplasmic protein which can exist in either a membrane-associated form or a soluble form (8, 20). We demonstrated that the membrane-associated form is an extrinsic membrane protein which is tightly associated with the cytoplasmic side of cellular membranes. Residues 172 to 192 were shown to be important for membrane association, indicating that membrane attachment is likely to be mediated by the C terminus.

In this study, we investigated the mechanism of membrane targeting of Vif and its role in Vif function. We demonstrate that membrane localization requires C-terminal basic domains and an interaction with a membrane-associated protein(s). We also show that membrane localization is required for the biological activity of Vif during HIV-1 replication in vivo.

MATERIALS AND METHODS

Plasmids. Plasmid pCDNA*vif* (8) expresses the *vif* gene of the HXB2 clone of HIV-1 (21, 26) under the control of the T7 and cytomegalovirus promoters (Invitrogen). Oligonucleotide-directed mutagenesis (17) was used to generate mutant pCDNA*vif* plasmids which were confirmed by DNA sequencing. Plasmid pHXB2 contains the infectious molecular clone HXB2 derived from HIV-1 IIIB (21, 26). Mutant HXB2 plasmids were made by oligonucleotide-directed mutagenesis of an *Eco*RI-to-*Eco*RI fragment (nucleotides 4648 to 5743) (26). Plasmid pHXBΔ*env*CAT contains an HIV-1 provirus with an in-frame deletion in *env* and a chloramphenicol acetyltransferase gene in place of *nef* (13). Plasmid pHXBΔAvrΔ*env*CAT contains a deletion between two *Avr*II restriction sites (nucleotides 5020 to 5240) in *vif* in pHXBΔ*env*CAT (5, 33). Plasmid pSVIII*env*

* Corresponding author. Mailing address: Division of Human Retrovirology, JF712, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115. Phone: (617) 632-2154. Fax: (617) 632-3113.

expresses HIV-1 *env* and *rev* (13). Plasmid pCDNA*gag* contains a *Sac*I-to-*Bal*I fragment (nucleotides 675 to 2619) (26) of HXB2 under the control of the T7 promoter in pCDNA1 (Invitrogen). Plasmids pSVL*vif* and pSVL*nef* express the HIV-1 Vif and Nef proteins, respectively (8, 44).

Antibodies. Rabbit polyclonal Vif antiserum was raised against Vif expressed in *Escherichia coli* and purified to >98% homogeneity (8). Rabbit polyclonal Nef antiserum was raised against Nef expressed in *E. coli* (44). Rabbit polyclonal HIV-1 p24 serum was obtained from American BioTechnologies, Inc. Rhodamine-conjugated sheep anti-rabbit immunoglobulin (Cappel) and fluorescein-conjugated goat anti-mouse immunoglobulin (Sigma) were used as secondary antibodies for indirect immunofluorescence.

Cell lines and DNA transfections. Infection of the CD4$^+$ T-lymphocyte cell lines SupT1, CEM, and H9 was initiated by DEAE-dextran transfection of 10^7 cells with 10 μg of wild-type or mutant pHXB2 DNA (25). SupT1, CEM, and H9 cultures were maintained in RPMI medium plus 10% fetal calf serum. Reverse transcriptase activity in the culture supernatants was measured as described previously (28). COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. COS-1 cells (2×10^6) transfected by the DEAE-dextran method with 5 μg of wild-type or mutant pHXB2 DNA were used to initiate infection of CEM cells in some experiments (see Fig. 2B) by cocultivation at 36 to 72 h after transfection as described previously (14).

In vitro membrane binding assay. The assay for membrane binding in vitro was performed as described previously (8). In vitro transcription and translation (TnT, T7 Coupled Reticulocyte Lysate System; Promega) of pCDNA*vif* or pCDNA*gag* were performed for 90 min at 30°C in methionine-deficient buffer supplemented with [^{35}S]methionine. The in vitro translation reaction mixture was then incubated with cycloheximide (0.05 μg/μl) for 15 min, and binding to canine pancreatic microsomal membranes (Promega) was carried out for 30 min at 30°C. The sedimentation of microsomes was performed as described previously (23). Briefly, the sample was diluted with 500 μl of physiological salt buffer (50 mM Tris-acetate [pH 7.5], 150 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM dithiothreitol), adjusted to 2 mM tetracaine hydrochloride, and incubated at room temperature for 5 min. The sample was then layered over a 1-ml cushion of 500 mM sucrose in physiological salt buffer, and the microsomes were sedimented in an SW51 rotor at 160,000 × *g* for 15 min at 4°C. The microsome pellets were rinsed gently with physiological salt buffer before being boiled in sample buffer and analyzed by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–PAGE). The supernatant fraction was immunoprecipitated with Vif antiserum before being boiled in sample buffer. The membrane pellet and supernatant fractions were quantitated by gel densitometry of the autoradiographs (Pharmacia LKB).

In vivo membrane association. Subcellular fractionation was performed as described previously (8) except for the following modifications. All steps were performed at 4°C in the presence of protease inhibitors (5 μg of leupeptin per ml, 50 μg of antipain per ml, 10 μg of aprotinin per ml, 5 μg of pepstatin per ml, and 100 μg of phenylmethylsulfonyl fluoride per ml). HIV-1-infected CEM cells were metabolically labeled with 100 μCi of [^{35}S]methionine per ml for 12 h. The cells were then washed, resuspended in hypotonic buffer (10 mM Tris-HCl [pH 7.9] containing 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol), incubated on ice for 10 min, homogenized with 20 strokes of a glass Dounce homogenizer (type B), and centrifuged at 500 × *g* for 5 min to yield a pellet consisting mainly of nuclei. The degree of cell breakage was monitored by phase-contrast microscopy and was optimized to disrupt all cells examined. The postnuclear supernatant fraction was further fractionated into cellular membrane (P100) and supernatant (S100) fractions by centrifugation at 100,000 × *g* for 60 min. Immunoprecipitation of the P100 and S100 fractions was performed after lysis in radioimmunoprecipitation assay (RIPA) buffer (0.15 M NaCl, 0.05 M Tris-HCl [pH 7.2], 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and immunoprecipitation with anti-Vif serum (1:100) (13). The immunoprecipitates were analyzed by SDS–15% PAGE and quantitated by gel densitometry of the autoradiographs.

