Subcellular Localization of the Vif Protein of Human Immunodeficiency Virus Type 1

JOAO GONCALVES,^{1,2} PRASAD JALLEPALLI,¹ AND DANA H. 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

Received 19 July 1993/Accepted 26 October 1993

The Vif (viral infectivity factor) protein of human immunodeficiency virus type 1 (HIV-1) has been shown to dramatically enhance the infectivity of HIV-1 virus particles during virus production. The subcellular localization of Vif was examined to elucidate cellular pathways which may be important for Vif function. Indirect immunofluorescence staining of Vif demonstrated a diffuse cytoplasmic distribution and showed that most Vif was not associated with the Golgi complex, a proposed site of localization (B. Guy, M. Geist, K. Dott, D. Spehner, M.-P. Kieny, and J.-P. Lecocq, *J. Virol.* 65:1325–1331, 1991). Subcellular fractionation of transfected COS cells and HIV-1-infected Jurkat and CEM cells demonstrated that Vif is a cytoplasmic protein which exists in both a soluble cytosolic form and membrane-associated form. The membrane-associated form of Vif is a peripheral membrane protein which is tightly associated with the cytoplasmic side of cellular membranes. The C terminus of Vif was required for the stable association of Vif with membranes. The C terminus was also essential for Vif function, suggesting that the association of Vif with membranes is likely to be important for its biological activity. The highly conserved regions at residues 103 to 115 and 142 to 150 were important for Vif function but did not affect membrane association, indicating that these regions are likely to be important for other, as-yet-unknown functions.

The Vif (viral infectivity factor) protein is one of several regulatory proteins encoded by human immunodeficiency virus type 1 (HIV-1) which regulate virus replication and infectivity. A *vif* open reading frame is present in the genomes of nearly all lentiviruses, including HIV-2, simian immunodeficiency virus (SIV), visna virus, caprine arthritis-encephalitis virus, and feline immunodeficiency virus (33, 34, 46). Previous studies have shown that Vif dramatically enhances the cell-free infectivity of HIV-1 virions (16, 44). Vif is expressed *in vivo* (3, 27, 31) and is required for efficient replication of HIV-1, HIV-2, and feline immunodeficiency virus in peripheral blood mononuclear cells (2, 6, 14, 18, 46). These observations indicate that Vif is likely to play an essential role during natural infections.

Previous studies have shown that Vif enhances viral infectivity during virus production, most likely by affecting virus assembly or maturation (6, 18, 39). *vif* mRNA is expressed late in the virus life cycle together with the *gag*, *pol*, and *env* mRNAs (19, 41), suggesting that its function may be important during the processing or assembly of virion structural proteins. It has been suggested that Vif may affect the processing, conformation, or virion incorporation of the HIV-1 envelope glycoproteins (22, 39). The 23-kDa Vif protein is present in infected cells but does not appear to be associated with the virus particle (16, 31, 40, 42). The demonstration 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, such as COS-1, SupT1, C8166, and Jurkat, but are absent in others, such as CEM and H9 (6, 18, 39).

To elucidate cellular pathways which may be important for Vif function, the subcellular localization of Vif was examined.

Biochemical characteristics of the association of Vif with cellular membranes and its membrane topology were also examined. These studies also investigate the roles of the N and C termini and highly conserved regions of Vif in membrane targeting.

MATERIALS AND METHODS

Plasmids. The pSVL*vif* plasmid expresses the *vif* gene of the HXB2 clone of HIV-1 (33, 37) under the control of the simian virus 40 late promoter (Pharmacia). The pCDNA*vif* plasmid expresses HXB2 *vif* under the control of the T7 and cytomegalovirus promoters (Invitrogen). The pHXB2 plasmid contains the infectious molecular clone HXB2 derived from the HIV-1 IIIB isolate (33, 37). The *vpr*, *vpu*, and *nef* genes of this strain are defective (11, 33). The pHXB2*vifstop* plasmid, which encodes a *vif*-defective provirus, was made by changing the sequence encoding Vif amino acids 21 and 22 to two in-frame stop codons, which results in a truncated 20-amino-acid Vif protein (18). The pHXBΔ*env*CAT plasmid contains an HIV-1 provirus with an in-frame deletion from the *Bgl*II to *Bgl*II sites (nucleotides 6620 and 7200) (37) in the *env* gene and a chloramphenicol acetyltransferase (CAT) gene replacing the *nef* gene (25, 45). The pHXBΔ*Avr*Δ*env*CAT plasmid was made by removing nucleotides 5020 to 5240 between two *Avr*II restriction sites in the pHXBΔ*env*CAT plasmid. The *Avr*II-to-*Avr*II deletion removes the 3' coding sequences of *vif* and the 5' coding sequences of *vpr*, which overlap *vif* (42), and results in the same phenotype as the point mutations in HXB2*vifstop* (18). The pSVII*env* plasmid expresses the HIV-1 *env* and *rev* genes of the HXB2 strain under the control of the HIV-1 long terminal repeat (24). Oligonucleotide-directed mutagenesis (30) was used to create the Δ(2–8), Δ(8–13), Δ(20–30), Δ(102–115), Δ(142–154), and Δ(171–192) mutant pCDNA*vif* plasmids. The Δ(20–30), Δ(102–115), and Δ(142–154) mutants contain the sequence Gly-Ala-Gly replacing the deleted se-

* Corresponding author. Mailing address: Division of Human Retrovirology, Dana-Farber Cancer Institute, JF711, 44 Binney St., Boston, MA 02115. Fax: (617) 632-3113. Electronic mail address: Dana_H_Gabuzda@HRV.DFCI.Harvard.edu.

quences. The construction of all mutant plasmids was confirmed by DNA sequencing.

Antibodies. Rabbit polyclonal Vif antiserum was raised against Vif expressed in *Escherichia coli* and purified to >98% homogeneity. JE4 is a mouse monoclonal antibody which is specific for the Golgi complex (8). Rhodamine-conjugated sheep anti-rabbit immunoglobulin (Cappel) and fluorescein-conjugated goat anti-mouse immunoglobulin (Sigma) were used as secondary antibodies for indirect immunofluorescence.

