Fully functional, naturally occurring and C-terminally truncated variant human immunodeficiency virus (HIV) Vif does not bind to HIV Gag but influences intermediate filament structure

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A variant human immunodeficiency virus type 1 (HIV-1) vif gene, vifA45-2, which encodes a protein lacking 19 amino acids at the C terminus but which is fully functional in supporting HIV replication in non-permissive cells has been described previously. By employing newly generated anti-VifA45 serum, further properties of VifA45 and its full-length counterpart, VifA45open, in comparison to Vif from HIV strain BH10 are reported in permissive HeLa and COS-7 cells. The results obtained using confocal microscopic localization studies and in vitro binding assays do not support a requirement for the direct interaction of HIV Gag with Vif. Furthermore and in contrast to previous conclusions, detergent solubility analyses do not demonstrate a role for the C terminus of Vif in mediating localization to the fraction containing cellular membrane proteins. Localization of Vif from HIV strain BH10 to perinuclear aggregates in a small fraction (about 10%) of transfected HeLa cells has been previously reported. The intermediate filament protein vimentin colocalizes to these structures. In contrast, VifA45 and VifA45open form perinuclear aggregates in nearly all transfected HeLa cells; vimentin as well as the cytoskeletal-bridging protein plectin, but not the microtubular protein tubulin, become relocalized to these structures. Interestingly, in COS-7 cells, all of the functional Vif proteins tested (Vif from strain BH10, VifA45 and VifA45open) predominantly localize in the cytoplasm but still induce dramatic aggregation of vimentin and plectin, i.e. in these cells the respective Vif proteins are influencing intermediate filament structure in the absence of colocalization.

Introduction

The human immunodeficiency virus type 1 (HIV-1) vif (viral infectivity factor) gene product is required for HIV-1 replication in vivo and in certain cell cultures (so-called non-permissive cells) but is dispensable in many cell lines in vitro (so-called permissive cells) (for reviews see Subbramanian & Cohen, 1994; Cullen, 1998). The molecular basis that determines the difference between permissive and non-permissive cells has not been elucidated. However, recent reports suggest that non-permissive cells may express a negative factor which has to be overcome by the action of the vif gene product in a species-specific manner (Madani & Kabat, 1998; Simon et al., 1998a, b). HIV-1 particles are produced in normal amounts in non-permissive cells in the absence of a functional vif gene but these particles are non-infectious and are blocked in an early replication step involving reverse transcription and/or stability of the preintegration complex (Sova & Volsky, 1993; von Schwedler et al., 1993; Borman et al., 1995; Courcoul et al., 1995; Simon & Malim, 1996). This block cannot be overcome by the presence of functional Vif in the target cell. Thus, a current hypothesis speculates that the function of Vif is to confer infectivity on the virus particle in the (non-permissive) producer cell, perhaps by modulating virus particle morphogenesis. The HIV Vif phenotype is, however, independent

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of the homologous Env glycoprotein and is also manifested when the glycoprotein of vesicular stomatitis virus is employed instead of HIV Env (Akari et al., 1999). Analyses of protein content and morphology of vif-defective virus particles have been conflicting. Several reports describe increased incorporation of unprocessed Gag precursor, Pr55<sup>RM</sup>, into virus particles and an increased proportion of aberrant particles as observed by electron microscopy (Hoglund et al., 1994; Borman et al., 1995; Simm et al., 1995), findings which could be compatible with the proposal that Vif may exert a regulatory effect on the HIV-1 protease (Kotler et al., 1997). However, more recent studies did not reproduce these results and no differences in either viral protein composition or particle morphology were detectable (Fouchier et al., 1996; Ochsenbauer et al., 1997), pointing to more subtle differences being responsible for the lack of infectivity of vif-defective virus particles. Another possibility is, of course, that the Vif protein itself is incorporated into virions and exerts its function there. In this regard, reported results are somewhat contradictory but in general indicate that if Vif is present in released virions at all then the amount is very low (Karczewski & Strebel, 1996; Dettenhofer & Yu, 1999).

The vif gene does not display significant sequence homology with any genes of known function so it has been difficult to gain insight into its mode of action. Immunofluorescence analysis of the subcellular distribution of Vif indicates that it is predominantly localized within the cytoplasmic region, with only weak staining of the nucleus (Goncalves et al., 1995; Karczewski & Strebel, 1996). In vitro assays indicate that Vif has an affinity for microsomal membranes (Goncalves et al., 1994, 1995). The C terminus of Vif, which contains a cluster of basic amino acids, has been implicated in this in vitro membrane association and, additionally, to be important for overall Vif function, as measured in infectivity assays (Goncalves et al., 1994, 1995). Fractionation analyses of transfected cells in various detergents have indicated that the Vif protein can be differentially solubilized. Thus, some cellular Vif is soluble in aqueous buffers, some can be solubilized in weak detergents and some can only be dissolved in strong ionic detergent solutions (Goncalves et al., 1994, 1995; Karczewski & Strebel, 1996; Simon et al., 1999). It is not clear whether these solubility properties reflect specific subcellular localizations of expressed Vif. Thus, it has been controversially discussed that Vif may be associated with cellular membranes (Goncalves et al., 1994, 1995), cytoskeletal components (Karczewski & Strebel, 1996) or insoluble components distinct from cytoskeletal components (Simon et al., 1999).

