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The Vif and Gag Proteins of Human Immunodeficiency Virus Type 1 Colocalize in Infected Human T Cells

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The Vif protein of human immunodeficiency virus type 1 (HIV-1) and other lentiviruses is required for efficient replication in primary cells and certain immortalized cell lines in vitro and, in all likelihood, for the establishment of pathogenic infections in vivo. Current hypotheses concerning Vif's mechanism of action posit that it operates in virus-expressing cells during virion assembly, budding, or maturation such that released virions are modified in a manner that enables them to undergo productive infection in subsequent viral challenges. To gain further insight into the mechanism of action of lentivirus Vif proteins, we have performed a variety of in situ localization and biochemical fractionation studies using cells in which Vif is essential for efficient replication. Double-label immunofluorescence analyses of cells productively infected with HIV-1 or feline immunodeficiency virus revealed dramatic patterns of colocalization between Vif and the virally encoded Gag proteins. Subcellular fractionations of human T cells expressing HIV-1 Vif performed in the absence of any detergent demonstrated that greater than 90% of Vif is associated with cellular membranes. Additional purification using a continuous density gradient indicated that the majority of the membrane-bound Vif copurifies with the plasma membrane. Taken together, these observations suggest that lentivirus Vif and Gag proteins colocalize at the plasma membrane as virion assembly and budding take place. As a result, Vif is able to exert its modulatory effect(s) on these late steps of the virus life cycle.

Among the additional genes that distinguish members of the lentivirus family of retroviruses from their oncovirus counterparts, *vif* is one of the more widely conserved (29). Indeed, only one lentivirus, equine infectious anemia virus, appears to lack a *vif* gene. In studies of human immunodeficiency virus type 1 (HIV-1), it has been shown that *vif* is required for productive replication in primary human T lymphocytes and certain immortalized T-lymphoid lines (2, 6, 10, 11, 15, 37, 39–42, 44, 47). It has also been demonstrated that expression of *vif*-deficient (Δ*vif*) proviruses in these cells directs the production of virus particles but that these may be at least 50-fold less infectious than wild-type virus (12, 39). Because *vif*-deficient infections cannot be rescued by the expression of Vif in target cells (2, 6, 15, 39, 47), it is likely that this dramatic infection phenotype is determined during the late stages of the life cycle, namely, during assembly, budding, maturation, or some combination thereof.

Compositional analyses of purified viral particles have failed to detect any Vif-dependent quantitative or qualitative difference in the Gag, Pol, or Env contents of virions that correlates with infectivity (12, 30). A low yet readily detectable amount of Vif is, however, present in virions (3, 12, 26); the significance of this remains unclear, as incorporation into the budding virions of an oncovirus, murine leukemia virus, that does not contain a *vif* gene also occurs (3). Interestingly, the expression levels of Vif in infected cells are much greater than in virions and, in fact, approach those of the viral Gag proteins (12). It is therefore tempting to speculate that Vif may function in virus-producing cells by influencing the conformation of Gag and/or the assembly of Gag precursors into viral cores. Not only could both of these effects impact on virion morphology, but they would also be consistent with the ultrastructural abnormalities that have been described by some groups for viral particles expressed in the absence of Vif (2, 24).

Analyses of the endpoint(s) of *vif*-deficient infections have revealed that provirus formation is very inefficient. Different groups have presented data suggesting that initial postpenetration reverse transcription is unaffected (39), reduced (42, 47), or abrogated (2, 6) in such infections. In an attempt to explain these observations, we proposed that Vif does not directly influence the enzymatic activity of reverse transcriptase but that it might be possible to attribute all of these Δ*vif* phenotypes to the instability of postentry viral nucleoprotein complexes (39). According to this model, experimental systems in which penetrating *vif*-deficient nucleoprotein complexes are very unstable would permit no DNA synthesis, whereas configurations in which these complexes are relatively stable would support substantial reverse transcription. Recent analyses of the effect of Vif on virion-associated reverse transcription in vitro are consistent with this notion. In particular, it has been demonstrated that Vif does not affect reverse transcriptase activity when exogenous template is added to the reactions, but that its expression is necessary for maximum activity when endogenous virion RNA serves as the template (20). In any event, it would be predicted that the postpenetration stability of viral nucleoprotein complexes could be predetermined by events that occur late in the viral life cycle and that are susceptible to modulation by Vif.

HIV-1 nucleoprotein core assembly is thought to be largely initiated by the association of Gag precursors ($p55^{Gag}$) with the inner leaflet of the plasma membrane. This is mediated by a bipartite signal that resides within the matrix (MA) component of $p55^{Gag}$ and comprises the amino-terminal myristoyl group and a cluster of positively charged amino acids (21, 43, 48, 50,

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51); as might be predicted, structural studies have revealed that these moieties are all located on one face of MA (23). Interactions between $p55^{Gag}$ monomers are then driven by sequences that, to a large extent but not exclusively, are located toward the carboxy terminus of the capsid (CA) portion of $p55^{Gag}$ (4, 5, 8, 9, 13, 14, 36, 46). Once the required number of Gag precursors have accumulated and are positioned appropriately, the immature core structure forms, and coincident with a pinching off of a small region of the plasma membrane, budding finally takes place (16). Interestingly, Gag-Gag interactions are not entirely restricted to the plasma membrane, as mutated Gag proteins deficient in membrane association can, together with wild-type Gag, still be incorporated into mixed core structures (50).

Prompted by its relative abundance in HIV-1-infected cells and by the postulated role in virus assembly, we wished to determine the subcellular localization of Vif. We considered that evidence in favor of substantial colocalization between Vif and other viral components could be viewed as being supportive of a function during virus assembly. In this study, we used confocal microscopy to demonstrate that there is a striking colocalization of Vif and $p55^{Gag}$ in human T cells productively infected with HIV-1. In addition, subcellular fractionation studies using T cells and disruption by nitrogen cavitation revealed, first, that the majority of HIV-1 Vif is associated with membranes, and second, that much of this Vif appears to copurify with the plasma membrane. Importantly, the close proximity of Vif and Gag was not a feature unique to HIV-1-infected cells; a marked colocalization of these proteins was also noted in feline cells productively infected with the distantly related nonprimate lentivirus, feline immunodeficiency virus (FIV).