Cell culture and transfections. COS-1 cells maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum were transfected by the DEAE-dextran method (10) with 5 μ g of pSVL_{vif} or 10 μ g of pCDNA_{vif}. Infection of the CD4⁺ T-lymphocyte cell line Jurkat was initiated by DEAE-dextran transfection (36) with 10 μ g of pHXB2 or pHXB2_{vifstop}. Infection of the CD4⁺ T-lymphocyte cell line CEM was initiated by DEAE-dextran transfection of COS-1 cells (10) with 5 μ g of the *vif*-positive or *vif*-negative HXB2 DNA followed by cocultivation with CEM cells at 36 to 72 h after transfection. The infected Jurkat and CEM cultures were maintained in RPMI 1640 supplemented with 10% fetal calf serum, with daily medium changes. Reverse transcriptase was measured as previously described (38).

Immunofluorescence staining. COS cells transfected with pSVL_{vif} were fixed in methanol at -20°C and washed with phosphate-buffered saline (PBS) plus 2% fetal calf serum before being stained. The fixed cells were incubated with Vif antiserum (1:200) for 90 min at 37°C, washed with PBS plus 2% fetal calf serum, incubated for 20 min at 37°C with rhodamine-conjugated sheep anti-rabbit immunoglobulin antibody (1:200), washed, and mounted on glass slides. For double staining, the fixed cells were incubated with the Vif antiserum (1:200) and JE4 monoclonal antibody (1:50) simultaneously for 90 min, and an additional incubation with fluorescein-conjugated goat anti-mouse immunoglobulin G (1:100) was performed.

Radioimmunoprecipitation. Transfected COS cells or infected Jurkat and CEM cells were metabolically labeled with 100 μ Ci of [³⁵S]methionine per ml for 12 h prior to lysis in RIPA lysis buffer (0.15 M NaCl, 0.05 M Tris-HCl [pH 7.2], 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) at 4°C, and immunoprecipitation with the Vif antiserum (1:100) was performed as previously described (25).

Subcellular fractionation. Subcellular fractionation was performed as described previously (24, 28, 29, 51) except for the following modifications. All steps were performed at 4°C in the presence of protease inhibitors (5 μ g of leupeptin, 50 μ g of antipain, 10 μ g of aprotinin, 5 μ g of pepstatin, and 100 μ g of phenylmethylsulfonyl fluoride per ml). Transfected COS cells and infected Jurkat and CEM cells were labeled with [³⁵S]methionine for 12 h. The cells were then washed, resuspended in hypotonic buffer (10 mM Tris-HCl [pH 7.9] containing 1.5 mM MgCl₂, 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 $\times$ g for 5 min to yield a pellet (P1) consisting mainly of nuclei. The degree of cell breakage was monitored by phase-contrast microscopy and optimized to disrupt all cells examined. The 500 $\times$ g supernatant fraction was further fractionated into cellular membrane (P50) and supernatant (S50) fractions by centrifugation at 50,000 $\times$ g for 20 min. The S50 supernatant fraction was centrifuged again for 60 min at 200,000 $\times$ g to pellet a heterogeneous population of microsomal vesicles and polyosomes (P200). The nuclear fraction was extracted as described previously (12) and clarified by centrifugation at 13,000 $\times$ g for 5 min to produce the nuclear membrane fraction. The assay for

5' nucleotidase activity was performed with aliquots of each fraction (1/10) as described previously (5), except that the reaction buffer contained 20 mM β -glycerophosphate and 1 mM adenosine (17).

In vitro transcription and translation. In vitro transcription and translation of Vif were performed by using the pCDNA_{vif} plasmid according to the manufacturer's instructions (TnT, T7 Coupled Reticulocyte Lysate System; Promega). The reaction was carried out at 30°C for 90 min in methionine-deficient buffer supplemented with [³⁵S]methionine in the absence or presence of canine microsomal membranes (Promega).

Sedimentation of microsomes. Following in vitro transcription and translation, the sedimentation of microsomes was performed as described previously (35). The sample was diluted with 500 μ l of physiological salt buffer (50 mM Tris-acetate [pH 7.5], 150 mM potassium acetate [KOAc], 2.5 mM MgOAc 1 mM dithiothreitol), adjusted to 2 mM tetra-caine 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 sedimentation of the microsomes was carried out in an SW51 rotor at 160,000 $\times$ g for 15 min at 4°C. The microsome pellets were rinsed gently once with physiological salt buffer before being boiled in sample buffer. The supernatant fraction was immunoprecipitated with Vif antiserum before being boiled in sample buffer.

Replication complementation assay. The transient complementation assay used to measure the ability of wild-type or mutant Vif proteins to complement a single round of HIV-1 replication *in trans* has been previously described (25). Briefly, CEM cells (10⁷) were cotransfected by the DEAE-dextran method (36) with 15 μ g of wild-type or mutant pCDNA_{vif}, 1 μ g of either pHXB Δ envCAT or pHXB Δ Avr Δ envCAT, and 1 μ g of pSVII_{env}. The ability of the wild-type or mutant Vif expresser plasmid to complement a single round of infection of the *vif*-negative virus *in trans* was measured by assaying for CAT activity in the transfected culture 9 or 10 days after transfection (18).

RESULTS

Localization of Vif by immunofluorescence. A high level of expression of HIV-1 Vif was achieved by using a transient expression system in COS cells. COS cells transfected with the Vif expresser plasmid pSVL_{vif} were metabolically labeled with [³⁵S]methionine and analyzed for Vif expression by immunoprecipitation and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoprecipitation of transfected COS cells with rabbit polyclonal Vif antiserum demonstrated the 23-kDa Vif protein (Fig. 1A), which comigrated with Vif expressed in HIV-infected Jurkat cells (Fig. 1B).