It is likely that Vif exerts its function by interacting with cellular and/or viral components. In this context, it is of note that in a subpopulation of transfected HeLa cells expressing Vif from a subvirus vector, the distribution of the intermediate filament protein, vimentin, was altered such that vimentin was present in perinuclear aggregates to which Vif also localized (Karczewski & Strebel, 1996). Concerning putative interactions with viral proteins, it has been reported that Vif colocalizes with Gag in infected cells (Simon et al., 1997, 1999), whereby more recent evidence suggests that this may reflect independent targeting to the same subcellular compartment without a specific interaction (Simon et al., 1999). Furthermore, it has been reported that Vif specifically and strongly interacts with Gag in vitro (Bouyac et al., 1997). The C terminus of Vif has also been implicated in Gag-binding and, again, this interaction has been proposed to be important for Vif function. We have previously described the properties of a patient-derived HIV-1 vif gene product, VifA45-2 (referred to here as VifA45; Ochsenbauer et al., 1996), which has a C-terminal truncation of 19 amino acids as a result of a premature stop codon. Despite the C-terminal truncation, VifA45 is functional and HIV virions, derived from pNL-VifA45, were infectious in both non-permissive H9 cells and peripheral blood mononuclear cells. This was somewhat surprising due to the proposed importance of the C-terminal region in mediating binding to cellular membranes and Gag, properties that had been previously implied to be essential to Vif function. It has previously not been possible to further analyse the properties of VifA45 since available antibodies, reactive with VifWt, do not recognize the variant VifA45 protein. We have generated specific antibodies to VifA45 and, in this study, we characterize further the properties of Vif with respect to intracellular distribution, putative interactions with Gag proteins and effects on components of the cellular intermediate filament network.

### Methods

#### Constructs

The patient-derived vif gene vifA45-2, which encodes a Vif protein lacking 19 C-terminal amino acids, and the respective provirus construct, which encodes vifA45-2 (referred to here as pNL-VifA45), have previously been described (Wieland et al., 1994; Ochsenbauer et al., 1996). vifA45open was generated using PCR technology by changing the premature stop codon TGA at codon position 174 back to TGG, which encodes tryptophan as in the full-length Vif of the same patient. The provirus vector encoding this gene is referred to as pNL-VifA45open. A provirus construct with a frame-shift mutation in the vif gene (filled-in NdeI site at position 5122, according to the pNL4-3 nucleotide numbering; Adachi et al., 1986) is referred to as pNL-Vif-minus.

The subvirus construct pNL-A1(CD4<sup>+</sup>) (Willey et al., 1992; Strebel et al., 1987) with the intron between SD1 and SA2 missing, i.e. nucleotide 743 has been joined to nucleotide 4913, contains vif as the first ORF and results in high-level expression of Vif (Strebel et al., 1987; Karczewski & Strebel, 1996). A BssHII–EcoRI fragment (nucleotides 711–5743) from this plasmid was replaced by BssHII/EcoRI-digested PCR fragments derived from the different pNL derivatives. The upstream chimeric primer employed, which contains the BssHII site, was from nucleotides 706–716 fused to nucleotides 4911–4933 and the downstream primer from nucleotides 5890–5869 within the tat gene. In comparison to pNL-A1(CD4<sup>+</sup>), this results in the deletion of 27 nucleotides downstream of BssHII and the insertion of two nucleotides upstream of SA2. These changes had no effect on Vif expression. The new Vif expression constructs based on pNL-A1(CD4<sup>+</sup>) are referred to as pNL-A1-VifWt [the vif sequence has been derived from HIV strain BH10 as in pNL-A1(CD4<sup>+</sup>)]. pNL-A1-VifA45, pNL-A1-VifA45open and pNL-A1-Vif-
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minus. This latter construct expresses all the gene products from pNL-A1(CD4), except Vif, i.e. including mutated Env. Expression of Gag and pol sequences in eukaryotic cells was achieved employing pK-R-gpV (Mergener et al., 1992).

Plasmids for in vitro transcription/translation were generated by cloning PCR-amplified copies of the respective vif genes into appropriate plasmids downstream from the bacteriophage T3 promoter. The vif gene from plasmid pNDK (Spire et al., 1990) was amplified as described (Bouyac et al., 1997) and cloned into a PCR-blunt cloning vector (Invitrogen) to give plasmid pT3-VifWt (strain DK). The respective genes from pNL-VifA45 and pNL-VifA45open were amplified and cloned into the pACT vector (Promega) to give plasmids pT3-VifA45 and pT3-VifA45open. Plasmids for bacterial expression of the glutathione S-transferase (GST) protein alone or as a fusion protein with Gag or NC have been described previously (Bouyac et al., 1997).

**Antiserum reactive with VifWt and VifA45.** Rabbit antiserum against wild-type Vif, strain B310 (Los Alamos; referred to as anti-Vif(VI)), was generated by inoculating a rabbit with a mixture of N- and C-terminally histidine-tagged full-length VifWt proteins. These proteins had been expressed in bacteria using commercially available expression plasmids (Qiagen) and had been purified, after denaturation, by nickel–agarose affinity purification. Antiserum reactive with VifA45 (referred to as anti-VifA45) was prepared by inoculating a rabbit with bacterially expressed denatured full-length VifA45 fused to GST, expressed from the commercial plasmid pGENX-2T (Pharmacia) and purified by solubility criteria and gel electrophoresis.