MATERIALS AND METHODS

Molecular clones. The wild-type and *vif*-deficient $HIV\text{-}1_{IIIB}$ proviral expression vectors, pIIIB and pIIIB/Δ*vif*, as well as the HIV-1 *rev* expression vector pcRev have been described previously (27, 40). The Rev-responsive *vif* expression vector pgVif contains a segment of pIIIB that extends from 15 nucleotides upstream of the ATG initiation codon for *vif* to the *Bam*HI site 51 nucleotides downstream from the stop codon for *tat* inserted between the cytomegalovirus immediate-early promoter and rat preproinsulin polyadenylation signal of the pBC12/CMV vector (7); this vector therefore contains the intron that separates the two coding exons of *tat* and harbors the Rev response element. The recombinant wild-type FIV proviral expression vector pFIV was derived by replacing the 1,128-bp *Bsp*E1-*Bgl*II fragment of pFIV-34TF10 (32) with the analogous fragment from pFIV-14-Petulama (31); this manipulation repaired the disrupted ORF-2 of pFIV-34TF10. The *vif*-deficient derivative pFIV/Δ*vif* carries a frameshift mutation after codon 32 of the *vif* gene (this creates nonsense codons at positions 35 and 40) and was generated by cleavage with *Bsp*E1, fill-in with Klenow fragment, and religation.

Cells, cell lines, and transfections. The human cell lines H9 and HeLa were maintained as described previously (28, 40). The H9/hVif line constitutively expresses HIV-1 Vif and was maintained in medium supplemented with G418 (1 mg/ml; GIBCO BRL Inc., Gaithersburg, Md.) (40). The feline embryonic brain cell line G355-5 (obtained from D. Blair) was maintained in McCoy's 5A medium supplemented with 15% fetal bovine serum and 50 μg of gentamicin sulfate per ml. All transient transfections were performed by using standard calcium phosphate precipitations.

Infections and virus replication. HIV-1 infections of H9 cells were initiated and maintained as described previously (40). Infections of G355-5 cells with FIV and FIV/Δ*vif* were initiated by transfection of monolayer cultures; the cells were passaged every 2 to 3 days, and virus production was monitored as levels of reverse transcriptase activity in the culture supernatants (18).

Antibodies. The following antibodies were used for the detection of viral and cellular proteins: for HIV-1 Vif, murine monoclonal antibody (MAb) 319 (40); for HIV-1 $p55^{Gag}$ and $p24^{Gag}$, a murine MAb (MAb 24-3) and a rabbit polyclonal antibody (PAb) generated by using standard protocols and purified recombinant $p24^{Gag}$ that carried an amino-terminal extension of Met-Arg-Gly-Ser-His_6-Ser (12); for FIV Vif, a rabbit PAb raised against purified recombinant protein that carried an amino-terminal extension of Met-Arg-Gly-Ser-His_6-Ser; for FIV $p24^{Gag}$, murine MAbs FIV 1-10 and PAK3-2C1, obtained from Custom Monoclonals International (West Sacramento, Calif.); for calreticulin, a rabbit PAb purchased from Affinity Bioreagents Inc. (Golden, Colo.); for transferrin receptor (TfR), murine MAb OKT9, provided by M. J. Birnbaum; for vimentin, a rabbit PAb provided by V. M. Lee (34); and for C1 and C2 proteins, murine MAb 4F4, provided by G. Dreyfuss (33).

Immunocytochemistry. H9 cells acutely infected with wild-type HIV-1 were washed twice in phosphate-buffered saline (PBS) and resuspended at 10^6 cells/ml in PBS. Then 5×10^5 cells were allowed to settle onto poly-D-lysine-coated 12-mm-diameter glass coverslips for 20 min at room temperature. The PBS was then aspirated, and the cells were fixed by addition of methanol for 3 min at −20°C. Adherent cells (transfected HeLa and G355-5) were cultured on glass coverslips prior to fixation with methanol. Immunostaining was performed as described previously (28), and antibodies that recognize the various proteins were detected as follows. The HIV-1 Vif-specific MAb 319 was purified from hybridoma culture supernatant by using a MAb TRAPGII column (Pharmacia Biotech Inc., Piscataway, N.J.) and conjugated directly to the Cy-3 fluorochrome as instructed by the manufacturer (Amersham Corp., Arlington Height, Ill.); the rabbit PAbs specific for HIV-1 $p24^{Gag}$, FIV Vif, and vimentin were detected by using goat anti-rabbit antibodies conjugated to fluorescein isothiocyanate or Texas red (FisherBiotech, Pittsburgh, Pa.); the cocktail of FIV $p24^{Gag}$-specific MAbs was detected with a goat anti-mouse antibody conjugated to Texas red (FisherBiotech). Samples were then visualized by epifluorescence, using a Nikon Microphot-SA microscope (Nikon Corp., Tokyo, Japan) at a magnification of ×400 or by laser scanning confocal microscopy using a Leica TCS 4D microscope (Leica Inc., Malvern, Pa.). In the latter case, 0.5 to 1.0-μm horizontal optical sections of stained cells were scanned, and the images were stored on a computer. Subsequent superimpositioning of the sections produced an image with an extended focus whereby the true three-dimensional localization of detected molecules could be visualized.

Subcellular fractionation by nitrogen cavitation, Percoll gradient centrifugation, and Western blotting. For the determination of total expression levels in transfected HeLa cultures, whole-cell lysates were prepared from 2×10^5 cells and subjected to standard Western blot analyses as described previously (40). For the fractionation studies, 3×10^8 H9/hVif cells were harvested by low-speed centrifugation, washed in detergent-free homogenization buffer [0.25 M sucrose, 10 mM Tris-HCl (pH 7.3), 1 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.)], and resuspended in 1.5 ml of homogenization buffer. The cells were then transferred to a stainless-steel cell disruption bomb (Parr Instrument Co., Moline, Ill.) and pressurized with nitrogen at 100 lb/in^2 for 10 min on ice. As the pressure was released, the suspension was extruded through a needle valve and the degree of cell disruption was monitored by phase-contrast microscopy; this was routinely in excess of 90%. The homogenate was centrifuged at $1{,}000 \times g$ for 10 min at 4°C to pellet unbroken cells and nuclei; the supernatant was removed and, having been combined with the supernatants of two nuclei washes (final volume of ~2 ml), treated in one of two ways.