Replication complementation assay. A transient complementation assay was used to provide a quantitative measure of the ability of wild-type or mutant Vif proteins to complement a single round of HIV-1 replication in *trans* (5, 8, 13). Briefly, CEM cells (10^7) were cotransfected by the DEAE-dextran method (25) with 15 μg of wild-type or mutant pCDNA*vif*, 1 μg of either pHXBΔ*env*CAT or pHXBΔAvrΔ*env*CAT, and 1 μg of pSVIII*env*. The ability of the wild-type or mutant Vif expressor plasmid to complement a single round of infection of the *vif*-negative virus in *trans* was measured by assaying for chloramphenicol acetyltransferase activity in the transfected culture at 9 or 10 days after transfection.

Membrane phospholipid binding. Liposome preparation and binding assays were performed as described previously (12, 45). To prepare phosphatidylcholine (PC) liposomes, lyophilized L-α-PC (9 mg) (Sigma) was resuspended in 2 ml of 0.2 M sucrose in 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.2)–20 mM KCl–2.5 mM magnesium acetate (final concentration, 6 mM PC) and sonicated until transparent. PC-phosphatidylserine (PS) liposomes (2:1 ratio) were prepared similarly after mixing of L-α-PC and L-α-PS (Sigma) in chloroform. Following sonication, the vesicle dispersion was centrifuged for 30 min at 13,000 × *g* to remove large multilamellar liposomes. The upper half of the supernatant, which contained unilamellar liposomes, was used for liposome binding studies. Trace amounts (300,000 cpm) of dipalmitoyl [^{3}H]PC were incorporated to determine the position of the liposomes in sucrose gradients. ^{35}S-labeled Vif or Gag proteins made by in vitro translation (100 μl) were incubated with PC or PC-PS liposomes (50 μl) for 30 min at 30°C and then adjusted to 0.2 M sucrose, 25 mM HEPES-KOH (pH 7.2), 20 mM KCl, and 2.5 mM magnesium acetate in a total volume of 500 μl before being mixed with 1.1 ml of 2.1 M sucrose. This solution (1.5 M sucrose) was overlaid with 1.5 ml of 1.2 M sucrose and 2.1 ml of 0.2 M sucrose. All sucrose solutions were made in the buffer described above. The sucrose gradients were centrifuged in an SW55 rotor at 180,000 × *g* for 12 h at 4°C. Fractions (0.25 ml) were collected from the bottom of the gradient, and the radioactivity in each fraction was determined by scintillation counting.

Immunofluorescence staining. COS cells transfected by the DEAE-dextran method (8) with 5 μg of pSVL*vif* or pSVL*nef* were fixed in phosphate-buffered saline (PBS) with 4% paraformaldehyde for 30 min at room temperature, permeabilized with PBS plus 0.1% Triton X-100 for 5 min, and washed with PBS plus 2% fetal calf serum before staining. The fixed cells were incubated with Vif antiserum (1:200) or Nef antiserum (1:200) for 90 min at 37°C, incubated with rhodamine-conjugated goat anti-rabbit immunoglobulin antibody (1:200) for 20 min at 37°C, washed, and mounted on glass slides.

Plasma membrane isolation. Plasma membrane isolation by colloidal gold adsorption and buoyant density shift was performed as described previously (36). All procedures were performed on ice. Colloidal gold (15-nm-diameter particles) was freshly prepared from $HAuCl_4$ (Sigma) as described previously (36). HIV-1-infected cells (10^8) were resuspended in 5 ml of lysis buffer (100 mM sucrose, 5 mM sodium glycinate, pH 8.5) and added to 150 ml of chilled colloidal gold (0.01% [wt/wt] solution). After the mixture was stirred for 2 min, 1 ml of polyethylene glycol (4 mg of polyethylene glycol 20,000 per ml) was added. After the mixture was stirred for 5 min, the cells were pelleted, resuspended to 5 ml in lysis buffer, and disrupted with a Dounce homogenizer (type B). The lysate was cleared by centrifugation at 1,000 rpm for 1 min (Sorvall 6000B), and the supernatant fraction was mixed with 25 ml of 5 mM sodium glycinate, pH 8.5, in 60% (wt/wt) sucrose. The cleared cell lysate was added to an SW27 tube and underlaid with a 5-ml cushion of 65% sucrose. After overnight centrifugation at 20,000 rpm (Beckman SW27 rotor), the plasma membrane pellet was resuspended in SDS sample buffer, boiled for 2 min, and cleared of gold by centrifugation at 13,000 × *g* for 30 min. Total cell lysate was prepared by lysis of 4 × 10^6 cells in RIPA buffer and boiling in SDS sample buffer for 2 min. The plasma membrane pellet and cell lysate were analyzed by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and analyzed by Western blotting (immunoblotting) using the ECL detection system (Amersham) with rabbit anti-Vif serum (1:2,500 dilution) or rabbit anti-p24 serum (1:2,000 dilution).

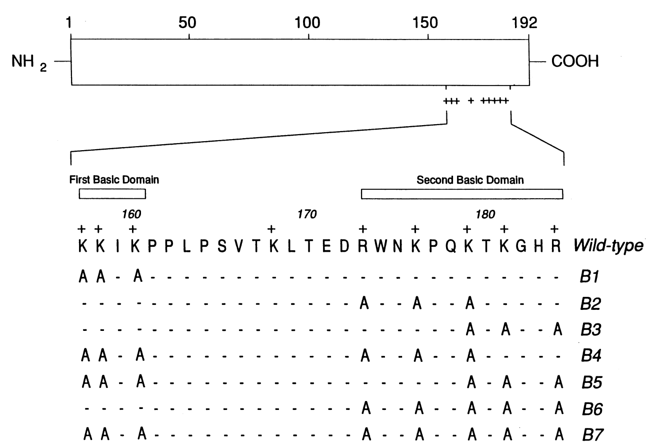

FIG. 1. Sequences of Vif mutants. The amino acid sequence of the wild-type HXB2 Vif C terminus is shown at the top. The positions of alanine substitutions for lysine and arginine residues within the basic domains are indicated for each mutant.