The subcellular distribution of Vif was examined in transfected COS cells by indirect immunofluorescence staining. Staining of Vif in transfected COS cells demonstrated an intense pattern of staining which was distributed throughout the cytoplasm (Fig. 2A and B). In most of the positively stained cells, a punctate pattern of cytoplasmic staining was observed at the cell periphery. Approximately 30% of cells expressing Vif also exhibited light staining of the nucleus. No staining was observed in COS cells transfected with the pSVL control plasmid, when the primary antibody was omitted, or when the cells were stained with preimmune serum (data not shown). Similar results were obtained with COS cells transfected with pCDNA_{vif}, although the staining was less intense because of lower levels of expression. The expression of Vif could not be detected in HIV-infected Jurkat cells by indirect immunoflu-

FIG. 1. Expression of Vif in transfected COS cells and infected Jurkat cells. Cells were metabolically labeled with [35 S]methionine for 12 h prior to immunoprecipitation with rabbit Vif antiserum and analysis by SDS-15% PAGE. (A) COS cells transfected with 5 μ g of the pSVL control plasmid (lane 1) or pSVLvif (lane 2). (B) Uninfected Jurkat cells (lane 1) or Jurkat cells infected with vif-positive HXB2 (lane 2). Numbers on the left are molecular masses in kilodaltons.

orescence staining, most likely because of the low level of Vif expressed in HIV-infected cells (19).

A previous report suggested that Vif may be associated with the Golgi complex (22). Double staining of transfected COS cells by using Vif antiserum and a Golgi marker showed that most Vif was not associated with the Golgi complex (Fig. 2C and D). Together with the results of the preceding experiment, this result suggests that Vif is predominantly localized in the cytoplasm. The appearance of a punctate pattern of staining at the cell periphery also raises the possibility that some Vif in the cytoplasm may be associated with vesicles.

Localization of Vif by subcellular fractionation. The subcellular distribution of Vif was examined by subcellular fractionation of transfected COS cells. Subcellular fractionation by the method of Hay (24) yields a fraction which contains most cellular organelles and membranes (plasma membranes, endo-

FIG. 2. Indirect immunofluorescence staining of Vif in transfected COS cells. (A and B) COS cells transfected with pSVLvif and stained with rabbit polyclonal Vif antiserum. (C and D) The same cell double stained with rabbit polyclonal Vif antiserum (C) and the Golgi marker, mouse monoclonal antibody JE4 (D). Most Vif appears to be in the cytoplasm. In some cells, a punctate pattern of staining at the periphery is seen.

plasmic reticulum, Golgi complex, mitochondria, and lysosomes) (P50); a microsome-polysome fraction which contains small vesicles derived from the endoplasmic reticulum and Golgi complex, endocytic vesicles, cytoplasmic vesicles, and polysomes (P200) (13, 28); and a supernatant fraction which contains soluble cytoplasmic components (S200). The enrichment for plasma membranes in each fraction was monitored by using a sensitive radioassay for 5' nucleotidase activity, a marker which is specific for plasma membranes (5, 17, 28) (see Table 1).

COS cells transfected with pSVLvif or the pSVL control plasmid were metabolically labeled with [35 S]methionine and fractionated as described in Materials and Methods. Immunoprecipitation of the subcellular fractions with Vif antiserum and analysis by SDS-PAGE demonstrated the 23-kDa Vif protein in COS cells transfected with the Vif expresser plasmid (Fig. 3A). Quantitation of the relative amount of Vif in each fraction by gel densitometry showed that most Vif was in the soluble cytosol fraction (49%) or P50 membrane fraction (23%) (Table 1). A significant amount of Vif (17%) was associated with the P200 fraction, which contains a heterogeneous population of microsomal vesicles and polysomes (13, 28) in addition to plasma membrane components (Table 1). Only a small fraction (5%) of Vif appeared to be associated with the nuclear membrane or nuclear extract fractions, which may have been due to a small amount of contamination with cellular debris or unbroken cells. Determination of the relative amount of Vif per relative amount of protein in each fraction demonstrated that the P200 fraction showed a sixfold enrichment in the concentration of Vif compared with that of other subcellular fractions (Table 1).

Previous studies have shown that Vif does not affect the infectivity of HIV-1 virus particles produced in COS cells (6, 18). In contrast, the CEM cell line used in our laboratory is nonpermissive for the replication of vif-negative viruses and shows a stringent requirement for Vif for the production of infectious virus particles (18). Jurkat cells show an intermediate phenotype, with partial complementation of the vif-negative phenotype (18). To examine the subcellular localization of Vif in CD4⁺ T-cell lines in which Vif has been shown to affect HIV-1 infectivity (18) and to determine whether the subcellular distribution of Vif is altered by the expression of other HIV-1 proteins, infection of Jurkat and CEM cells was initiated by transfection with vif-positive or vif-negative HXB2 DNA. After peak levels of reverse transcriptase in the culture supernatants were achieved, the cells were metabolically labeled with [35 S]methionine and the localization of Vif was examined by subcellular fractionation as described above. Immunoprecipitation and analysis by SDS-PAGE demonstrated the 23-kDa Vif protein in subcellular fractions of vif-positive but not vif-negative HIV-infected Jurkat and CEM cells (Fig. 3B and C). Quantitation of the relative amount of Vif in each fraction and determination of the relative amount of Vif per relative amount of protein demonstrated that the subcellular localization of Vif in infected Jurkat and CEM cells was essentially similar to that observed in transfected COS cells (Table 1). The percentage of Vif in the P200 microsome-polysome fraction was twofold higher in infected Jurkat and CEM cells than in transfected COS cells. The higher concentration of Vif in the nuclear membrane and nuclear extract fractions observed in both Jurkat and CEM cells may have resulted from either the very low amount of protein present in these fractions or possible contamination with a small amount of unbroken cells or cellular debris. Together with the results of the immunofluorescence staining, these results suggest that Vif is predominantly a cytoplasmic protein which can exist in

FIG. 3. Subcellular distribution of Vif in transfected COS cells and HIV-1-infected Jurkat and CEM cells. Subcellular fractionation was performed as described in Materials and Methods; this was followed by immunoprecipitation of the fractions with Vif antiserum and analysis by SDS-15% PAGE. (A) Subcellular fractionation of Vif in transfected COS cells. Shown are COS cells transfected with pSVL^{vif} (left panel) or with the pSVL control plasmid (right panel). (B and C) Subcellular fractionation of Vif in infected Jurkat (B) and CEM (C) cells. Shown are cells infected with *vif*-positive HXB2 (left panel) or *vif*-negative HXB2^{vifstop} (right panel). Numbers on the left are molecular masses in kilodaltons.

both a soluble cytosolic form and a membrane-associated form. These results also indicate that the subcellular localization of Vif does not appear to be significantly altered by the coexpression of other HIV-1 proteins or CD4.