**Expression and infectivity of provirus constructs with different Vif alleles.** Permissive 293T cells were transfected with 10 µg of the different provirus DNA constructs using standard calcium phosphate procedures. Equal amounts of virions, quantified by ELISA for HIV CA (Innogenetics), released into the media at 48 h post-transfection (p.t.) were employed to infect fresh cultures of permissive MT-4 cells or non-permissive H9 cells. Infected cells were washed 16 h post-infection (p.i.) to remove input virus and fed with fresh medium. Subsequently, samples of medium were collected every 3 days and the newly synthesized virions were quantified for HIV CA by ELISA.

**Expression of different Vif alleles from subvirus expression plasmids in permissive HeLa and COS-7 cells.** Transfection with 2 µg subgenomic pNL-A1-Vif expression plasmids in the presence or absence of 2 µg pk-R-gpV was achieved in HeLa cells employing Lipofectamine (Life Technologies) and in COS-7 cells employing FuGENE (Roche Diagnostics), as described by the manufacturers. Transfected cells growing on glass cover slips were fixed and permeabilized with acetone–methanol (1:1) at 40 h p.i. Indirect immunofluorescence was then carried out using combinations of rabbit anti-Vif(VI), rabbit anti-VifA45, rabbit anti-HIV gp120 (Bosch & Pawlita, 1990; human anti-HIV serum, mouse anti-vimentin 3B4 (Progen) (Herrmann et al., 1993), mouse anti-tubulin B-5-1-2 (Sigma), guinea pig anti-pectin P1 (Schroder et al., 1999) or culture supernatant from mouse hybridoma 183 cells (Chesebro et al., 1992) containing antibody specific for HIV p24 Gag. The respective secondary antibodies labelled with fluorescein or rhodamine were purchased from Dianova. The fluorescent labelled cells were analysed by confocal fluorescence microscopy using a Carl Zeiss (Jena) LSM 510 UV laser scanning microscope equipped with a HeNe (543 nm wavelength) and an argon ion (488 nm wavelength) laser as light sources and the corresponding beam splitters and barrier filters. The microscope was used in the multitracking scanning mode to avoid bleed-through of the fluorescent dyes, i.e. each scan-line is alternately illuminated by only one laser.

**In vitro Vif–Gag interaction.** The different pT3-Vif plasmids or T3-luciferase control DNA (Promega) were used for in vitro transcription/translation in the presence of [35S]methionine (> 1000 Ci/mmol; Amersham) using the Tnt T3 Wheat Germ Extract system (Promega), as recommended by the manufacturer. Radio-labelled proteins were resolved by SDS–PAGE on a 12% gel and revealed by autoradiography using Kodak X-Omat films. Bacteria expressing the GST, GST–Gag and GST–NC proteins were kindly provided by B. Spire (Bouyac et al., 1997). The different proteins were prepared essentially as follows. Briefly, transformed E. coli Top 10 cells were grown at 37 °C to an OD600 of about 1.0. Expression of the fusion proteins was then induced with 1 mM IPTG for 3 h at 30 °C. Bacterial cultures were pelleted by centrifugation at 5000 g for 15 min at 4 °C and then resuspended in 1/10 vol. of MT–PBS (150 mM NaCl, 125 mM Na2HPO4, 2.5 mM KH2PO4, 100 mM EDTA, pH 7.5). Bacteria were lysed on ice by mild sonication, the lysates were adjusted to 1% Triton X-100 and incubated on ice for 30 min before clearing by centrifugation at 15000 g for 30 min at 4 °C. Glutathione–agarose beads (Sigma), previously resuspended in MT–PBS, were added to the cleared supernatant for 1 h at 4 °C. Beads were then extensively washed in 1 M NaCl and then in PBS–0.1% Triton X-100 supplemented with a protease inhibitor cocktail (1 µg/ml each of aprotinin, leupeptin, pepstatin A and antipain, 100 µg/ml PMSF, 200 µg/ml pefabloc). Beads were then resuspended in SDS–PAGE sample buffer and the bound GST fusion proteins were resolved by SDS–PAGE on a 12% gel. Proteins were visualized by Coomassie-blue staining. Equal amounts of GST or GST fusion proteins bound to glutathione–agarose beads were incubated in vitro with [35S]methionine-labelled Vif proteins in Tnt binding buffer containing 50 mM Tris–HCl, pH 7.6, 0.2% Tween 20 and 150 mM NaCl, in the presence of BSA (100 µg/ml) for 1 h at 4 °C. Beads were washed in Tnt buffer and resuspended in SDS–PAGE sample buffer. The bound proteins were resolved by SDS–PAGE on a 12% gel, revealed by autoradiography, quantified with a Fuji phosphorimager and the data analysed by MacBas version 2.5 software (Fuji Photo Film).