First, the pooled supernatant fraction was centrifuged at $200{,}000 \times g$ for 60 min at 4°C in a TLA100.2 rotor (Beckman Instruments Inc., Fullerton, Calif.) to pellet all cellular membranes away from the cytosol. The nuclear and membrane samples were each resuspended in 1 ml of radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 250 μM phenylmethylsulfonyl fluoride) while the cytosol was adjusted to 1× radioimmunoprecipitation assay buffer. The total amount of protein present in each of the three samples was shown to be similar by Coomassie blue staining of electrophoresed material. Aliquots corresponding to equivalent numbers of cells were then analyzed by Western blotting. Second, the pooled supernatant (2 ml) was layered onto 26.5 ml of a 17% (vol/vol) Percoll solution (Pharmacia Biotech) over a 1.5-ml cushion of 2.5 M sucrose and centrifuged at $40{,}000 \times g$ for 40 min at 4°C in an SS34 rotor (Sorvall Du Pont, Wilmington, Del.) to form a continuous density gradient. Fractions of 2 ml were collected from the bottom of the gradient, and 50-μl samples were analyzed directly by Western blotting. To determine which fractions contained plasma and endosomal membranes, 10^8 H9/hVif cells were incubated with OKT9 hybridoma supernatant at 4°C and then either maintained at 4°C (TfR remains at the plasma membrane) or incubated at 37°C for 60 min (TfR is internalized to endosomes). Cells were then disrupted by nitrogen cavitation, and the $1{,}000 \times g$ supernatants were fractionated on Percoll gradients and subjected to Western blotting. Transferred proteins were initially hybridized with antibodies specific for HIV-1 Vif, calreticulin (a chaperone that resides in the endoplasmic reticulum [ER]), TfR (an integral membrane protein; in this case electrophoresis was carried out under nonreducing conditions), the nuclear C1 and C2 proteins, or a goat anti-mouse antibody (to detect OKT9). Bound antibodies were visualized by secondary hybridization with appropriate species-specific horseradish peroxidase-conjugated antibodies, enhanced chemiluminescence, and autoradiography.

RESULTS

The Gag and Vif proteins of HIV-1 colocalize in infected H9 T cells. One of the principal objectives of this study was to evaluate the subcellular distribution of HIV-1 Vif in cells that require its expression for a productive infection. By definition,

cells with this phenotype do not support the replication of *vif*-deficient virus and are referred to as nonpermissive; examples include peripheral human blood lymphocytes and the H9 T-cell line. In contrast, other T-cell lines such as CEM-SS, C8166, and Sup-T1 allow the replication of *vif*-deficient viruses and are termed permissive (2, 6, 10, 11, 15, 37, 39–42, 44, 47). Since H9 cells accurately recreate the Δ*vif* phenotype observed in peripheral blood lymphocytes, it is most likely that analyses performed with these cells will reflect in vivo Vif function whereas experiments carried out in permissive cell lines will not. With this in mind, H9 cells were infected with wild-type HIV-1 and, at a point when virus production was close to its maximum, fixed and subjected to immunocytochemistry, using antibodies specific for a variety of viral and cellular proteins (Fig. 1).

In the first analysis, samples were initially hybridized with a murine MAb specific for Vif together with a rabbit PAb that recognizes the CA component of $p55^{Gag}$ (Fig. 1A). To eliminate any cross-reactivity during processing, bound antibodies were detected by secondary hybridization with a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody and by direct labeling of the Vif-specific antibody with Cy-3. The specificity of this method for detecting Vif was confirmed by the lack of a signal that was obtained when cells expressing *vif*-deficient proviruses were hybridized with the Cy-3-labeled Vif-specific MAb (data not shown).

The samples were ultimately visualized by using a confocal microscope and optical sectioning; the staining patterns for Vif and $p55^{Gag}$ in a representative syncytium are shown (Fig. 1A, panels i and ii) along with a computer-generated superimposition of the two images (Fig. 1A, panel iii). Vif displayed a diffuse pattern of cytoplasmic localization in which no striking areas of local concentration could be discerned but a clear exclusion from the nuclei was evident. The distribution of $p55^{Gag}$ was somewhat similar to that of Vif, although there did appear to be a greater confinement toward the upper right section of this syncytium. Combining these two images revealed a dramatic pattern of colocalization whereby almost all of the areas that stained positive for Gag also contained Vif (seen as yellow in Fig. 1A, panel iii). Conversely, it appeared that some areas of Vif staining did extend beyond those containing the Gag protein. Single (unfused) cells that expressed viral antigens were also examined in this analysis. However, both the Vif- and Gag-specific signals tended to be evident throughout the cytoplasm; this therefore rendered visualization of such cells less informative in terms of compartmentalization and colocalization (data not shown).

These results stand in contrast to a recent report on Vif localization in which substantial juxtapositioning of Vif and the cytoplasmic intermediate filaments (cIFs) that are composed of vimentin was described (25). To determine whether this particular observation would hold true in our infected T-cell system, double-label immunofluorescence was performed with the Vif-specific MAb and a rabbit PAb that recognizes vimentin (Fig. 1B). In this case, the samples were examined by epifluorescence and conventional microscopy. As before, Vif was relatively evenly dispersed throughout the cytoplasm of a typical syncytium (Fig. 1B, panel i). Vimentin, on the other hand, was predominantly localized to a filamentous network-like structures that resided primarily at the cell periphery (Fig. 1B, panel ii). Thus, no discernible colocalization of Vif and the cytoskeletal component vimentin could be detected in nonpermissive T cells productively infected with HIV-1.

Examination of Vif expression in transfected HeLa cultures. As noted above, our finding that Vif does not appear to localize to vimentin cIFs is inconsistent with an earlier analysis (25). A major point of difference between these two studies is that we have used infected H9 T cells, whereas transiently transfected HeLa cells were utilized previously. It is plausible that the choice of cell type, the mode of gene expression (viral infection versus transfection), or the expression level of protein per cell could affect the pattern of Vif localization. To investigate these possibilities, a time course experiment was carried out in which Vif was expressed in HeLa cells following transient transfection with (i) the wild-type HIV-1 expression vector pIIIB, (ii) the pIIIB/Δ*vif* variant in combination with the subgenomic vector pgVif, or (iii) pgVif in the absence of any viral structural genes. At each time point, matched samples were subjected to Western analysis (Fig. 2) and double-label indirect immunofluorescence (Fig. 3).