Vif is tightly associated with membranes. Understanding the nature of the association of a protein with membranes can provide insights into determining a putative function. The biochemical characteristics of the association of Vif with membranes were examined by using a cell-free system. Vif was expressed *in vitro* under the control of the T7 promoter in pCDNA^{vif} by using a coupled transcription-translation system. Analysis of the *in vitro* transcription-translation product by

SDS-PAGE demonstrated the 23-kDa Vif protein, which comigrated with Vif expressed in HIV-1-infected Jurkat cells and could be immunoprecipitated with Vif antiserum (Fig. 4A).

The association of Vif with membranes was examined by *in vitro* transcription-translation in the presence of canine microsomal membranes. Sedimentation of the microsomes after translation and analysis of the pellet and supernatant fractions by SDS-PAGE and gel densitometry demonstrated that 60% of Vif was associated with the pellet fraction when microsomal membranes were present during translation (Fig. 4B). In contrast, when Vif was translated in the absence of membranes, only 10% was present in the pellet fraction, most likely as a result of protein aggregation. Disruption of the microsomal membranes with 2% Triton X-100 reduced the amount of Vif associated with the pellet fraction to the amount which could be pelleted in the absence of membranes (Fig. 4B). These results confirm the results of our earlier experiments which suggested that Vif is a membrane-associated protein and indicate that approximately 50% of Vif translated *in vitro* in the presence of microsomal membranes is membrane associated.

The subcellular fractionation experiments showed that Vif is enriched in the P200 fraction, which contains a heterogeneous population of microsomal vesicles and polysomes (translation complexes of ribosomes). This result and the observation that a small fraction of Vif was associated with the pellet fraction when membranes were not present during *in vitro* translation raised the possibility that Vif could be associated with ribosomes. To examine this possibility, Vif translated *in vitro* in the presence of microsomal membranes was treated with puromycin in the absence or the presence of a high salt concentration (21). Treatment with puromycin alone results in dissociation of the nascent mRNA chain from ribosomes. When the salt concentration is raised to 1 M after treatment with puromycin, the ribosome dissociates into its two subunits (1). To determine whether Vif could be associated with ribosomes either via the ribosome-associated nascent mRNA chain or by a direct interaction with the ribosomal subunits, Vif was translated *in vitro* in the presence of microsomal membranes. The membranes were solubilized with digitonin (3%), the ribosomes were isolated, and treatment with puromycin (1 mM) with or without subsequent 1 M KOAc was performed (21). The amount of Vif in the pellet fraction was unchanged after treatment either with puromycin alone or with both puromycin and 1 M KOAc, indicating that Vif does not appear to be associated with ribosomal subunits or with the ribosome-associated nascent mRNA chain (data not shown).

The association of membrane proteins with cellular membranes can occur either during or after translation. Some proteins require a cotranslational process for membrane association because the formation of a molten globule structural intermediate during or immediately after translation can expose discontinuous hydrophobic regions of the protein which can interact with membranes (48, 50). To determine whether the association of Vif with membranes requires a cotranslational process, microsomal membranes were added either during or after translation (35). When membranes were added after translation, the quantity of Vif in the pellet fraction was similar to the quantity observed when membranes were present during translation (Fig. 4C). These results indicate that the association of Vif with membranes can occur either during or after translation and does not require a cotranslational process.

To examine the biochemical characteristics of the association of Vif with membranes and to determine whether Vif is an

TABLE 1. Subcellular distribution of Vif^a

Subcellular fraction	Vif (%) ^b in:			Protein (%) ^c in:			Vif (%) ^b /protein (%) ^c in:		
	COS-1	Jurkat	CEM	COS-1	Jurkat	CEM	COS-1	Jurkat	CEM
Cytosol	49	33	42	60	54	43	0.8	0.6	1
Membranes	23	24	20	28	38	50	0.8	0.6	0.4
Microsomes and polysomes	17	32	32	3	5	5	6	6	6
Nuclear membranes	5	8	4	5	2	1	1	4	5
Nuclear extract	6	3	2	4	1	1	1	3	5
Total	100	100	100	100	100	100			

^a Subcellular fractionation of COS cells transfected with pSVL_{vif} and of HIV-infected Jurkat and CEM cells was performed as described in Materials and Methods. The P50 membrane and P200 microsome-polysome fractions contained 91 and 9%, respectively, of the total 5' nucleotidase activity, a marker specific for plasma membranes (5, 17).

^b Percentage of the total amount of Vif as determined by gel densitometry of radioimmunoprecipitates (Fig. 3).

^c Percentage of the total amount of protein as determined by a method similar to the Lowry assay (Bio-Rad DC protein assay).

integral membrane protein, the effect of high salt concentration, 50 mM EDTA, or high pH on membrane association was examined. The association of Vif with membranes was partially resistant to extraction with a high salt concentration (1.5 M NaOAc) (Fig. 4D) or 50 mM EDTA (data not shown), conditions sufficient to extract most peripheral cellular membrane proteins (9, 21). However, extraction with 100 mM Na₂CO₃ at pH 11.5 resulted in the release of 80% of Vif from the membrane fraction as assessed by gel densitometry (Fig.

4D), indicating that Vif is not an integral membrane protein (21). Extraction of the pellet fraction with 1% Triton X-114 resulted in the partitioning of Vif into the aqueous phase (Fig. 4D) (7). Together, these results suggest that Vif is tightly associated with membranes but is not a transmembrane or integral membrane protein.

The membrane topology of Vif was examined by using a proteinase K protection assay (35). Proteinase K treatment of Vif translated in vitro in the presence of microsomal membranes demonstrated a complete lack of protection from digestion with proteinase K (Fig. 4E), indicating that Vif is not likely to be translocated across membranes during translation. Instead, this result suggests that Vif associated with membranes is likely to be oriented towards the cytoplasmic side.