RESULTS

Basic domains in the Vif C terminus are important signals for membrane localization. We previously demonstrated that the C terminus of Vif is important for membrane binding (8). The Vif C terminus contains a high density of basic amino acids which are clustered predominantly at positions 157 to 160 and 173 to 184 (Fig. 1). These two basic domains are highly conserved among different strains of HIV-1, although some strains contain conservative K→R or R→K substitutions (21, 34, 42). Site-directed mutagenesis was used to replace clusters

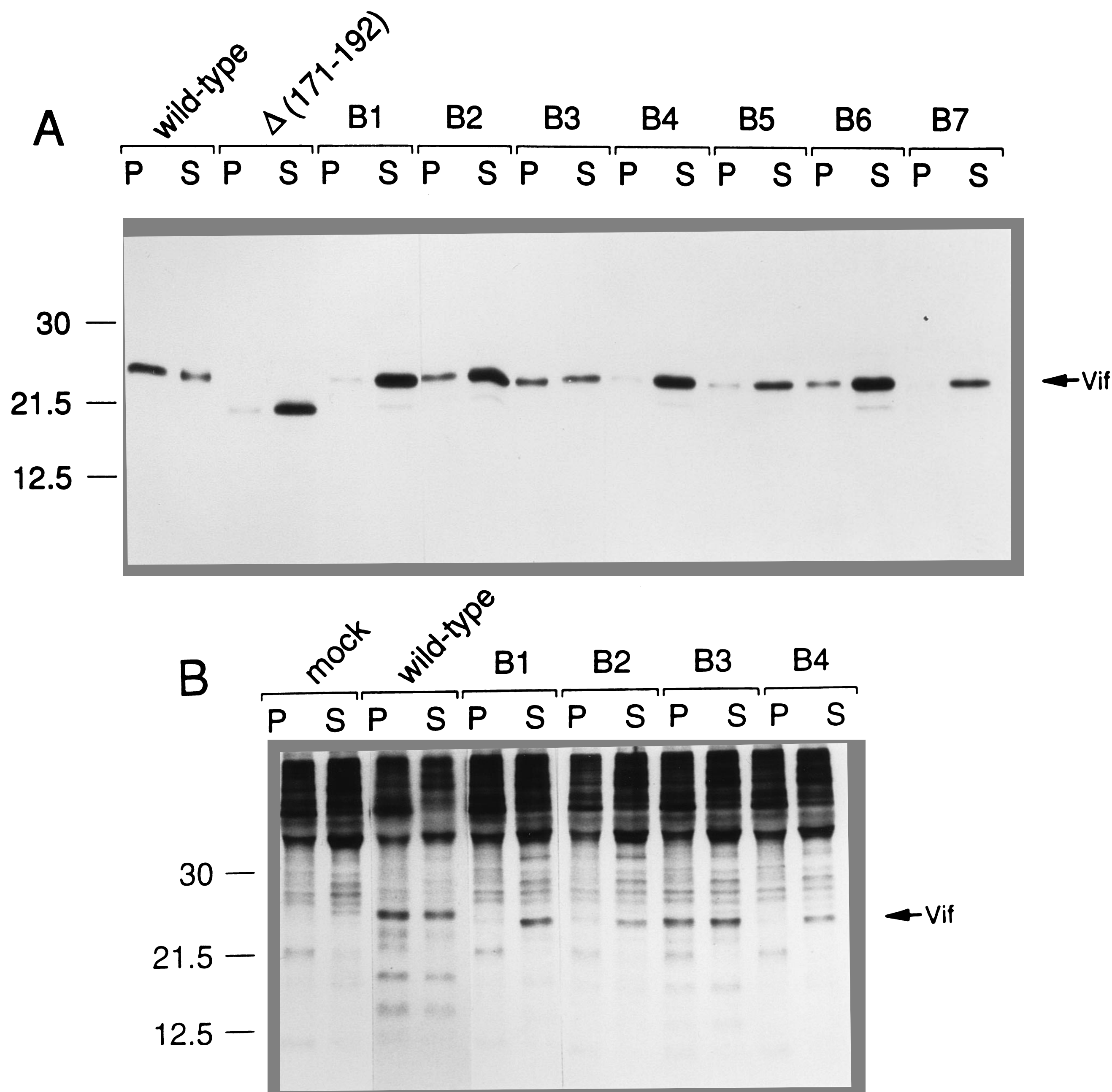

FIG. 2. Membrane binding of wild-type and mutant Vif proteins in vitro and in vivo. (A) Membrane-associated (P) and supernatant (S) fractions after in vitro translation and binding to microsomal membranes. Similar results were obtained in three independent experiments (see Fig. 3). (B) Subcellular fractionation of HIV-1-infected CEM cells. Uninfected CEM cells (mock) or CEM cells infected with wild-type or mutant HIV-1 were metabolically labeled with [^{35}S]methionine and fractionated into P100 (P) and S100 (S) fractions after removal of the nuclear fraction. The fractions were immunoprecipitated with rabbit Vif antiserum and analyzed by SDS-PAGE and autoradiography. Molecular mass markers are indicated on the left in kilodaltons.

of basic amino acids with uncharged alanine residues. A series of such Vif mutants was made (Fig. 1) and used for studies of membrane localization and Vif function.

To investigate the role of C-terminal basic domains in membrane association, wild-type or mutant Vif was translated in vitro and bound to microsomal membranes, and the microsomes were sedimented. The membrane pellet and supernatant fractions were analyzed by SDS-PAGE and quantitated by gel densitometry (Fig. 2A and 3). Sixty-two percent of the wild-type Vif was associated with the membrane pellet, and 28% remained soluble. The remaining 10% was associated with the pellet fraction in the absence of microsomal membranes, most likely because of the formation of insoluble protein aggregates. Deletion of residues 171 to 192 at the C terminus reduced the membrane-bound fraction to 23% ± 2%, consistent with our previous study (8). A larger deletion of residues 156 to 192 caused the formation of insoluble protein aggregates in >50% of the in vitro-translated protein and was not used for further studies.