**Differential solubility of VifWt, VifA45 and VifA45open.** HeLa or COS-7 cells transfected with pNL-A1-VifWt, pNL-A1-VifA45 or pNL-A1-VifA45open were metabolically labelled for 5 h at different time-points p.t. with 100 µCi/ml [35S]methionine and [35S]cysteine (Promix, Amersham). Subsequently, labelled cells were suspended in PBS and detergent extracted, essentially as described by Karczewski & Strebel (1996). Briefly, cells were lysed by four cycles of freezing and thawing (3 min each at −70 °C and 37 °C). The supernatants containing soluble cytoplasmic proteins (referred to here as the soluble fraction, S) were separated by centrifugation at 15000 g for 5 min. Insoluble material was extracted with CHAPS/DOC buffer (50 mM Tris–HCl, pH 8.5, 5 mM EDTA, 100 mM NaCl, 0.5% CHAPS, 0.2% deoxycholate) by incubation for 5 min at room temperature and centrifugation for 5 min at 15000 g. The resulting supernatant is referred to as the membrane fraction (M), since it contains membrane proteins. However, this should not imply that all proteins in this fraction necessarily originate from cellular membranes. Detergent-resistant material was solubilized by boiling the samples in 1% SDS, 2.5% mercaptoethanol, 2.5% glyceral and 0.031 M Tris–HCl, pH 8.0 for 15 min at 80 °C. This sample, referred to as the insoluble fraction (I) was clarified by centrifugation at 15000 g for 10 min. The buffers in all three fractions were adjusted to contain 1% Triton, 0.5% deoxycholate and 0.1% SDS in PBS (RIPA buffer) and immunoprecipitation performed using anti-ViWt, anti-VifA45 or anti-gp120 sera plus protein A-Sepharose (Pharmacia). Immunoprecipitates were analysed by PAGE on a 15% gel and autoradiographed as described previously (Pfeiffer et al., 1997).
Results

Infectivities of HIV virions encoding VifWt, VifA45, VifA45open and Vif-minus in H9 cells

The infection kinetics initiated by equal amounts of virions with different Vif alleles in non-permissive H9 cells are shown in Fig. 1. As we have previously described, there is no significant difference in the infection kinetics employing pNL4-3 and pNL-VifA45, showing that the 19 C-terminal amino acids, which are missing from VifA45, are not required for Vif function (Ochsenbauer et al., 1996). Furthermore, as has been demonstrated by sequencing of integrated provirus DNA, infection does not result in reversion of the premature stop codon in vifA45 (Ochsenbauer et al., 1996). The infection kinetics with pNL-VifA45open, now containing the 19 C-terminal amino acids missing in VifA45, are also not significantly different from pNL4-3, which shows that the vifA45open gene product is also as functional as the vifA45 gene. On the other hand, as previously demonstrated, virus encoded by pNL-Vif-minus does not give rise to a spreading infection in H9 cells. Thus, although both vifA45 and vifA45open show quite divergent sequences in comparison to pNL-Vif, both encode functional Vif proteins, since virions containing these genes are capable of supporting HIV infection in non-permissive H9 cells.

We had previously been unable to efficiently detect VifA45 protein in transfected or infected cells using any of a variety of available antisera prepared against VifWt. This is presumably due to the fact that, in comparison to Vif from pNL4-3 or BH10, VifA45 contains a non-conservative amino acid substitution (K→E) in the first Vif antigenic domain (aa 87–94) (Wieland et al., 1991) and, additionally, five out of the seven amino acids of the major second antigenic domain (aa 172–179) are missing. Thus, new rabbit antisera specific for VifWt (BH10) and VifA45, respectively, were generated and now allow the immunological detection of VifWt, VifA45 and VifA45open. In immunofluorescence analyses, neither antiserum showed any reactivity with untransfected cells. Anti-VifWt serum reacted with expressed VifWt, VifA45open and, less strongly, with VifA45, whereas anti-VifA45 serum reacted with VifA45open and, less strongly, with VifWt (data not shown).

Confocal microscopy analyses of VifWt, VifA45, VifA45open and Gag in transfected HeLa cells

It has been implied that the basic C-terminal region of VifWt plays a role in the subcellular localization and putative interactions of Vif with cellular and viral components. Thus, making use of the newly generated antisera, we initially employed indirect immunofluorescence and confocal microscopy to analyse the subcellular distributions of the different Vif proteins in HeLa cells, and compared these to the subcellular distribution of coexpressed Gag protein. Fig. 2 illustrates the different types of Vif protein localizations that were observed and the percentages of cells, transfected with pNL-A1-VifWt, pNL-A1-VifA45 and pNL-A1-VifA45open, respectively, which exhibit these distributions. The bulk of the cells (approximately 80–90%) transfected with pNL-A1-VifA45 exhibited a somewhat punctate distribution of VifWt throughout the cytoplasmic region and a weaker fluorescence signal in the nucleus (Fig. 2, left). This intracellular distribution was not affected by the fixation procedure, as has been reported to be the case with the vif gene product from feline immunodeficiency virus (Chatterji et al., 2000). Thus, our usual fixation procedure in either acetone–methanol or paraformaldehyde yielded similar results. However, in agreement with previously published results (Karczewski & Strebel, 1996), about 10–20% of the cells expressing VifWt showed a different distribution of the Vif protein; in these cases, VifWt exhibited either ‘stringy’ aggregation on a background of diffuse cytoplasmic staining (Fig. 2, middle) or almost complete aggregation to a perinuclear structure (Fig. 2, right). In strong contrast to VifWt, most of the cells (80–90%) expressing VifA45 exhibit stringy or perinuclear Vif staining. The localization to stringy or perinuclear aggregates is even more obvious in the case of VifA45open and virtually all of the transfected cells (99%) exhibit this Vif localization. This localization was not a property of the anti-VifA45 serum but was also observed with anti-VifWt serum, which also reacts well with VifA45open. This distinct localization in comparison to VifWt is thus independent of the C-terminal 19 amino acids and must be due to one or a combination of further sequence differences that exist between VifWt and VifA45open (Wieland et al., 1994).