Roles of the N terminus, C terminus, and highly conserved regions of Vif in membrane association. Many cellular or viral proteins have been shown to associate with membranes through motifs within the amino or carboxyl terminus (17, 23, 32, 47). To determine whether the N or C terminus of Vif is important for membrane association, site-directed mutagenesis was used to create deletion mutations in these regions (Fig. 5) and the effect on membrane association was examined. The role of regions of Vif which are highly conserved among HIV-1, HIV-2, and SIV_{mac} (Fig. 5) in membrane association was also examined.

The wild-type pCDNA_{vif} plasmid or a mutant plasmid containing a deletion in the N terminus [Δ (2–8) and Δ (8–13)],

FIG. 4. Transcription-translation of Vif in vitro and association with microsomal membranes. Shown in panels B through D are the membrane-associated (P) and supernatant (S) fractions after the sedimentation of microsomes. (A) Transcription-translation of Vif in vitro (lane 1) and immunoprecipitation with rabbit polyclonal Vif antiserum (lane 2). (B) Association of Vif with microsomal membranes in vitro and disruption of membrane association by Triton X-100 (2%). (C) Vif translated in vitro with microsomal membranes added during or after translation. (D) Effect of high pH, high salt concentration, or extraction with Triton X-114 on the association of Vif with membranes. The membrane-associated fraction was isolated by sedimentation of microsomes prior to extraction with 0.1 M Na₂CO₃ (pH 11.5) or 1% Triton X-114 as described previously (7, 15, 17). Extraction with 1.5 M NaOAc was performed on the total translation reaction (15). (E) Membrane topology of Vif. Vif translated in vitro in the presence of microsomal membranes prior to extraction with 100 μ g of proteinase K per ml. For each panel, similar results were obtained in at least three independent experiments. Numbers on the left are molecular masses in kilodaltons.

FIG. 5. Deletion mutations in HIV-1 Vif. Shown in black are the regions of Vif (amino acids at positions 21 to 30, 103 to 115, and 142 to 150) which are most highly conserved among HIV-1, HIV-2, and SIV_{mac} (33, 34). The amino acids at positions 144 to 149 in HXB2 (SLQXLA) are highly conserved among 34 of 38 lentiviruses (34).

FIG. 6. Effects of mutations in Vif on membrane association. Shown are the membrane-associated (P) and supernatant (S) fractions after *in vitro* translation of wild-type or mutant pCDNA_{vif} and sedimentation of microsomes. Similar results were obtained in at least three independent experiments. Numbers on the left are molecular masses in kilodaltons.

C terminus [$\Delta(171-192)$], or one of the highly conserved regions [$\Delta(20-30)$, $\Delta(102-115)$], or $\Delta(142-154)$] was used for *in vitro* transcription-translation of *vif* in the presence of microsomal membranes. The mutant Vif proteins were expressed *in vitro* at levels similar to that of the wild type when corrected for the number of methionine residues (Fig. 6), except for the $\Delta(2-8)$ and $\Delta(20-30)$ mutants. The $\Delta(2-8)$ and $\Delta(20-30)$ Vif mutants were expressed at only 15 and 50% of the wild-type level, respectively, most likely because of an effect on mRNA or protein stability (Fig. 6 and data not shown). Deletion of residues 171 to 192 at the C terminus resulted in an approximately threefold reduction, from 50 to 15%, in the membrane-associated fraction of Vif as determined by gel densitometry (Fig. 6). Deletion of residues 2 to 8 or 8 to 13 in the hydrophilic or hydrophobic region of the N terminus, respectively, or deletion of residues 20 to 30, 102 to 115, or 142 to 154 did not have a significant effect on membrane association (Fig. 6 and data not shown). The mutant Vif proteins exhibited the expected alteration in the apparent molecular weight, except for the $\Delta(2-8)$ and $\Delta(20-30)$ Vif mutants, which exhibited a lower-than-expected apparent molecular weight, most likely as a result of altered protein conformation. These results indicate that the C terminus of Vif is important for association with membranes.

Effect of mutations in the N terminus, C terminus, and highly conserved regions of Vif during a single round of virus replication. To determine whether regions important for the association of Vif with membranes are also important for function, 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* (18, 25). In a previous study, we have shown that the expression of Vif *in trans* in this assay restores the efficiency of a single round of replication of a *vif*-defective virus to the wild-type level (18). The Vif expresser plasmid is cotransfected with a *vif*-positive or *vif*-negative *env*-defective HXB2 provirus plasmid which expresses CAT and with an HIV-1 envelope expresser plasmid. The HIV-1 virus particles produced in this assay result in only a single round of infection, as the packaged viral genome is defective for Env production. The efficiency of a single round of virus replication is quantitated by measuring the level of CAT enzyme activity in the infected cultures. Although both cell-free and cell-to-cell virus transmission occur, most of the virus transmission measured in the assay probably results from cell-to-cell spread, since the levels of p24 antigen in the transfected culture supernatants are <1 ng/ml.

The ability of the wild-type or mutant Vif expresser plasmid to complement a single round of replication of the *vif*-negative CAT virus *in trans* was examined. The assay was performed

FIG. 7. Effect of mutations in Vif on *trans* complementation during a single round of virus replication. CAT assays were performed with CEM lysates 9 or 10 days after cotransfection of 15 μ g of either the pCDNA1 control plasmid, wild-type pCDNA_{vif} plasmid, or a mutant pCDNA_{vif} plasmid with 1 μ g of pSVIII_{env} and 1 μ g of either pXHBΔ_{env}CAT (open bars) or pXHBΔ_{Avr}Δ_{env}CAT (solid bars). Values shown represent the percentage of replication complementation relative to the value obtained for the wild-type Vif expresser plasmid. The values shown below the transfected Vif expresser plasmid represent the fold enhancement of transmission of the *vif*-positive virus relative to that of the *vif*-negative virus for each mutant Vif protein. When the envelope expresser plasmid is not cotransfected, the values obtained are less than 3% of the level achieved with pSVIII_{env} (18). Similar results were obtained in at least three independent experiments.