The analyses of Vif and Gag expression levels were performed with whole-cell lysates prepared 18, 24, and 40 h after the addition of DNA precipitates to the HeLa cell monolayers. As would be predicted, both Vif and Gag continued to accumulate for the duration of the experiment (Fig. 2, upper and lower panels, respectively); clearly, however, the most significant increases in steady-state expression levels had taken place by 24 h posttransfection. Importantly, the levels of Vif expression were markedly greater in the samples that contained pgVif than in those that received the *vif* gene in the context of the pIIIB provirus. This was to be expected, however, as the mRNAs that encode Vif comprise a relatively small fraction of the total viral mRNA content of provirus-expressing cells (35).

Immunofluorescence analyses of pIIIB-transfected cultures at 40 h posttransfection revealed that, as in T cells, Vif and Gag were distributed across the cytoplasm, with no particular areas of local concentration (Fig. 3A, panels i to iii). In contrast, vimentin was primarily confined to filamentous structures that, to a certain degree, appeared to radiate from a point close to the cytoplasmic face of the nuclear envelope (Fig. 3A, panels v and vi); no accumulation of Vif in the proximity of these structures could be detected (Fig. 3A, panel iv). Examination of similar cultures at 18 h posttransfection failed to detect Vif (data not shown), presumably due to the low levels of protein that had accumulated at that time (Fig. 2). Analyses of cells cotransfected with pIIIB/Δ*vif* and pgVif revealed rather different patterns of Vif localization; Gag, however, did retain its diffuse cytoplasmic distribution (Fig. 3B, panels ii, iii, v, and vi). At 18 h posttransfection, Vif appeared to be expressed in the nucleus as well as the cytoplasm (Fig. 3B and C, panels i). By 40 h, however, Vif was tending toward a more filamentous staining pattern with a well-defined region of accumulation close to the nucleus (Fig. 3B and C, panels iv). Double staining using the vimentin-specific antibody demonstrated that this area of local concentration coincided with the vimentin-rich region (Fig. 3C, panel v). Importantly, the expression levels of Vif in these cultures were significantly greater than in corresponding pIIIB-transfected cultures, particularly at the 40-h time point (Fig. 2). Cells transfected with pgVif in the absence of Gag expressed the highest levels of Vif and displayed numerous localized areas of Vif accumulation that frequently colocalized with vimentin (Fig. 3D). Taken together, these data imply that increased intracellular accumulation of Vif correlates with more defined patterns of subcellular localization and with significant juxtapositioning with the vimentin cIFs. Of note, only the samples that received pIIIB, and that therefore contained levels of Vif that likely mimicked those encountered during viral infection, exhibited patterns of localization that resembled those observed in infected T cells.

Membrane association of HIV-1 Vif in T cells: cell disruption by nitrogen cavitation. Following synthesis and cotranslational myristoylation, $p55^{Gag}$ associates with membranes and is

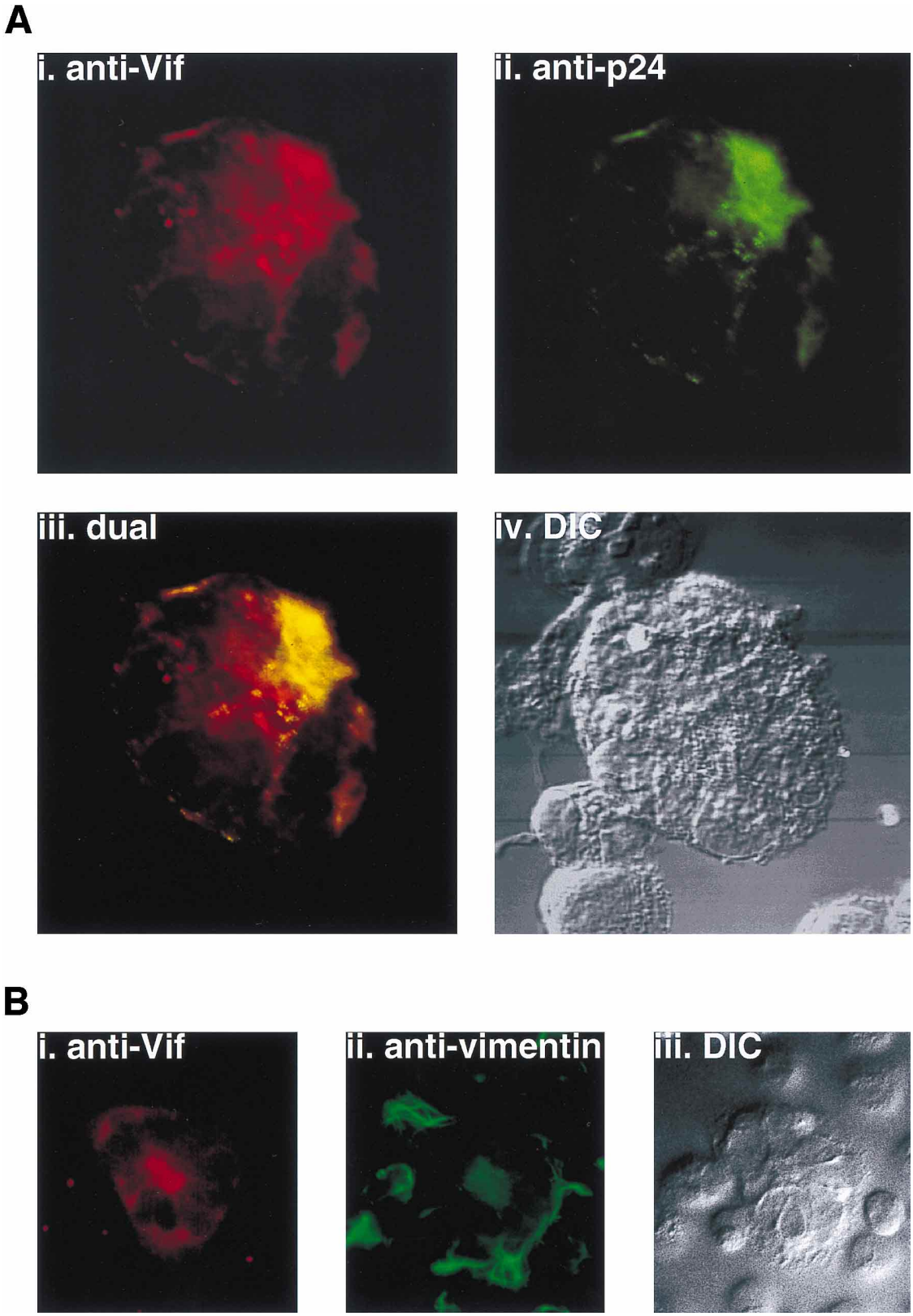

FIG. 1. The Vif and Gag proteins of HIV-1 colocalize in infected human T cells. H9 cells were infected with wild-type HIV-1 and, at a time when virus production was close to its peak, fixed and analyzed by double-label immunofluorescence. (A) Cells were incubated with the Vif-specific MAb 319 (panel i) and Gag-specific PAb raised in rabbits ($p24^{Gag}$; panel ii), and a representative syncytium was visualized by laser scanning confocal microscopy. The superimposed Vif and Gag images are also shown (panel iii) together with the corresponding differential interference contrast (DIC) image (panel iv). (B) Cells were incubated with antibodies specific for Vif (panel i) and vimentin (panel ii) and visualized by epifluorescence; the corresponding DIC image is also shown (panel iii).