Fig. 3 shows indirect double immunofluorescence of HeLa cells coexpressing Vif and Gag. The upper panels (A–C) show a cell expressing VifWt, which is distributed predominantly within the cytoplasmic region of the cell (A), and Gag, which is similarly distributed in the cytoplasm (B), i.e. in these cells there is a rough colocalization of the bulk of VifWt and Gag.
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Fig. 2. Indirect immunofluorescence and confocal microscopy of the different possible distributions of Vif protein in transfected HeLa cells at 40 h p.t. Cytoplasmic distribution (left) as exemplified by VifWt, stringy aggregation (middle) and perinuclear aggregation (right) as exemplified by VifA45. The percentages of transfected cells exhibiting the respective Vif-distributions are given below each panel for VifWt, VifA45 and VifA45open.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cytoplasmic</th>
<th>Stringy Aggregation</th>
<th>Perinuclear Aggregation</th>
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<tr>
<td>VifWt</td>
<td>88%</td>
<td>4%</td>
<td>8%</td>
</tr>
<tr>
<td>VifA45</td>
<td>13%</td>
<td>3%</td>
<td>84%</td>
</tr>
<tr>
<td>VifA45open</td>
<td>1%</td>
<td>1%</td>
<td>98%</td>
</tr>
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</table>

Fig. 3. HeLa cells cotransfected with pNL-A1-Vif/BH10 (A)–(C) or pNL-A1-VifA45 (D)–(F) and pK-R-gpV were fixed at 40 h p.t. and subjected to indirect double immunofluorescence and confocal microscopy employing rabbit anti-VifWt (A), anti-VifA45 (D) and mouse monoclonal anti-CA (B, E). Merged images of (A) and (B), and (D) and (E) are shown in (C) and (F), respectively. Secondary anti-rabbit antibodies were labelled with fluorescein and anti-mouse antibodies with rhodamine.

Fig. 4. The in vitro association of Vif proteins with Gag. Percentages of input radioactive protein, which has remained bound to GST (columns a), GST–NC (columns b) and GST–Gag (columns c), are given for luciferase (columns 1), VifWt (columns 2), VifA45 (columns 3) and VifA45open (columns 4). Averages from three experiments are given.

(C). In contrast, the lower panels (D–F) show a cell expressing VifA45 present as a perinuclear aggregate (D), and Gag, which is still distributed within the cytoplasmic region of the cell (E), i.e. in these cells most of the expressed Gag protein does not colocalize with VifA45 (F). This complete lack of colocalization of Vif and Gag was also observed in all cells expressing VifA45open (not shown). Since both VifA45 and VifA45open are functional (Fig. 1), these results make it unlikely that
colocalization of Vif with Gag is important for its mode of action.

**In vitro interaction of Vif proteins with Gag**

Since VifA45 is fully functional in supporting HIV infection in non-permissive cells (above and Ochsenbauer et al., 1996), but shows little colocalization with Gag in transfected cells, we were interested in examining its association with Gag in an in vitro binding assay. For this purpose, the genes for VifWt, in this case from strain NDK (see Methods), VifA45, VifA45open and, as control, the luciferase gene were in vitro transcribed and translated in the presence of radioactive methionine. The $^3$H-labelled proteins were allowed to interact with GST–Gag, GST–NC and, as a negative control, GST bound to glutathione beads. After washing, bound radioactive material and the respective input radioactive protein preparations were subjected to gel electrophoresis. Radioactivity in the respective samples was quantified and the percentage of bound proteins (VifWt, VifA45, VifA45open and luciferase) was calculated in comparison to the input (Fig. 4). In line with published data (Bouyac et al., 1997), it can be seen that VifWt exhibits strong binding to GST–Gag ($\sim 80\%$ of input) as well as to GST–NC ($\sim 25\%$ of input). Binding to GST alone is very low ($\sim 2\%$ of input). In contrast, the binding of both radioactive VifA45 and VifA45open to GST–Gag ($\sim 15\%$ of input) and GST–NC ($\sim 8\%$ of input) is much weaker than that of VifWt. It is, in fact, only about threefold higher than the binding of metabolically-labelled luciferase protein, which serves as a negative control for unspecific binding. These data show that binding of VifA45 to GST–Gag and GST–NC is much lower than that of VifWt and, as demonstrated in the case of VifA45open, the presence of an intact C-terminal domain does not improve binding of Gag. Thus we can conclude that strong binding of Vif to Gag is not a requirement for Vif function and, in the case of VifA45open, is not mediated by the basic C-terminal domain.