with the CD4⁺ T-lymphocyte cell line CEM, since Vif is required for HIV-1 replication in this cell line (6, 18). In the absence of Vif expressed *in trans*, replication of the *vif*-positive CAT virus was approximately sixfold greater than that of the *vif*-negative CAT virus (Fig. 7). Expression of wild-type Vif *in trans* resulted in restoration of the replication of the *vif*-negative CAT virus to the level of the *vif*-positive CAT virus. The $\Delta(102-115)$, $\Delta(142-154)$, and $\Delta(171-192)$ mutations resulted in a significant reduction in the ability of Vif to complement replication of the *vif*-negative CAT virus *in trans* (Fig. 7). In contrast, deletion of residues 2 to 8 or 8 to 13 in the N terminus did not have a significant effect on *trans* complementation by Vif in this assay system. Deletion of residues 20 to 30 caused a slight reduction in Vif *trans* complementation, but this may have resulted from an effect on protein conformation (Fig. 6). These results demonstrate that the C terminus of Vif is likely to play a role in both membrane association and protein function. In addition, the results suggest that highly conserved residues within the $\Delta(102-115)$ and $\Delta(142-154)$ deleted sequences are also likely to be essential for Vif function. The finding that these regions were not important for membrane association suggests that they may be important for other, as-yet-unknown functions of Vif.

DISCUSSION

The Vif protein of HIV-1 enhances virus infectivity during virus production in the host cell (6, 18, 39, 49). Vif is likely to be important for virus assembly or maturation, but its biological function and cellular mechanism of action are unknown. The results of this study demonstrate that Vif is a cytoplasmic protein which exists in both a soluble cytosolic form and a membrane-associated form. The membrane-associated form of

Vif is not a transmembrane or integral membrane protein but instead appears to be a peripheral membrane protein which is tightly associated with the cytoplasmic side of cellular membranes. Mutagenesis studies showed that residues 171 to 192 at the C terminus of Vif are required for stable association with membranes. The demonstration that the C terminus was also important for Vif function in *trans* during a single round of HIV-1 replication suggests that the association of Vif with membranes is likely to play a role in its biological function. The finding that HIV-1 Vif is predominantly localized to the cytoplasm is consistent with a previous study which demonstrated that the Q protein of visna virus, which is analogous to Vif, is located in the cytosol (4). Although the authors of that paper concluded that the Q protein is not associated with cellular membranes, this conclusion was based only on cell surface immunofluorescence staining. Further studies are required to determine whether the Q protein of visna virus or the Vif proteins of other lentiviruses are associated with membranes.

A previous study suggested that HIV-1 Vif is associated with the Golgi complex (21). The results of the present study show that most Vif does not appear to be associated with the Golgi complex. However, the possibility that a small amount may be associated with the Golgi complex cannot be excluded. The concentration of Vif was significantly enriched in the P200 fraction, which has been shown to contain a heterogeneous population of microsomal vesicles and polysomes (13, 28). The possibility that Vif may be associated with cytoplasmic vesicles was suggested by a previous study (22) and would be consistent with the punctate pattern of staining observed in the cytoplasmic periphery (Fig. 2). Whether Vif is associated with cytoplasmic vesicles or other specific subcellular organelles remains to be determined.

Conditions sufficient to extract most peripheral membrane proteins, including a high salt concentration and 50 mM EDTA, did not significantly disrupt the association of Vif with membranes (9, 15, 17). These results indicate that Vif is a tightly bound membrane protein that most likely does not associate with membranes through divalent cation bridge formation (9). The disruption of membrane association by high pH or Triton X-114 indicates that Vif is not an integral membrane protein but instead appears to be a peripheral membrane protein which forms an extremely tight noncovalent interaction either with membrane lipids or with a protein in the lipid bilayer. In vitro studies showed that Vif can associate with membranes either during or after translation, indicating that a cotranslational process is not required. The observation that the subcellular localization of Vif is not altered by the expression of other HIV-1 proteins indicates that the association of Vif with membranes is not dependent on other HIV-1 membrane-associated proteins, such as Gag or Env, and raises the possibility that Vif itself may contain a membrane-targeting domain. Further studies are required to confirm this hypothesis and to determine whether the association of Vif with membranes is mediated by protein-lipid or protein-protein interactions.

Many cellular and viral proteins associate with membranes via their N or C terminus, frequently through posttranslational modifications such as myristoylation or prenylation (23, 32, 47). The C terminus but not the N terminus of Vif appears to be important for association with membranes. Twenty of 22 residues in the C terminus are conserved in 12 North American and African HIV-1 isolates; of note, however, is that this region is not highly conserved among HIV-1, HIV-2, and SIV and is widely divergent among the nonprimate lentiviruses (33, 34). The C terminus does not contain any motifs known to be

important for interaction with membranes. A lipid modification is unlikely, since the C terminus does not contain any known motifs for modification by fatty acids (i.e., CAAX, CXC, or CC) (23, 32). Approximately 80% of the 22 residues at the C terminus are hydrophilic, including about 35% which are positively charged. One possibility is that the high density of positively charged amino acids in the C terminus may be involved at least in part in mediating an interaction with membranes, as in plasma membrane targeting of Ras proteins (23). Presumably, this would result from electrostatic interactions with negatively charged phospholipid phosphates. Further studies are required to determine whether the role of the C terminus in membrane association is a result of membrane targeting or of an effect on secondary structure.

Deletion of the C terminus of Vif did not completely abolish membrane association, suggesting that another region of the protein may also play a role. Computer searches using the BLAST, FIND, PIR, and FASTA programs did not show any regions of significant homology between Vif and any other viral or cellular protein present in the data base (20, 34). Computer alignments show that Vif contains three regions which are highly conserved among HIV-1, HIV-2, and SIV_{mac} (residues 21 to 30, 103 to 115, and 142 to 150 in HXB2) (33, 34). The most hydrophobic region in HIV-1 Vif, residues 142 to 154, contains the region most highly conserved among all lentivirus Vif proteins (SLQXLA), which is conserved among 34 of 38 lentiviruses (Fig. 5) (34). The hydrophobic region near the SLQXLA motif was predicted to be important for membrane association on the basis of the finding that similar motifs were detected in several other proteins which are membrane associated (34). However, our data show that residues 142 to 154, which include the SLQXLA motif, do not appear to be important for membrane association. The highly conserved residues at positions 21 to 30 and 103 to 115 also do not appear to be important for membrane association. Residues 142 to 154 and 102 to 115 were required for complementation of Vif function in *trans*, suggesting that these highly conserved regions are likely to be important for other functions of Vif which remain to be determined.