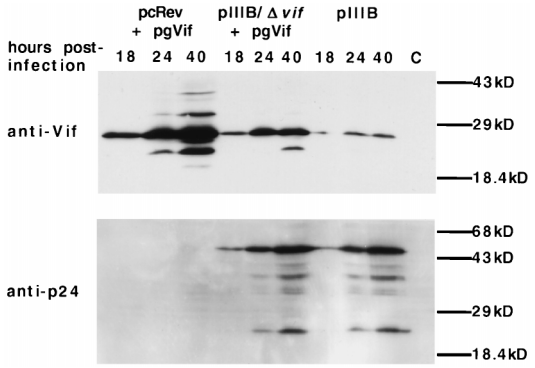

FIG. 2. Western analysis of HIV-1 Vif and Gag expression in transfected HeLa cells. HeLa cells monolayers (35-mm diameter) were transfected with 4 μg of pIIIB, 2 μg of pIIIB/Δ*vif* plus 2 μg of pgVif, 2 μg of pcRev plus 2 μg of pgVif, or 4 μg of negative control plasmid (lane C) by using calcium phosphate. At 18, 24, and 40 h posttransfection, whole-cell lysates were prepared, loaded on SDS-12% polyacrylamide gels, and analyzed by Western blotting using the Vif-specific MAb 319 (upper panel) or the Gag-specific MAb 24-3 (lower panel). Bound antibodies were detected by enhanced chemiluminescence. The positions of prestained protein molecular mass standards are indicated to the right.

transported to the plasma membrane. Given that a substantial fraction of Vif colocalizes with Gag (Fig. 1), we wished to determine whether Vif itself is also associated with cellular membranes. Rather than employ the more commonly used techniques of detergent extraction or hypotonic swelling followed by Dounce homogenization for cell disruption, we took an alternative approach, gaseous shearing (also known as nitrogen cavitation) (49). Here, cells are initially pressurized under nitrogen in a closed stainless-steel vessel. As the sample is then returned to atmospheric pressure, there is an explosive formation of nitrogen bubbles which, together with rapid extrusion through a narrow tube, results in extensive disruption of the plasma membrane in the absence of sample warming. Subsequent centrifugational processing of the cleared lysate in the absence of any detergent can then be used to evaluate association with specific cellular membranes.

H9 T cells that stably express the HIV-1 Vif protein, H9/hVif, were therefore disrupted in this manner, the nuclei were removed by low-speed centrifugation, and the supernatant was separated into cytosolic and membrane-containing fractions by high-speed centrifugation (200,000 × *g*). All three samples—cytosol, membranes, and nuclei—were then subjected to Western analyses using a Vif-specific MAb and a variety of anti-