**Solubility properties of Vif proteins in transfected cells**

The basic C-terminal region of VifWt has been implicated in Vif binding to cellular membranes in vitro (Goncalves et al., 1994, 1995). We were thus interested in comparing VifWt, VifA45 and VifA45open in transfected cells with respect to their solubility in aqueous and detergent buffers. For this purpose, HeLa or COS-7 cells were transfected with pNL-A1-VifWt, pNL-A1-VifA45 and pNL-A1-VifA45open in transfected cells with respect to their solubility in aqueous and detergent buffers. For this purpose, HeLa or COS-7 cells were transfected with pNL-A1-VifWt, pNL-A1-VifA45 or pNL-A1-VifA45open and metabolically labelled at 48–52 h p.t. Additionally, transfected COS-7 cells, which in our hands were more efficiently transfected than HeLa cells, were metabolically labelled at 20–25 h p.t. Cells were processed to yield a fraction soluble in aqueous buffer (S fraction), a fraction soluble in CHAPS/DOC buffer (M fraction) and an insoluble fraction (I fraction). Gel electrophoreses and quantification of immunoprecipitated proteins from the respective fractions are shown in Fig. 5. It is important that Env, which is coexpressed from all of the pNL-A1 constructs, localizes exclusively to the detergent (M) fraction (data not shown), confirming that this fraction contains membrane proteins. In both transfected HeLa and COS-7 cells, the various Vif species were present in all the fractions and no
Fig. 6. (A)–(C) and (D)–(F) show indirect double immunofluorescence for Vif protein (shown alone in A and D) and vimentin (shown alone in B and E) with the respective merged images shown in (C) and (F), respectively. The cell shown in (A)–(C) exhibits cytoplasmic staining of VifWt and the cell shown in (D)–(F) exhibits perinuclear aggregation of VifA45. (H)–(J) and (K)–(M) show indirect double immunofluorescence for Vif protein (shown alone in H and K) and plectin (shown alone in I and L), with the respective merged images shown in (J) and (M), respectively. The cell shown in (H)–(J) exhibits cytoplasmic staining of VifWt and the cell shown in (K)–(M) exhibits perinuclear aggregation of VifA45. (O)–(Q) and (R)–(T) show indirect double immunofluorescence for Vif protein (shown alone in O and R) and tubulin (shown alone in P and S), with the respective merged images shown in (Q) and (T), respectively. The cell shown in (O)–(Q) exhibits cytoplasmic staining of VifWt and the cell shown in (R)–(T) exhibits perinuclear aggregation of VifA45. The negative controls in (G), (N) and (U) of cells transfected with pNL-A1-Vif-minus are merged images after double-staining with anti-HIV (red fluorescence) plus anti-vimentin (green fluorescence) (G), anti-plectin (N) or anti-tubulin (U), respectively. All transfected cells were examined at 40 h p.t.
### Table 1. Distribution of vimentin and plectin in HeLa and COS-7 cells expressing VifWt, VifA45, VifA45open or Vif-minus

(a) Percentages of cells expressing VifWt, VifA45, VifA45open and Vif-minus and displaying a cytoplasmic network (N), stringy (S) or aggregated (A) distribution of vimentin or plectin are given.

<table>
<thead>
<tr>
<th>Protein</th>
<th>VifWt</th>
<th>VifA45</th>
<th>VifA45open</th>
<th>Vif-minus</th>
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<tr>
<td></td>
<td>N</td>
<td>S</td>
<td>A</td>
<td>N</td>
</tr>
<tr>
<td>Vimentin</td>
<td>94</td>
<td>1</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Plectin</td>
<td>83</td>
<td>8</td>
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</table>

(b) Percentage of COS-7 cells expressing VifWt, VifA45, VifA45open or Vif-minus and displaying aggregated distributions of vimentin and plectin.

<table>
<thead>
<tr>
<th>Protein</th>
<th>VifWt</th>
<th>VifA45</th>
<th>VifA45open</th>
<th>Vif-minus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin</td>
<td>79</td>
<td>93</td>
<td>98</td>
<td>6</td>
</tr>
<tr>
<td>Plectin</td>
<td>91</td>
<td>95</td>
<td>99</td>
<td>16</td>
</tr>
</tbody>
</table>

Dramatic differences in the relative distributions of VifWt, VifA45 and VifA45open within the S, M and I fractions were discernible. In all cases, approximately equivalent amounts (~10–20%) of the Vif proteins were present in the M fraction. A minor but reproducible difference was that in both HeLa and COS-7 cells at 20 h p.t., both VifA45 and VifA45open were slightly less soluble (i.e. less in the S fraction and more in the I fraction) in comparison to VifWt. A further reproducible observation was that in COS-7 cells labelled at 48 h p.t., the amount of Vif in the S fraction was less than that observed at 20 h p.t. There were no significant differences in the distributions of VifA45 and VifA45open, indicating that the C-terminal region of the Vif protein, which is missing in VifA45, does not play a major role in determining localization of VifA45open to the S, M or I fractions.

### Effect of expression of the different Vif proteins on the distribution of vimentin, plectin and tubulin in HeLa cells

Karczewski & Strebel (1996) have previously reported that in HeLa cells transfected with pNL-A1-VifWt, Vif and the intermediate filament protein vimentin appear to colocalize and that, in a subpopulation of cells, both appear to be present in a perinuclear aggregation. In view of the distinctly different subcellular localizations of VifA45 and VifA45open in comparison to VifWt, as observed by confocal microscopy of transfected HeLa cells (Fig. 2), it was of interest to examine the effects of their expression on the localization of vimentin, tubulin and the cytoskeleton-bridging protein plectin (Herrmann & Aebi, 2000). The negative controls for these analyses were cells transfected with the construct pNL-A1-Vif-minus, which, apart from vif, expresses the same gene products as pNL-A1-VifWt, including the mutated env gene product unable to bind to CD4. Transfection of and expression from this construct were analysed using anti-HIV serum. In transfected HeLa cells, as has been previously reported for VifWt (Karczewski & Strebel, 1996), the immunofluorescence patterns for cellular vimentin roughly overlapped those of the different coexpressed Vif proteins. Thus, in the case of most cells expressing VifWt, the distribution of vimentin remained unaltered and exhibited a relatively even cytoplasmic distribution as does the VifWt protein itself (Fig. 6A–C). However, in those cells in which the Vif protein was localized to a stringy or perinuclear aggregate, i.e. in a subpopulation of cells expressing VifWt, in most cells expressing VifA45 and in all cells expressing VifA45open, vimentin was no longer distributed throughout the cytoplasm but rather colocalized to the same structures as Vif (Fig. 6D–F). We have extended our analyses to an examination of possible effects of Vif expression on the localization of plectin, a protein implicated in cytoskeleton network organization (Herrmann & Aebi, 2000).
Interestingly, plectin also exhibited the same behaviour as vimentin, i.e. in those cells in which Vif was localized in the cytoplasm, plectin was also localized in the cytoplasm with a network appearance (Fig. 6H–J). However, in cells in which Vif exhibited stringy or perinuclear aggregation, plectin no longer localized to a cytoplasmic network but became relocalized to these same structures (Fig. 6K–M). Expression of the different Vif proteins did not affect the localization of tubulin and, irrespective of whether Vif was distributed throughout the cytoplasmic region (Fig. 6O–Q) or in a perinuclear aggregate (Fig. 6R–T), the pattern of tubulin localization remained unaltered. In the negative controls, i.e. in cells transfected with pNL-A1-Vif-minus, there were, for the most part, no detectable changes in the localizations of vimentin, plectin or tubulin. In these cases, the immunofluorescence patterns obtained roughly overlapped the staining pattern that was obtained using anti-HIV serum. This latter serum detects the defective env gene product, encoded by pNL-A1 which have resulted in the very significant effects on the vimentin and plectin localizations observed. Table 1(a) summarizes the distributions of vimentin and plectin in HeLa cells expressing VifWt, VifA45, VifA45open and Vif-minus.