The association of proteins with membranes frequently plays an important role in their biological function (17, 23, 32, 47). The final steps of HIV-1 virion assembly occur at the inner surface of the plasma membrane. Previous studies have demonstrated that Vif appears to be associated with effects on the HIV-1 envelope glycoproteins (22, 39). The demonstration that Vif is predominantly a cytoplasmic protein which is tightly associated with the cytoplasmic side of membranes raises the possibility that Vif may be important for an effect on the Gag proteins or may play a role during the final stages of virion assembly at the plasma membrane. These possibilities would be consistent with the observations that Vif is associated with an effect on the morphology of the virus core (26) and serves a function which is important for the completion of viral DNA synthesis following virus entry (43, 49). The requirement for Vif during virus replication differs among established cell lines (6, 14, 18, 43, 49). Some cell lines, such as CEM and H9, require Vif during virus production, whereas other cell lines, such as SupT1 and COS-1, do not (6, 14, 18). The explanation for the differing requirements for Vif among established cell lines remains unknown, but the finding that the subcellular distribution of Vif is similar in cell types which differ in their requirement for Vif suggests that it is not likely to result from altered subcellular localization. Understanding the biological role of Vif in virus replication and the nature of its interaction with the host cell may provide insights into new therapeutic

strategies which could be used to reduce HIV-1 infectivity and transmission.

ACKNOWLEDGMENTS

We gratefully acknowledge Jie-Oh Lee and Robert Liddington for providing purified recombinant HXB2 Vif protein and Lan Bo Chen for providing monoclonal antibody JE4.

This work was supported by grants from the National Institutes of Health (AI01017 and AI33837), a Claudia A. Barr Investigator award, the G. Harold and Leila Y. Mathers Charitable Foundation, and the Milton Fund. We also acknowledge grants from the Center for AIDS Research (AI28691) and Center for Cancer Research (AO6516) for supporting necessary core facilities. J.G. is the recipient of a doctoral fellowship by Junta Nacional De Investigação Científica, Portugal.

REFERENCES

- Adelman, M. R., D. D. Sabatini, and G. Blobel. 1973. Ribosome-membrane interaction. Nondestructive disassembly of rat liver rough microsomes into ribosomal and membranous components. *J. Cell. Biol.* **56**:206–229.
- Akari, H., J. Sakuragi, Y. Takebe, K. Tomonaga, M. Kawamura, M. Fukasawa, T. Miura, J. Shinjo, and M. Hayami. 1992. Biological characterization of human immunodeficiency virus type 1 and type 2 mutants in human peripheral blood mononuclear cells. *Arch. Virol.* **123**:157–167.
- Arya, S. K., and R. C. Gallo. 1986. Three novel genes of human T-lymphotropic virus type III: immune reactivity of their products with sera from acquired immune deficiency syndrome patients. *Proc. Natl. Acad. Sci. USA* **83**:2209–2213.
- Audoly, G., N. Sauze, G. Harkiss, C. Vitu, P. Russo, G. Querat, M. Suzan, and R. Vigne. 1992. Identification and subcellular localization of the *Q* gene product of visna virus. *Virology* **189**:734–739.
- Avruch, J., and D. F. H. Wallach. 1971. Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. *Biochim. Biophys. Acta* **233**:334–347.
- Blanc, D., C. Patience, T. F. Schultz, R. Weiss, and B. Spire. 1993. Transcomplementation of Vif[−] HIV-1 mutants in CEM cells suggests that Vif affects late steps of the viral life cycle. *Virology* **193**:186–192.
- Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256**:1604–1607.
- Chen, L. B. Unpublished observations.
- Chong, L. D., and J. K. Rose. 1993. Membrane association of functional vesicular stomatitis virus matrix protein in vivo. *J. Virol.* **67**:407–414.
- Cullen, B. R. 1987. Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol.* **152**:684–703.
- Dedera, D., W. Hu, N. Heyden, and L. Ratner. 1987. Viral protein R of human immunodeficiency virus type 1 and 2 is dispensable for replication and cytopathicity in lymphoid cells. *J. Virol.* **63**:3205–3208.
- Dignam, J. D., R. M. Lebowitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**:1475–1489.
- Evans, W. H. 1992. Isolation and characterization of membranes and cell organelles, p. 233–270. In D. Rickwood (ed.), *Preparative centrifugation—a practical approach*. Oxford Press, Oxford.
- Fan, L., and K. Peden. 1992. Cell-free transmission of Vif mutants of HIV-1. *Virology* **190**:19–29.
- Fath, K. R., and D. R. Burgess. 1993. Golgi-derived vesicles from developing epithelial cells bind actin filaments and possess myosin-I as a cytoplasmically oriented peripheral membrane protein. *J. Cell Biol.* **120**:117–127.
- Fisher, A. G., B. Ensoli, L. Ivanoff, M. Chamberlain, S. Petteway, L. Ratner, R. C. Gallo, and F. Wong-Staal. 1987. The *sor* gene of HIV-1 is required for efficient virus transmission in vitro. *Science* **237**:888–893.
- Frangioni, J. V., P. H. Beahm, V. Shifrin, C. A. Jost, and B. G. Neel. 1992. The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. *Cell* **68**:545–560.
- Gabuzda, D. H., K. Lawrence, E. Langhoff, E. Terwilliger, T. Dorfman, W. Haseltine, and J. Sodroski. 1992. Role of vif in replication of human immunodeficiency virus type 1 in CD4⁺ T lymphocytes. *J. Virol.* **66**:6489–6495.
- Garrett, E. D., L. Tiley, and B. Cullen. 1991. Rev activates expression of the human immunodeficiency virus type 1 *vif* and *vpr* gene products. *J. Virol.* **65**:1653–1657.
- Goncalves, J., P. Jallepalli, and D. Gabuzda. Unpublished observations.
- Gorlich, D., P. Siegfried, E. Hartmann, K.-U. Kalies, and T. A. Rapoport. 1992. A mammalian homolog of SEC61p and SECYp is associated with ribosomes and nascent polypeptides during translocation. *Cell* **71**:489–503.
- Guy, B., M. Geist, K. Dott, D. Spehner, M.-P. Kieny, and J.-P. Lecocq. 1991. A specific inhibitor of cysteine proteases impairs a Vif-dependent modification of human immunodeficiency virus type 1 Env protein. *J. Virol.* **65**:1325–1331.
- Hancock, J. F., K. Cadwallader, H. Paterson, and C. J. Marshall. 1991. A CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins. *EMBO J.* **10**:4033–4039.
- Hay, A. J. 1974. Studies on the formation of the influenza virus envelope. *Virology* **60**:398–418.
- Helseth, E., M. Kowalski, D. Gabuzda, U. Olshevsky, W. Haseltine, and J. Sodroski. 1990. Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. *J. Virol.* **64**:2416–2420.
- Höglund, S., Å. Öhagen, K. Lawrence, and D. Gabuzda. Role of Vif during packing of the core of HIV-1. Submitted for publication.
- Kan, N. C., G. Franchini, F. Wong-Staal, G. DuBois, W. Robey, J. Lautenberger, and T. Papas. 1986. Identification of HTLV-III/LAV *sor* gene product and detection of antibodies in human sera. *Science* **231**:1553–1555.
- Krueger, J. G., E. Garber, and A. R. Goldberg. 1983. Subcellular localization of pp60^{src} in RSV-transformed cells. *Curr. Top. Microbiol. Immunol.* **107**:51–123.
- Krueger, J. G., E. Wang, E. A. Garber, and A. R. Goldberg. 1980. Differences in intracellular location of pp60^{src} in rat and chicken cells transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA* **77**:4142–4146.
- Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367–382.
- Lee, T. H., J. E. Coligan, J. S. Allan, M. F. McLane, J. E. Groopman, and M. Essex. 1986. A new HTLV-III/LAV protein encoded by a gene found in cytopathic retroviruses. *Science* **231**:1546–1549.
- Marshall, C. J. 1993. Protein prenylation: a mediator of protein-protein interactions. *Science* **259**:1865–1866.
- Myers, G., S. F. Josephs, A. B. Rabson, T. I. Smith, and F. Wong-Staal (ed.). 1988. Human retroviruses and AIDS. Los Alamos National Laboratory, Los Alamos, N.Mex.
- Oberste, M. S., and M. A. Gonda. 1992. Conservation of amino-acid sequence motifs in lentivirus Vif proteins. *Virus Genes* **6**:95–112.
- Ooi, C. E., and J. Weiss. 1992. Bidirectional movement of a nascent polypeptide across microsomal membranes reveals requirements for vectorial translocation of proteins. *Cell* **71**:87–96.
- Queen, C., and D. Baltimore. 1983. Immunoglobulin gene transcription is activated by downstream sequence elements. *Cell* **33**:741–748.
- Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghayeh, N. T. Chang, R. C. Gallo, and F. Wong-Staal. 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature (London)* **313**:277–284.
- Rho, H. M., B. Poesz, W. Ruscetti, and R. C. Gallo. 1981. Characterization of the reverse transcriptase from a new retrovirus (HTLV) produced by a human cutaneous T-cell lymphoma cell line. *Virology* **112**:355–360.