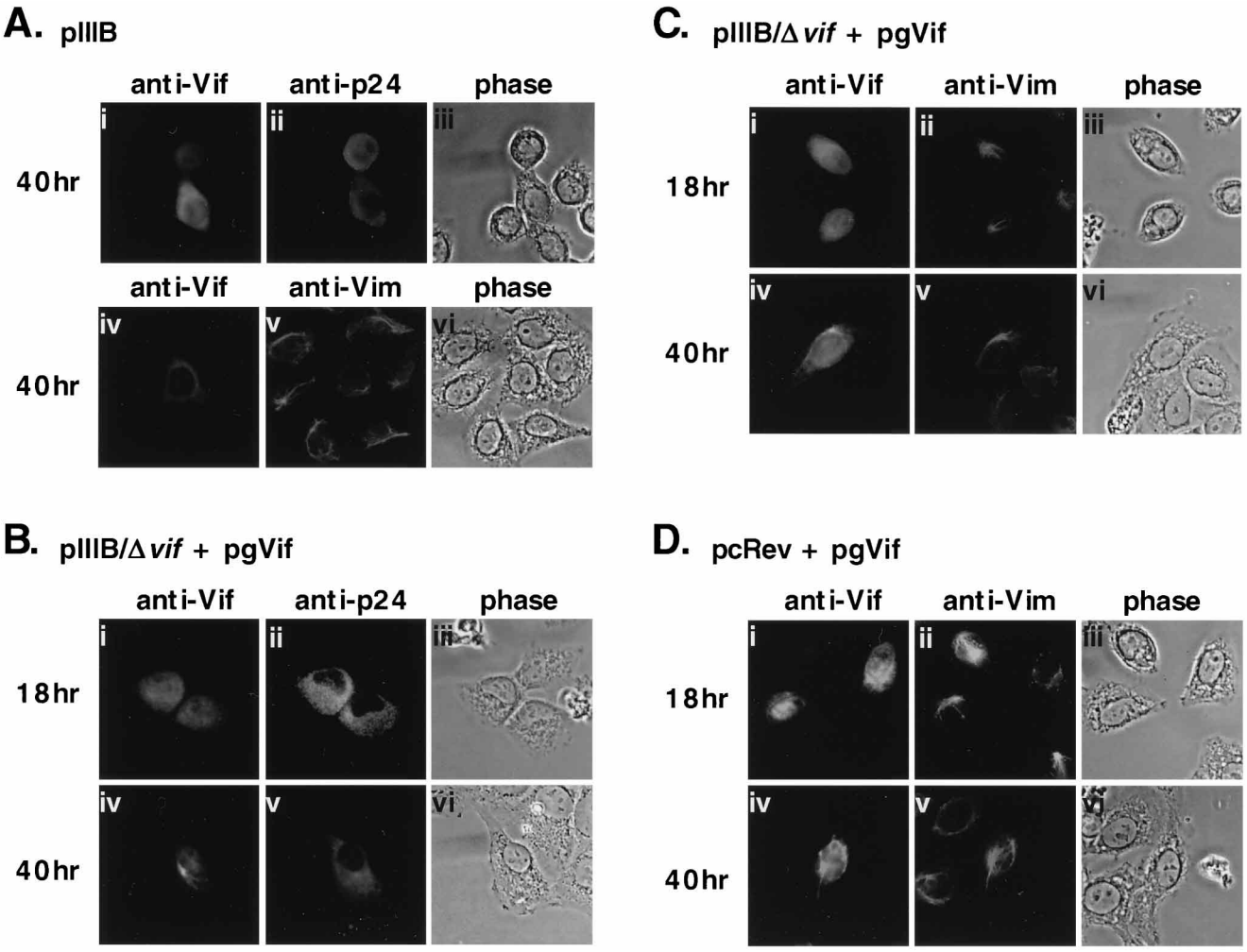

FIG. 3. Subcellular localization of HIV-1 Vif, HIV-1 Gag, and vimentin in HeLa cells. Cultures were transfected as for Fig. 2 with pIIIB (A), pIIIB/Δ*vif* plus pgVif (B and C), or pcRev plus pgVif (D) and analyzed by double-label immunofluorescence using the Vif-specific MAb 319, a Gag-specific PAb, or a vimentin-specific PAb at various times posttransfection as indicated. Also indicated are the corresponding phase-contrast images.

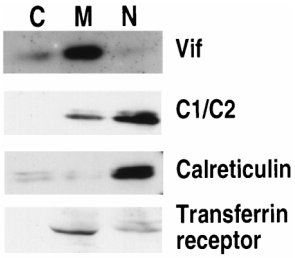

FIG. 4. HIV-1 Vif is associated with cellular membranes in human T cells. H9/hVif cells (3×10^8) were disrupted by nitrogen cavitation and separated into cytosolic (lane C), membrane (lane M), and nuclear (lane N) fractions by differential centrifugation. Aliquots corresponding to equivalent cell numbers were applied to SDS-polyacrylamide gels and analyzed by Western blotting using the Vif-specific MAb 319, the hnRNP C1/C2-specific MAb 4F4, a calreticulin-specific PAb, or the TfR-specific MAb OKT9 for primary hybridization. Detection of bound antibodies was performed as for Fig. 2.

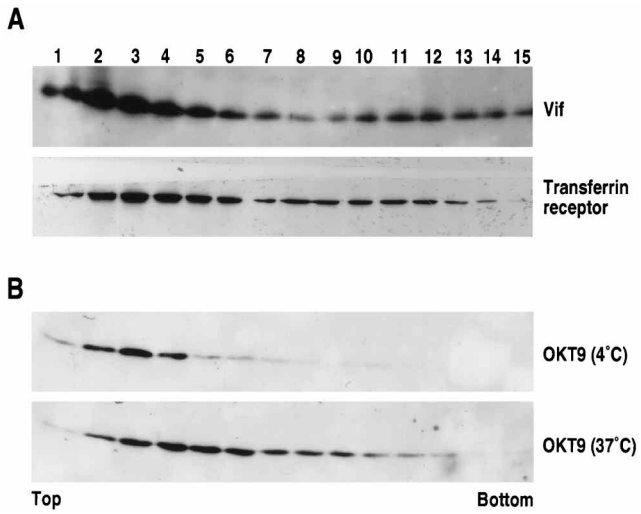

FIG. 5. HIV-1 Vif copurifies with the plasma membrane in Percoll gradients. (A) The combined cytosol and membrane fraction from 3×10^8 H9/hVif cells that had been disrupted by nitrogen cavitation (Fig. 4) were applied to the top of a 17% (vol/vol) Percoll solution, and a continuous gradient was established by centrifugation. Samples from each fraction (1 = top, 15 = bottom) were analyzed by Western blotting using MAb 319 (upper panel) and OKT9 (lower panel). (B) H9/hVif cells (10^8) were incubated with OKT9 at 4°C and either maintained at 4°C (upper panel) or incubated at 37°C for 60 min. The cells were then disrupted, and the cytosol-membrane supernatants were fractionated on Percoll gradients and analyzed by Western blotting using a goat anti-mouse antibody. The bands that are shown correspond to the immunoglobulin G light chains.

bodies that each recognize cellular proteins (Fig. 4). Inspection of the data revealed that at least 90% of Vif is associated with cellular membranes that are distinct from the nuclear membranes (Fig. 4, upper panel). Analyses of the samples with the other antibodies established that the cells had been fractionated appropriately. In particular, the heterogeneous nuclear ribonucleoprotein particle (hnRNP) C1 and C2 proteins were predominantly retained in the nuclear fraction, whereas TfR, an integral membrane protein, was found almost exclusively in the membrane fraction. The detection of calreticulin, a chaperone of the ER, in the nuclear pellet was also anticipated, as the ER membrane is continuous with the outer membrane of the nuclear envelope. Importantly, the cytosolic fraction was confirmed as possessing a total protein content at least as great as that of the membrane and nuclear fractions by standard Coomassie blue staining of electrophoresed samples (data not shown).

In an attempt to identify, with some degree of accuracy, which cellular membranes are bound by Vif, further separations using density gradients were performed. Accordingly, the combined membrane-cytosol extract of disrupted H9/hVif cells was fractionated on the basis of velocity through a continuous Percoll gradient (Fig. 5A). Collected fractions were then analyzed by Western blotting for the presence of Vif and TfR. To establish where certain membranes, specifically the plasma and endosomal membranes of H9/hVif cells, sediment in such gradients, two control fractionations were carried out (Fig. 5B). Thus, the TfR-specific MAb OKT9 was allowed to bind to TfR on the surface of H9/hVif cells by incubation at 4°C; subsequent maintenance at 4°C restricted OKT9 to the plasma membrane, whereas warming to 37°C for 60 min resulted in its active internalization and trafficking to endosomes. By determining the gradient profiles of OKT9 following cell disruption, fractions 2 to 4 were therefore identified as being enriched in plasma membrane (Fig. 5B, upper panel), and a much broader peak of fractions 3 to 9 was identified as containing the majority of endosomes (Fig. 5B, lower panel).