**Effect of expression of VifWt, VifA45 and VifA45open on the distribution of vimentin, plectin and tubulin in COS-7 cells**

Fig. 7 illustrates the subcellular distribution of the different Vif protein species in COS-7 cells and the effects of their expression on vimentin, plectin and tubulin localization. In contrast to the distribution in HeLa cells, all of the Vif proteins exhibit relatively even cytoplasmic localizations. However, despite the relatively even distribution of the different Vif proteins in the cytoplasm, vimentin (Fig. 7A–C) and plectin (Fig. 7E–G) but not tubulin (Fig. 7I–K) exhibited localizations to perinuclear aggregates in most of the transfected cells. The negative controls in which pNL-Vif-minus was expressed did not exhibit alterations in vimentin, plectin or tubulin localizations (Fig. 7D, H, L), showing that the effects observed were due to expression of the respective Vif proteins. Thus, in COS-7 cells, the bulk of the respective Vif proteins does not
colocalize with aggregated vimentin or plectin, as occurs in HeLa cells. This makes it unlikely that the aggregation is a result of a direct interaction between Vif and these intermediate filament components. Table 1 summarizes the distributions of vimentin and plectin in COS-7 cells expressing VifWt, VifA45, VifA45open and Vif-minus.

**Discussion**

A current hypothesis suggests that Vif is required in non-permissive producer cells to positively influence virus particle morphogenesis such that released virions are infectious. The observations that VifWt and Gag appear to colocalize in infected cells (Simon et al., 1997) and that Vif exhibits strong binding to GST–Gag in *in vitro* assays (Bouyac et al., 1997) had been interpreted as pointing to a direct effect of Vif on Gag structural proteins. *In vitro* mutagenesis had indicated that the basic C terminus of Vif may be involved in both the binding of Gag and overall Vif function (Bouyac et al., 1997). However, our previous description of the functionality of the variant Vif protein, VifA45, already indicated that at least the C-terminal 19 amino acids were not essential for Vif function, at least in the context of the VifA45 molecule. It is of note that VifA45 exhibits further significant amino acid changes in comparison to Vif molecules from HIV-1 strain IIIB and HIV-1 strain NDK used previously (Bouyac et al., 1997; Goncalves et al., 1994, 1995); it is likely that some or all of these changes, which have arisen *in vivo*, contribute to maintaining the functionality of VifA45. By employing a newly generated antiserum reactive with VifA45, we have demonstrated that VifA45 does not colocalize with Gag in transfected HeLa cells. Furthermore, in a *similar in vitro* binding assay as that described by Bouyac et al. (1997), VifA45 exhibited only weak binding to GST–Gag, in fact only marginally greater than the binding of an irrelevant protein (luciferase) to GST–Gag. It is difficult to rigorously establish if this remaining weak binding of VifA45 to Gag is specific and of importance for Vif function or if it simply reflects a somewhat higher non-specific association to Gag than that observed with the negative control. Interestingly, the C-terminal region of VifA45 when present, as is the case in VifA45open, does not increase the binding of Gag. Thus, it is conceivable that, also in the case of Vif from strain NDK, which was employed in the previously described *in vitro* binding assays (Bouyac et al., 1997) and is also shown here to bind strongly to GST–Gag, deletion of the C terminus does not actually remove the Gag-binding domain of Vif but rather leads to the generation of an inactive Vif protein (strain NDK) that has lost both Gag-binding activity and function. It is of note in this context that in the caprine arthritis–encephalitis virus (CAEV) model system, binding of CAEV-Vif to CAEV-Gag does not correlate with CAEV-Vif function (Seroude et al., 2001) and is independent of the presence of 24 C-terminal amino acids of Vif (M. Suzan & G. Audoly, unpublished results).