39. Sakai, H., R. Shibata, J.-I. Sakuragi, S. Sakuragi, M. Kawamura, and A. Adachi. 1993. Cell-dependent requirement of human immunodeficiency virus type 1 *Vif* protein for maturation of virus particles. *J. Virol.* **67**:1663–1666.
40. Sakai, K., X. Ma, I. Gordienko, and D. Volsky. 1991. Recombinational analysis of a natural noncytopathic human immunodeficiency virus type 1 (HIV-1) isolate: role of the *vif* gene in HIV-1 infection kinetics and cytopathicity. *J. Virol.* **65**:5765–5773.
41. Schwartz, S., B. Felber, and G. Pavlakis. 1991. Expression of human immunodeficiency virus type 1 *vif* and *vpr* mRNAs is rev-dependent and regulated by splicing. *Virology* **183**:677–686.
42. Sodroski, J. W. C. Goh, C. Rosen, A. Tartar, D. Portetelle, A. Burny, and W. Haseltine. 1986. Replicative and cytopathic potential of HTLV-III/LAV with *sor* gene deletions. *Science* **231**:1549–1551.
43. Sova, P., and D. Volsky. 1993. Efficiency of viral DNA synthesis during infection of permissive and nonpermissive cells with *vif*-negative human immunodeficiency virus type 1. *J. Virol.* **67**:6322–6326.
44. Strebel, K., D. Daugherty, K. Clouse, D. Cohen, T. Folks, and M. A. Martin. 1987. The HIV 'A' (*sor*) gene product is essential for virus infectivity. *Nature (London)* **328**:728–730.
45. Terwilliger, E., B. Godin, J. Sodroski, and W. Haseltine. 1989. Construction and use of replication-competent human immunodeficiency virus that expresses the CAT enzyme. *Proc. Natl. Acad. Sci. USA* **86**:3857–3861.
46. Tomonaga, K., J. Norimine, Y.-S. Shin, M. Fukasawa, T. Miyazawa, A. Adachi, T. Toyosaki, Y. Kawaguchi, C. Kai, and T. Mikami. 1992. Identification of a feline immunodeficiency virus gene which is essential for cell-free virus infectivity. *J. Virol.* **66**:6181–6185.
47. Towler, D. A., and J. I. Gordon. 1988. The biology and enzymology of eukaryotic protein acylation. *Annu. Rev. Biochem.* **57**:69–99.
48. van der Goot, F. G., J. M. Gonzales-Manas, J. G. Lakey, and F. Pattus. 1991. A 'molten-globule' membrane-insertion intermediate of the pore-forming domain of colicin A. *Nature (London)* **354**:408–410.
49. von Schwedler, U., J. Song, C. Aiken, and D. Trono. 1993. *vif* is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. *J. Virol.* **67**:4945–4955.
50. Walter, P., and G. Blobel. 1983. Preparation of microsomal membranes for cotranslational protein translocation. *Methods Enzymol.* **96**:84–93.
51. Yu, G., and R. Felsted. 1992. Effect of myristoylation on p27^{nif} subcellular distribution and suppression of HIV-LTR transcription. *Virology* **187**:46–55.