The steady-state distribution of TfR (Fig. 5A, lower panel) was close to what would be expected for combining the plasma and endosomal membrane-specific profiles. Specifically, there was both a distinct peak at the top of the gradient (fractions 2 to 6) and a trailing down throughout most of the gradient. One point of difference between the lower region of this gradient and the endosome profile was that fractions 10 to 13 still contained readily detectable levels of TfR. In contrast to TfR, Vif displayed a marked double-peak pattern of membrane association (Fig. 5A, upper panel). Vif was clearly most abundant in the plasma membrane-enriched portion of the gradient (fractions 2 to 5); it was least abundant in the center of the gradient (for example, fractions 8 and 9) and then peaked again toward the bottom (fractions 10 to 15). The identities of the membranes that comprise this second peak of Vif expression are not known at this time, though they appear to be distinct from the endosomes and may also contain significant levels of TfR. Consistent with our earlier analysis (Fig. 4), only relatively low levels of Vif and TfR were detected in the cytosol-containing portion of the gradient (fraction 1). In conclusion, these biochemical experiments strongly suggest that the majority of HIV-1 Vif is associated with, and is consequently in the proximity of, the plasma membrane of T cells. This finding is consistent with Vif's colocalization with the Gag proteins, which accumulate at the plasma membrane for the purposes of viral assembly and budding.

Colocalization of Vif and Gag in FIV-infected cells. As discussed above, *vif* genes have been identified in almost all lentiviruses examined to date (29). If, as is generally thought, the function of Vif is conserved among this family of viruses, then one might expect that certain features of Vif expression would also be conserved. For this reason, we wished to determine the subcellular localization of the Vif protein of a lentivirus derived from a nonprimate host, in this case FIV. Prior to performing this analysis, it was important to establish that our chosen cell system was appropriate for evaluating FIV Vif function. Monolayers of the feline embryonic brain cell line G355-5 were therefore transiently transfected with the pFIV or pFIV/Δ*vif* provirus expression vectors, and virus replication was measured over time as the accumulation of reverse transcriptase activity in the culture medium (Fig. 6). Wild-type FIV yielded a clear peak of virus production by 6 days posttransfection, indicative of a rapidly spreading infection. In contrast, and consistent with the findings of others (38), the *vif*-deficient

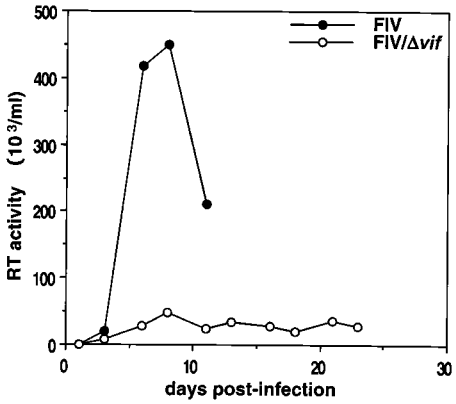

FIG. 6. Vif is required for efficient FIV replication in G355-5 cells. Infections with wild-type FIV and FIV/Δ*vif* were initiated by transient transfection of 2×10^5 cells by using calcium phosphate. Cultures were maintained by serial passage every 2 to 3 days, and virus production was measured as levels of supernatant reverse transcriptase (RT) activity.

virus was able to sustain only a low level of virus production over the 3-week time period of this experiment. In conclusion, G355-5 cells are nonpermissive to a highly productive infection by FIV/Δ*vif* and are therefore likely to represent a suitable cell line for the analysis of FIV Vif.

The localization of FIV Vif was determined in G355-5 cells infected with wild-type FIV by double-label immunofluorescence and confocal microscopy (Fig. 7). As before, a combination of antibodies specific for Vif and $p24^{Gag}$ was used for the initial hybridizations, and representative results are shown both individually (Fig. 7, panels i and ii) and as a superimposition (Fig. 7, panel iii). Vif was expressed throughout the interior of these adherent cells but, unlike HIV-1 Vif, did not appear to be excluded from the nucleus (compare Fig. 7, panel i, with Fig. 1A, panel i). Moreover, the staining pattern, rather than being even, was visibly punctate, with many small areas appearing to possess relatively high concentrations of protein. In contrast, Gag was localized exclusively to the cytoplasm, with the bulk of the staining being proximal to the cell periphery (Fig. 7, panel ii). Superimposing these two images demon-