We then examined the membrane binding of Vif mutants in which clusters of basic residues in the C terminus were replaced with uncharged alanine residues (Fig. 2A and 3). Com-

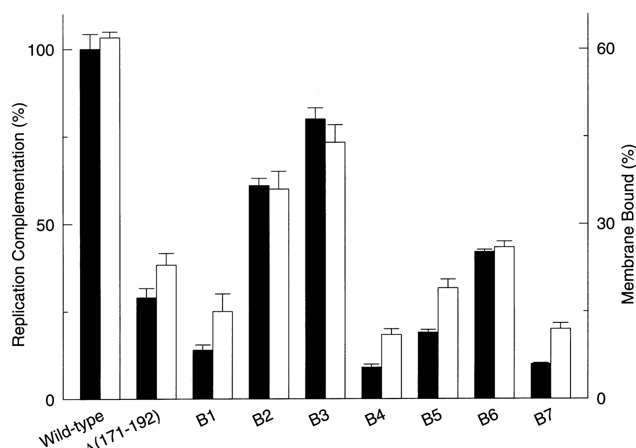

FIG. 3. Correlation between membrane binding and Vif function in a *trans*-complementation assay. Values shown by the black bars represent the percentages of replication complementation relative to the value obtained for the wild type. CEM cells were cotransfected with a wild-type or mutant Vif expressor plasmid, either pHXBΔenvCAT or pHXBΔAvrΔenvCAT, and pSVIIIenv as described in Materials and Methods. Replication complementation for each Vif protein was determined by the level of chloramphenicol acetyltransferase activity in CEM cells cotransfected with pHXBΔAvrΔenvCAT relative to that obtained with pHXBΔenvCAT. Background levels obtained when pSVIIIenv was not cotransfected were 10% ± 2%. Values shown by the white bars represent the percentage of each Vif protein which was membrane associated as determined by in vitro translation and binding to microsomal membranes (Fig. 2A). Results shown are the means ± standard errors of the means for three independent experiments.

plete replacement of eight lysine and arginine residues in the B7 mutant reduced the membrane-bound fraction of Vif to 12% ± 1%. Replacement of the positively charged residues in the first or second basic domains of the B1 and B6 mutants (positions 157 to 160 and 173 to 181) reduced membrane binding to 15% ± 3% and 24% ± 1%, respectively. For the second basic domain, substitutions of the clustered basic residues in the B2 and B3 mutants (positions 173 to 179 and 179 to 184) reduced membrane binding to 36% ± 3% and 44% ± 3%, respectively. These results demonstrate that as the C terminus becomes progressively uncharged, Vif becomes increasingly localized to the soluble fraction. However, the differential effects associated with the B1, B2, and B3 mutations indicate that the positions rather than the total number of basic residues determine the extent of membrane binding.

To determine whether C-terminal basic domains are required for membrane binding of Vif in vivo, similar mutations were introduced into an infectious HIV-1 molecular clone. An infection of CEM cells was established by cocultivation with transfected COS-1 cells to bypass the replication defect which occurs when *vif*-negative viral DNA is directly transfected into this nonpermissive cell line (see Fig. 4). After 36 h of cocultivation, the nonadherent CEM cells were removed and cultured for 18 days. On day 18 after infection, the cultures were metabolically labeled with [^{35}S]methionine for 12 h following normalization for cell number and reverse transcriptase activity. Following subcellular fractionation of the postnuclear supernatant, the amounts of Vif in the membrane (P100) and soluble (S100) fractions were determined by immunoprecipitation of the fractions with rabbit anti-Vif serum and analysis by SDS-PAGE (Fig. 2B). Quantitation of the P100 and S100 fractions by gel densitometry demonstrated that 65% of the wild-type Vif was associated with the membrane fraction in vivo, similar to the results observed in vitro. Similar results were obtained with the NL4-3 HIV-1 molecular clone, which contains functional open reading frames for all of the HIV-1 accessory genes (data not shown). The B1 and B4 HIV-1 mutants showed nearly complete disruption of membrane binding, with 95% of the Vif in the soluble fraction (Fig. 2B). For the B2 and B3 HIV-1 mutants, the membrane-associated fraction of Vif was reduced to 15 and 51%, respectively. These results are similar to those observed in vitro, except that the disruption of membrane binding by the B2 mutation was much greater in the in vivo assay. Expression of the B1, B2, and B4 mutant Vif proteins was reduced to approximately 40% of the wild-type level, an effect which may reflect diminished immunoreactivity with the anti-Vif serum (9). These results indicate that C-terminal basic domains are required for binding of Vif to membranes in HIV-1-infected cells in vivo. As in the case of the in vitro studies, this experiment also demonstrates that different clusters of basic residues are associated with differential effects on membrane association, indicating that the positions of basic residues rather than their total number determine the extent of membrane binding.

Membrane localization is required for Vif function during HIV-1 replication. To provide a quantitative measure of the biological activity of the mutant Vif proteins, a transient complementation assay was used to examine the abilities of the mutant Vif proteins to complement a single round of HIV-1 replication in *trans* (Fig. 3) (5, 13). The expression of wild-type Vif in *trans* in this assay restores the replication of a *vif*-defective virus to the wild-type level (5, 8). In the absence of Vif expressed in *trans*, replication of the *vif*-positive virus was approximately 10-fold greater than that of the *vif*-negative virus. Deletion of residues 171 to 192 at the C terminus reduced replication complementation to 29% ± 3% of the wild-type level. The B1, B4, and B7 mutations, which have in common substitutions in the clustered lysine residues at positions 157 to 160, reduced replication complementation to near background levels. In contrast, the B2, B3, and B6 mutations, which contain substitutions in clusters of basic residues between positions 173 to 184, caused less-significant reductions in replication complementation to 61% ± 2%, 80% ± 3%, and 46% ± 1% of the wild-type level, respectively. The biological activities of the mutant Vif proteins correlated directly with their membrane binding activities in vitro (Fig. 3), indicating that Vif function is likely to require membrane localization.

The experiments described above examined the biological activity of mutant Vif proteins in a transient assay under conditions in which most virus transmission occurs by cell-to-cell spread. To investigate whether membrane localization is required for Vif function during HIV-1 replication in vivo, the B1, B2, B3, and B4 mutations were introduced into the HIV-1 infectious molecular clone HXB2. For these experiments, three cell lines with different requirements for Vif function were used to demonstrate the characteristic cell-type-dependent phenotype of Vif (1, 5, 29, 41). Previous studies have shown that SupT1 cells are completely permissive for the replication of *vif*-defective viruses, whereas H9 cells and some CEM cell lines are nonpermissive (5, 41). The wild-type and *vif* mutant HIV-1 proviral DNAs were transfected directly into SupT1, CEM, and H9 cells. Virus replication was monitored by measuring the reverse transcriptase activity in the culture supernatant. SupT1 cells supported the replication of the wild-type and the *vif* mutant viruses equally well, indicating that the mutant viruses show wild-type replication kinetics in a permissive cell line (Fig. 4A). In CEM cells, the wild-type virus and the B3 mutant virus replicated to similar high levels but the B1, B2, and B4 mutant viruses showed severe defects in replication, with peak levels of virus production at <10% of the wild-type level (Fig. 4B). The most dramatic effects were ob-

served in H9 cells (Fig. 4C). The wild-type virus and the B3 mutant virus replicated to similar high levels. However, no virus replication was detected in H9 cultures transfected with the B1, B2, or B4 HIV-1 mutant virus DNAs. These results confirm the absence or near absence of Vif function in the B1, B2, and B4 mutants during HIV-1 replication in nonpermissive cells. Moreover, the direct correlation between the loss of biological activity and the loss of membrane binding of Vif during HIV-1 replication in infected cells (Fig. 2B) indicates that membrane localization is likely to be required for Vif function in vivo.

Inhibition of binding of Vif to membranes by polyanions. The preceding experiments indicate that charge interactions are likely to be important for the membrane localization of Vif. To further examine this possibility, we examined the effect of polyanions on the binding of Vif to microsomal membranes in vitro. Vif translated in vitro was preincubated with heparin sulfate or dextran sulfate prior to membrane binding (18). Heparin sulfate and dextran sulfate both caused a concentration-dependent inhibition of membrane binding, with 90 and 85% inhibition at 10 μM and 1 μg/ml, respectively (Fig. 5A). These results indicate that electrostatic interactions are likely to be important for binding of Vif to membranes.

Binding of Vif to membranes requires a membrane-associated protein. To investigate whether membrane binding of Vif requires interaction with a membrane-associated protein, the effect of trypsin on membrane binding was examined (24, 31). The HIV-1 $p55^{gag}$ precursor, which does not require protein-protein interaction for membrane association (45), was used as a negative control. Preincubation of microsomal membranes with 1 μg of trypsin per ml followed by inactivation with soybean trypsin inhibitor (STI) reduced membrane binding of Vif to approximately 50% of control levels (Fig. 5B). At 100 μg of trypsin per ml, membrane binding of Vif was almost completely abolished. In contrast, trypsin treatment did not affect membrane binding of HIV-1 $p55^{gag}$ (Fig. 5B). To exclude a nonspecific inhibitory effect of trypsin or STI on membrane binding of Vif, preincubation of the membranes with both trypsin and STI prior to the in vitro binding reaction was included as a control (24, 31). Also, trypsin treatment did not produce an inhibitor of Vif binding, since membrane binding was similar when trypsin-treated membranes were added to mock-trypsin-treated membranes (31). Experiments using microsomal membranes isolated from CEM cells gave similar results (data not shown). These results suggest that an interaction with a membrane-associated protein is likely to be required for the association of Vif with membranes.

Binding of Vif to liposomes. To study the interaction between Vif and membrane phospholipids, we examined its binding to unilamellar liposomes composed of electrically neutral (PC) or neutral plus acidic (PC-PS) phospholipids (Fig. 6). PC liposomes were prepared from L-α-PC and labeled with trace amounts of dipalmitoyl [^{3}H]PC. Vif translated in vitro in the presence of [^{35}S]methionine was incubated with PC liposomes. The liposome and soluble fractions were then separated on sucrose gradients, in which liposomes and free proteins are separated according to their differences in density. The HIV-1 $p55^{gag}$ precursor, which interacts with membrane phospholipids via its N-terminal myristoyl group and an adjacent basic domain (45), was used as a positive control. There was no Vif protein found together with PC liposomes near the top of the gradient (Fig. 6A). In contrast, a significant amount of HIV-1 $p55^{gag}$ was associated with the PC liposome fractions. Vif, therefore, does not show affinity for neutral phospholipids.

To examine the interaction between Vif and negatively charged phospholipids, similar experiments were performed

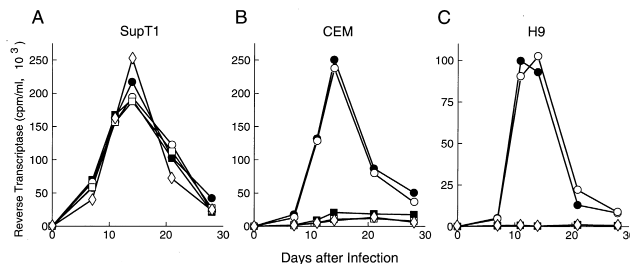

FIG. 4. Effects of *vif* mutations on HIV-1 replication in SupT1, CEM, and H9 cells. HIV-1 infection was initiated by transfection of the cell lines with full-length proviral DNA in wild-type or mutant HXB2 plasmids. Virus replication was monitored by measuring the levels of reverse transcriptase activity in the culture supernatants at each time point. ○, wild type; □, B1; ■, B2; ●, B3; ◇, B4.

with PC-PS liposomes, which contain a mixture of neutral and acidic (2:1 PC/PS ratio) phospholipids (Fig. 6B). Less than 10% of the Vif in vitro translation reaction mixture was found together with the PC-PS liposome fractions. In contrast, a significant amount of HIV-1 $p55^{gag}$ was associated with the PC-PS liposome fractions. The B1, B2, and B3 Vif mutant proteins showed binding to PC-PS liposomes similar to that of the wild type (Fig. 6C), indicating that the removal of three positively charged residues does not significantly reduce the association of Vif with acidic phospholipids. However, the removal of eight positively charged residues in the B7 mutant almost completely abolished the association between Vif and PC-PS liposomes (Fig. 6C). These results indicate that electrostatic interactions between C-terminal basic residues and acidic phospholipids may contribute to the binding of Vif to membranes. However, the relatively low level of Vif associated with PC-PS liposomes raises the possibility that a direct interaction with membrane phospholipids is not sufficient to account for its membrane localization.