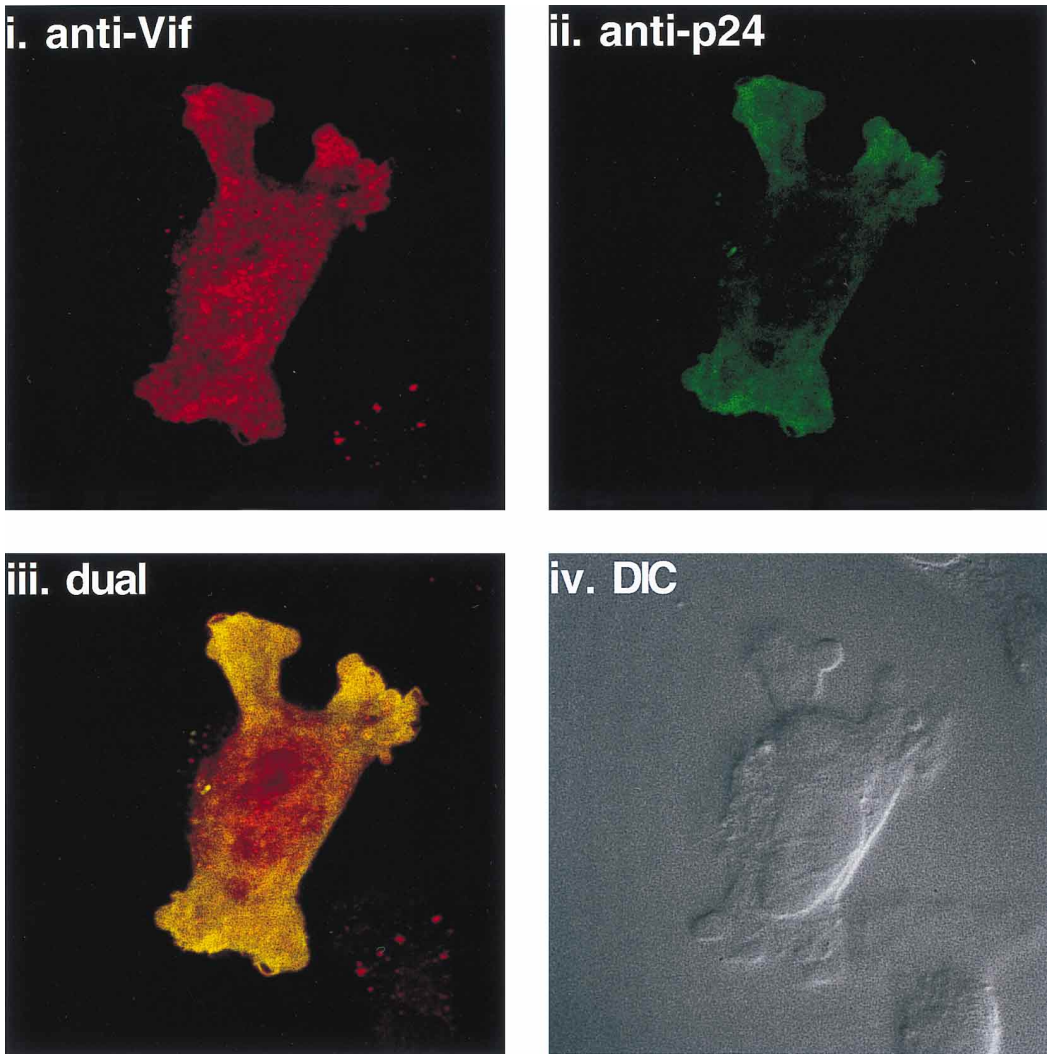

FIG. 7. The Vif and Gag proteins of FIV colocalize in infected feline G355-5 cells. Cells were infected with wild-type FIV and, when virus production was close to its peak, fixed and analyzed by double-label immunofluorescence using a Vif-specific PAb (panel i) and a cocktail of Gag-specific MAbs ($p24^{Gag}$; panel ii). Samples were viewed by laser scanning confocal microscopy, and the superimposed Vif and Gag images (panel iii) and the differential interference contrast (DIC) image (panel iv) are shown.

strated that Vif was present in the majority of the regions that contained Gag (Fig. 7, panel iii). As with HIV-1, there were regions that clearly harbored Vif in the absence of any detectable Gag. Thus, the Vif and Gag proteins of a second lentivirus, FIV, also colocalize to a significant degree in virally infected cells. These findings may be consistent with the notion that Vif proteins modulate virus infectivity by influencing virus assembly.

DISCUSSION

The lentivirus *vif* genes have been established as critical regulators of virus replication (11, 17, 22, 41, 44, 45). However, in spite of their clear importance, an appreciation of Vif's mechanism of action has lagged well behind that of some of the other additional or regulatory proteins of this virus family. To characterize this protein further, we have performed a number of in situ and cell fractionation studies to determine its localization in expressing cells. First, using H9 T cells productively infected with HIV-1, indirect double-label immunofluorescence, and confocal microscopy, it was found that a substantial proportion of intracellular Vif is colocalized with the viral Gag proteins (Fig. 1). Second, using H9 cells stably expressing the HIV-1 Vif protein and subcellular fractionation following disruption by nitrogen cavitation, it was found that the majority of Vif is associated with membranes and that the remainder (less than 10%) appears to be cytosolic (Fig. 4). Third, additional separation using velocity centrifugation through a continuous Percoll gradient revealed that a substantial amount of membrane-bound HIV-1 Vif copurifies with the plasma membrane (Fig. 5). We also found that a significant fraction of Vif appeared to be associated with membranes distinct from the plasma membrane and endosomes; a more accurate description of these membranes will likely be of future interest. Fourth, immunofluorescence analysis of feline cells productively infected with FIV demonstrated that the Vif and Gag proteins of this nonprimate virus also colocalize (Fig. 6).

Some of these points have been addressed previously for HIV-1 by other groups. Goncalves et al. (19) used antibody staining of transiently transfected COS cells and visualization by epifluorescence to show that HIV-1 Vif accumulated in the cytoplasm, and they used Dounce homogenization followed by differential centrifugation to indicate that Vif was present in membrane-associated and soluble cytosolic forms. In contrast, Karczewski and Strebel (25) used staining of transfected HeLa cells and confocal microscopy, as well as detergent-mediated extractions, to show that HIV-1 Vif existed in soluble and cytoskeleton-associated forms. Our findings appear to be more in line with the former analysis in that we were also able to demonstrate an association between Vif and cellular membranes (Fig. 4 and 5) but were unable to detect an association between Vif and vimentin cIFs in cells expressing HIV-1 proviruses (Fig. 1 and 3A); however, it is worth noting that these groups did not use nonpermissive T cells for their studies, nor did they examine expression patterns in virus-producing cells. In contrast, Audoly et al. (1) have examined Vif expression in sheep choroid plexus cells infected with the ungulate lentivirus visna virus. In this system, Vif was also found to be cytoplasmic, with many regions displaying higher levels of local accumulation. Importantly, and unlike the results presented here, none of these earlier analyses addressed the localization of Vif relative to that of other viral proteins.

With respect to the apparent association of Vif (which is not an integral membrane protein) with the plasma membrane of T cells (Fig. 5), further experimentation will be required to determine whether the interaction is direct or is mediated via bridging molecules. Of interest, it has been shown that in vitro-translated Vif is able to associate directly with membranes either cotranslationally or posttranslationally and that deletion of its carboxyl-terminal 22 amino acids (residues 171 to 192) can disrupt this interaction (19). Conversely, the resistance of Vif (when overexpressed in transfected HeLa cells) to detergent extraction has been taken as evidence in favor of a direct association with the cytoskeleton (25). One possible explanation that could reconcile some of these data might be that Vif binds to cytoskeletal elements that are, themselves, associated with the membranes. We are currently exploring these issues further.

As discussed earlier, the current consensus view of Vif function is that it modifies virions prior to the initiation of infection. Hypothetically, regulation could take place during assembly, budding, or postrelease maturation. Based on the far greater abundance of Vif in infected cells than in cell-free virions, its indiscriminate incorporation into murine leukemia virus particles, and its dispensability for the production of infectious virus in certain cells (2, 6, 15, 39, 47), we previously proposed that the step of the life cycle at which Vif principally acts may be virion assembly (12, 39). If this were the case, one might predict that Vif would accumulate in regions of the cell at which lentivirus nucleoprotein cores are formed. Here, we have presented two complementary lines of evidence that are consistent with this prediction: first, Vif strongly colocalizes with a major virion component, the viral Gag precursor (HIV-1 and FIV), in productively infected cells; and, second, a substantial fraction of Vif appears to be associated with the plasma membrane, the predominant site for Gag-Gag interactions and the assembly of viral nucleoprotein complexes (HIV-1) (16). Critical future directions for enhancing our understanding of the molecular basis for Vif function therefore include the identification of the interacting cellular and viral factors that dictate its subcellular localization.

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