Cellular localization of Vif. In a previous study, we examined the cellular localization of Vif by immunofluorescence staining and reported that Vif is predominantly a cytoplasmic protein (8). To determine whether Vif is localized to the plasma membrane, we compared its immunofluorescence staining in transfected COS-1 cells with that of HIV-1 Nef under conditions optimized for plasma membrane staining (Fig. 7A). Nef is a myristoylated protein which is localized to the plasma membrane, cytoplasm, and nucleus (43). As expected, Nef showed intense plasma membrane immunofluorescence staining in addition to cytoplasmic and nuclear staining. In contrast, Vif showed predominantly cytoplasmic staining. Some cells also showed light staining of Vif in the nucleus.

The preceding experiment raises the possibility that the membrane-associated form of Vif targeted predominantly to intracellular membranes rather than to the plasma membrane. To investigate this possibility, we examined the association of Vif with the plasma membrane fraction (Fig. 7B). HIV-1 $p55^{gag}$, which associates with the inner leaflet of the lipid bilayer (45), was used as a positive control for plasma membrane association. The plasma membrane fraction was isolated from HIV-1-infected CEM cells by colloidal gold adsorption and buoyant density shift (36). This method utilizes colloidal gold binding to the cell surface to isolate gold-coated plasma membranes by virtue of their increased buoyant density. The relative amounts of Vif and $p55^{gag}$ in the plasma membrane fraction were determined by Western blotting and quantitation of the autoradiograms by gel densitometry after normalization for the same cell equivalents. Approximately 10% of the Vif was detected in the plasma membrane fraction (Fig. 7B). In

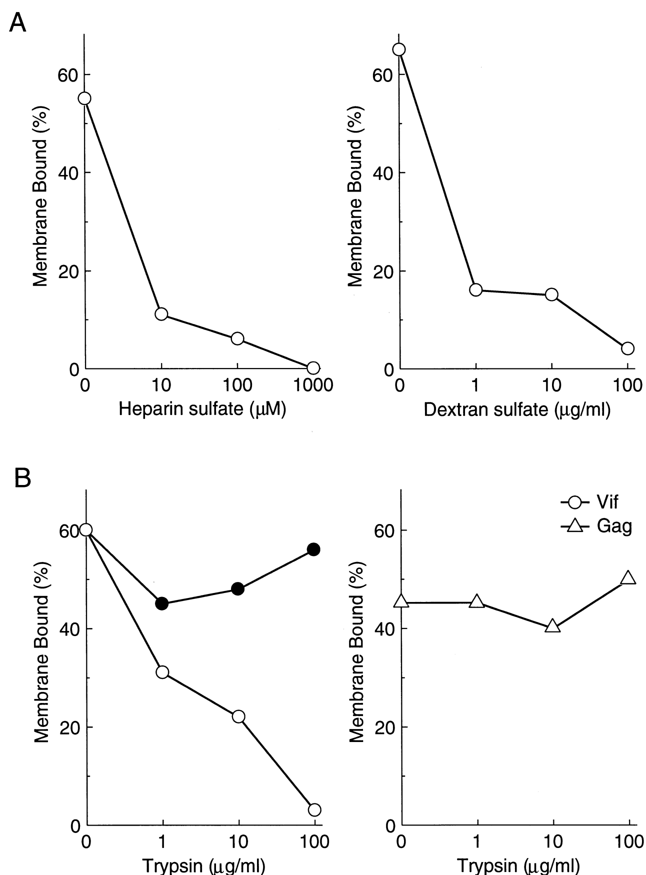

FIG. 5. Effects of heparin sulfate, dextran sulfate, and trypsin on binding of Vif to membranes. (A) Vif translated in vitro was pretreated with heparin sulfate or dextran sulfate for 30 min at 30°C prior to incubation with microsomal membranes for 30 min at 30°C. (B) Microsomal membranes were pretreated with trypsin in the absence (open circles) or presence (dark circles) of STI for 15 min at 37°C. The reaction was then stopped by the addition of 1 mg of STI per ml for 15 min at 37°C prior to incubation of the membranes with in vitro-translated Vif or HIV-1 p55^{gag} (Gag) for 30 min at 30°C. For panels A and B, incubation of Vif or p55^{gag} with microsomal membranes was followed by sedimentation of the microsomes and analysis of the pellet and supernatant fractions by SDS-PAGE. The pellet and supernatant fractions were quantitated by densitometry of the autoradiographs.

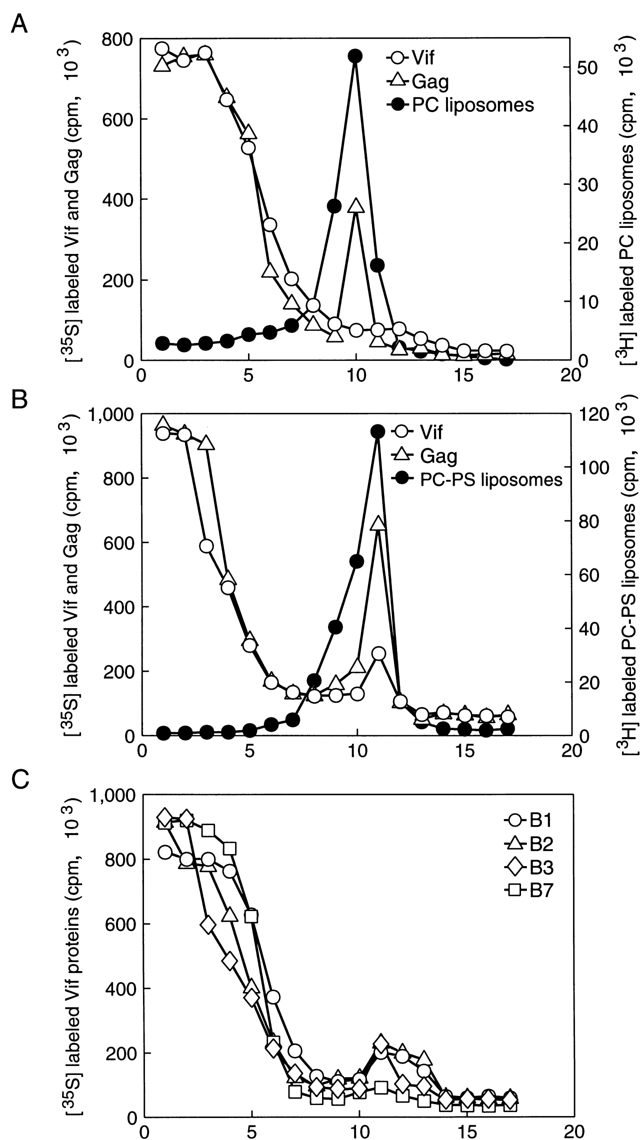

FIG. 6. Separation of liposomes on sucrose gradients after incubation with Vif. Unlabeled PC liposomes containing neutral phospholipid (A) or PC-PS liposomes containing a 2:1 ratio of neutral to acidic phospholipid (B and C) were incubated with in vitro-translated ^{35}S-labeled Vif or HIV-1 p55^{gag} for 30 min at 30°C prior to fractionation on sucrose gradients. The ^{35}S counts per minute represent the total in vitro translation reaction. Separation of PC and PC-PS liposomes labeled by incorporation of trace amounts of dipalmitoyl [^{3}H]phosphatidylcholine is superimposed in panels A and B. Radioactivity in each fraction was quantitated by liquid scintillation counting.

contrast, 45% of the p55^{gag} was associated with the plasma membrane. These results provide evidence that the membrane-associated form of Vif is predominantly localized to an internal cytoplasmic compartment rather than to the plasma membrane.

DISCUSSION

This study demonstrates that membrane targeting of Vif mediated by C-terminal basic domains is required for its biological activity during HIV-1 replication in vivo. The mechanism of membrane targeting of Vif appears to be different in some respects from that used by other extrinsic membrane proteins, including members of the Ras and Src protein families, the HIV-1 Gag precursor, the myristylated alanine-rich protein kinase C substrate (MARCKS), and the G-protein-coupled receptor kinase (GRK) family, which utilize basic domains in the membrane targeting signal. These other extrinsic membrane proteins all contain N- or C-terminal lipid modifications (i.e., myristoylation, palmitoylation, or prenylation) which act in concert with adjacent basic domains to mediate membrane targeting (10, 11, 27, 32, 37, 39, 45). The proposed model for membrane association is that the fatty acids or prenyl groups interact hydrophobically with the lipid bilayer and the basic residues interact electrostatically with acidic phospholipids. Specific protein-protein interactions may also be involved in membrane attachment of at least some of these proteins (16, 37). Like these other proteins, Vif contains a basic domain which is required for membrane targeting. However, Vif does not appear to contain an N- or C-terminal lipid modification, as it lacks a motif for N-terminal myristoylation or a C-terminal lipid modification (i.e., CAAX, CXC, or CC) (19, 30). Further evidence which supports the lack of a lipid modification is the localization of Vif to the aqueous phase

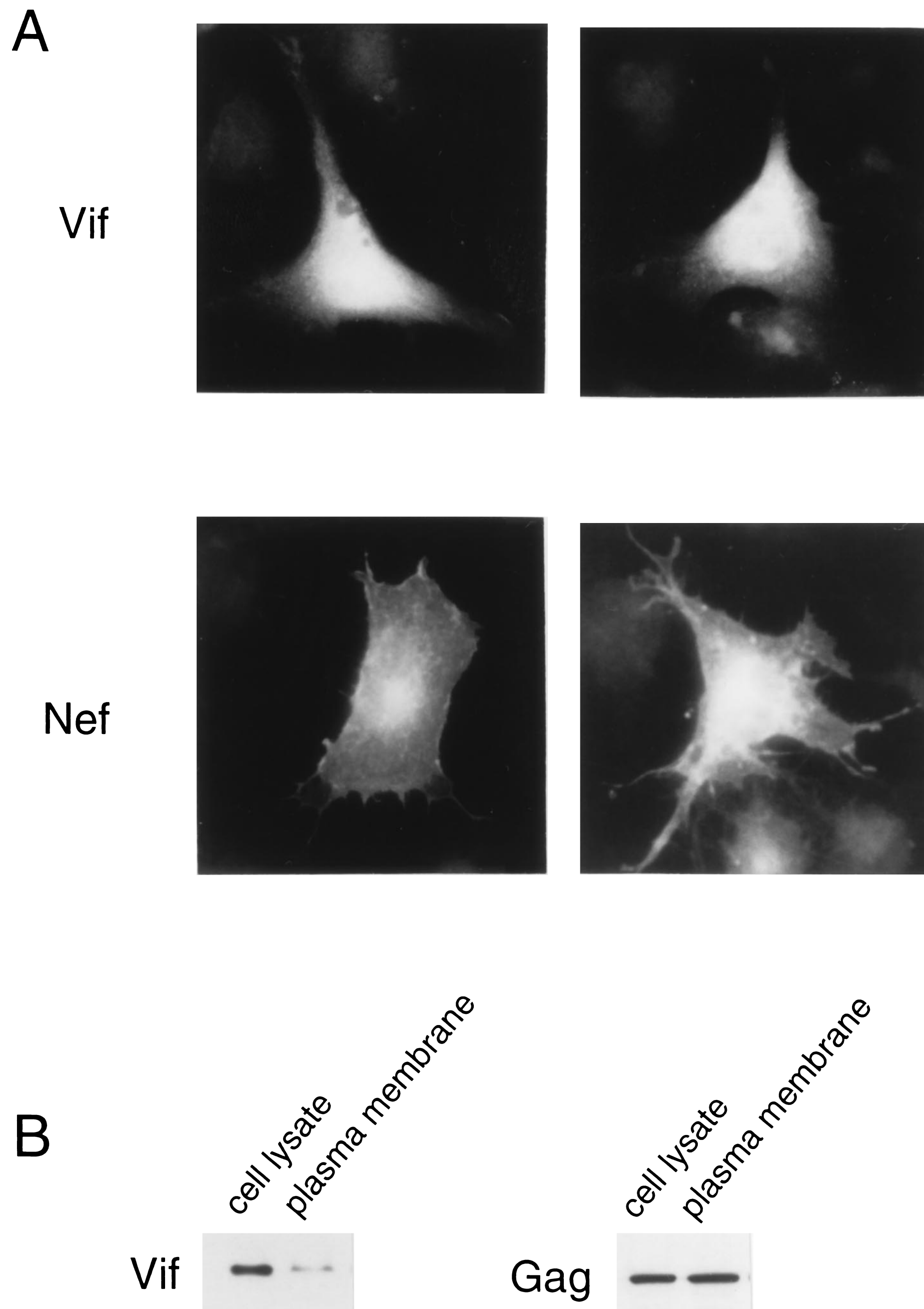

FIG. 7. Cellular localization of Vif. (A) Indirect immunofluorescence staining. Transfected COS cells expressing Vif or the myristoylated HIV-1 Nef protein were stained with anti-Vif or anti-Nef serum as described in Materials and Methods. (Top) Vif staining in the cytoplasm. Some cells also show light nuclear staining. (Bottom) Nef staining in the plasma membrane, cytoplasm, and nucleus. Note the intense plasma membrane staining of Nef, which is not evident for Vif. (B) Analysis of plasma membrane fraction. Shown are Vif and HIV-1 $p55^{gag}$ (Gag) detected in cell lysate or plasma membrane fractions by Western blotting using anti-Vif or anti-p24 serum. Plasma membranes were isolated from HIV-1-infected CEM cells by colloidal gold adsorption and buoyant density shifting (36), boiled in SDS sample buffer, and analyzed by SDS-PAGE. Each lane was loaded with total cell lysate from 10^6 cells or the plasma membrane fraction from 2×10^6 cell equivalents.

following Triton X-114 extraction (8, 11, 40) and its lack of [^{3}H]myristate labeling (7). The low level of binding of Vif to trypsin-treated membranes indicates that a direct interaction with membrane phospholipids is not likely to account for its membrane localization. Rather, membrane association of Vif appears to require an interaction with a membrane-associated protein(s). This interaction is likely to play an important role in the HIV-1 life cycle, since membrane localization of Vif appears to be required for HIV-1 replication in vivo.

The membrane targeting signal of Vif appears to reside in its highly basic C terminus. Progressive reduction of the number of positively charged residues was associated with a reduction in membrane binding. However, differential effects were associated with different clusters of basic residues. These results indicate that a strong basic character is necessary, but the positions of basic residues rather than their total number determines the extent of membrane binding. These findings, together with the dose-dependent inhibition of membrane bind-

ing by polyanions, indicate that membrane binding is likely to involve electrostatic interactions mediated by positively charged side chains of the lysine and arginine residues. Other types of biochemical interactions are also likely to be involved, since high salt concentrations only partially disrupt membrane binding (8). By computer analysis using Chou-Fasman and GOR algorithms, the secondary structure of the C terminus is predicted to be heterogeneous, consisting of random coils and β-turns (9). There are no predicted regions of amphiphilic α-helix or amphiphilic β-sheet, secondary structures which have been shown to promote the interaction of peptides with membranes (15). The hydrophobic region at positions 142 to 154 and the proline-rich motif at positions 161 to 164 do not appear to be important for membrane binding (7, 8). However, these regions are very highly conserved (21, 22, 34, 42) and are required for Vif function in the *trans*-complementation assay, raising the possibility that these adjacent parts of the molecule have important functions which are regulated by membrane association. The HIV-1 molecular clone used in our study is defective for the Vpr, Vpu, and Nef accessory gene functions, and we therefore cannot exclude the possibility that the described mutations would exhibit a different phenotype in the context of a fully competent HIV-1 molecular clone. However, this possibility seems unlikely since the Vif phenotype is independent of the other accessory genes (4–6, 33, 38, 41). Further studies are required to identify other regions of Vif which may be important for membrane association and to localize the entire membrane targeting motif.

Our data suggest that a protein-protein interaction with a membrane-associated protein(s) is likely to be required for localization of Vif to membranes. Evidence which supports this possibility includes the lack of binding to trypsin-treated membranes (12, 31) and the previous demonstration that membrane binding is partially resistant to conditions sufficient to extract most extrinsic membrane proteins, including high salt concentrations and 50 mM EDTA (8). Moreover, the relatively low level of Vif associated with PC-PS liposomes raises the possibility that a direct interaction with membrane phospholipids is not sufficient to account for its membrane localization. Furthermore, the observation that several triple mutations in clusters of C-terminal basic residues significantly disrupted membrane association but did not reduce binding of Vif to acidic liposomes supports the possibility that C-terminal basic domains interact with a membrane-associated protein(s). Identification of the specific host cell protein(s) involved in membrane attachment of Vif and whether Vif interacts with membrane-associated cytoskeletal components are currently under investigation.

Our data are consistent with the possibility that the interaction of Vif with a membrane-associated protein directs it to a specific cellular site. Immunofluorescence staining and plasma membrane fractionation studies indicate that Vif is localized predominantly to an internal cytoplasmic compartment rather than to the plasma membrane. A fraction of Vif may also be associated with the nucleus or nuclear membrane (8). We previously showed that Vif is not associated with the Golgi complex (8) or with clathrin-coated vesicles (7). In addition, the distribution of Vif staining is different from the reticular pattern characteristic of the endoplasmic reticulum. Rather, the punctate pattern of cytoplasmic staining (8) and association of Vif with membranes raise the possibility that Vif is associated with cytoplasmic vesicles or membrane-associated cytoskeletal components. The observation that 30% of the Vif in the P100 fraction of HIV-1-infected CEM cells was resistant to extraction with 1% Nonidet P-40 (7) provides evidence for the latter possibility. Thus, Vif function may be important for a step in the HIV-1 life cycle which occurs in a cytoplasmic compartment, such as the posttranslational modification of one of the Gag or Gag-Pol proteins. The observation that Vif is associated with an effect on the structure of the virion core would be consistent with this possibility (14). Additional studies are required to identify the specific cellular site(s) of Vif localization and to identify the viral targets for its function.

We propose a model for membrane targeting of Vif in which C-terminal basic domains may direct Vif to cellular membranes via electrostatic interactions with acidic phospholipids. Once Vif is recruited to membranes, a protein-protein interaction between Vif are membrane-associated protein(s) may then serve to stably anchor Vif to the membrane surface. Our data are consistent with the possibility that active and inactive forms of Vif are associated with membrane and soluble cytoplasmic compartments, respectively. The explanation for the presence of both soluble and membrane-associated forms and whether membrane association is reversible remain to be determined. Identification of the factors which determine membrane association of Vif may provide new insights into the HIV-1 life cycle and may also lead to the discovery of new potential therapeutic targets.

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