By employing an *in vitro* membrane-binding assay, it was previously reported that Vif has an affinity for membranes *in vitro* and that this association is mediated by the basic C-terminal region (Goncalves et al., 1994, 1995). In cell fractionation analyses, a fraction of the Vif protein is soluble in aqueous buffers, another fraction is soluble in weak detergent buffers and a last fraction is insoluble in weak detergent. The fact that the detergent buffer fraction contains integral and peripheral membrane proteins like Env, which localizes exclusively to this fraction (data not shown), further suggests that a fraction of Vif could be associated with cellular membranes. However, our observation that approximately the same amounts of Vif, which represents about 10–20% of the total VifWt, VifA45 and VifA45open, are present in the detergent fraction of transfected HeLa and COS-7 cells, indicates that regions of the protein other than the C terminus mediate solubility in weak detergent. In confocal microscopy analyses, both VifA45 and VifA45open, expressed in HeLa cells, exhibit distinctly different immunofluorescence patterns as compared to VifWt. In most transfected cells, VifA45 and VifA45open are localized in stringy or perinuclear aggregates, whereas VifWt is localized and more evenly distributed within the cytoplasmic region. This distinct localization is thus not related to the presence or absence of the 19 C-terminal Vif amino acids but is presumably mediated by one or several other amino acids which differ between VifA45 and VifWt. It is perhaps surprising that this distinct localization, observed by confocal microscopy, does not result in a more significant difference in the solubility of VifA45 and VifA45open in HeLa cells in comparison to VifWt. In fact, the only difference observable is a slight decrease in the solubility of VifA45 and VifA45open in comparison to VifWt, so that slightly less of these components are present in the S fraction and slightly more in the I fraction.

In contrast to the localizations in HeLa cells, confocal microscopy analyses demonstrate that VifWt, VifA45 and VifA45open all exhibit a relatively even distribution within the cytoplasmic region of monkey COS-7 cells. This cell-specific difference in the distributions of VifA45 and VifA45open in HeLa and COS-7 cells, as observed by confocal microscopy, is intriguing but its significance, if any, is unclear.

In eukaryotic cells, three distinct yet interconnected filament systems are of central importance for the mechanical stability and dynamic behaviour of the cytoarchitecture. These are microfilaments consisting of actin, microtubules made from α/β-tubulin subunits and intermediate filaments made from fibrous proteins, including vimentin. Plectin and its isoforms are ubiquitous cytoskeletal cross-bridging proteins of very large size with characterized binding domains for all three types of filament systems (for review see Herrmann & Aebi, 2000). In both HeLa and COS-7 cells, the cellular localizations of vimentin and plectin but not microtubular protein tubulin are affected by coexpression of Vif protein from the pNL-A1 constructs. The fact that the microtubular network has remained unaffected shows that Vif does not exert a det-
rimental effect on all of the cellular filament systems. There are again, however, intriguing cell-specific differences. In HeLa cells, when the respective Vif protein species forms aggregates (as is especially prominent for VifA45 and VifA45open), vimentin and plectin also aggregate to similar stringy or perinuclear locations. Thus, there appears to be colocalization between Vif and vimentin or plectin in transfected HeLa cells. In contrast, although the respective Vif proteins all localize throughout the cytoplasmic region in COS-7 cells, vimentin and plectin form perinuclear aggregates, i.e. in these cells, there is very little colocalization, if any, between the respective Vif proteins and aggregated vimentin and plectin. These results in COS-7 cells suggest an indirect mechanism of action of Vif on vimentin and plectin organization whereby the nature of this putative indirect effect can only be speculated upon. It is known that intermediate filaments undergo dynamic changes during the cell-cycle and that this may be regulated by phosphorylation of vimentin, which is a substrate for several kinases involved in cell-cycle regulation (reviewed by Herrmann & Aebi, 2000). In another virus system, frog virus 3, phosphorylation of vimentin has been shown to be involved in virus-induced intermediate filament reorganization (Chen et al. 1991). On the other hand, it has recently been shown that in an early step of apoptosis, plectin is proteolytically cleaved at a defined position by caspase-8 and that this leads to a reorganization of the microfilament system (Stegh et al., 2000). Further investigations will be required to establish if any of these types of mechanism also play a role in the effect of HIV-1 Vif on intermediate filament organization shown here.

The dramatic effects on vimentin and plectin cellular localization, which have been shown here after transfection of cells with pNL-A1 constructs, were not encountered in HeLa or COS-7 cells transfected with provirus (pNL4-3) constructs from which Vif is less strongly expressed. Only occasionally, although significantly, were aggregated vimentin and plectin structures observed in some COS-7 cells expressing pNL-VifA45 (data not shown). It is also of importance to keep in mind that both HeLa and COS-7 cells are ‘permissive’ with respect to Vif, i.e. infectious virus is still released from these cells in the absence of any Vif protein. Nevertheless, we find it conceivable that the observations on intermediate filament relocalization do still reflect an essential, even if not such a dramatically visible, function of Vif in ‘non-permissive’ cells.

In this context, it is of interest that several studies report on effects of virus infection on cytoskeletal structure as well as on associations of viral proteins with cytoskeletal components. For example, as mentioned above, frog virus 3 interacts with the cytomatrix (Chen et al., 1986), the E1-E4 protein of human papillomavirus type 16 has been reported to bind to, and cause the collapse of, the cytokeratin matrix (Doorbar et al., 1991) and the E1B 19 kDa protein of adenovirus causes disruption of the intermediate filament network (White & Cipriani, 1990). More recently, Theiler’s murine encephalomyelitis virus, a picornavirus, has been shown to bind to desmin and vimentin and cause a structural rearrangement in the intermediate filament network (Nedellec et al., 1998). Also in these cases, the importance of these interactions and their putative functional involvement in virus replication and/or pathogenesis have not yet been elucidated.

In the case of HIV-1, it is intriguing that cytoskeletal proteins have also been detected inside released virions (Ott et al., 1996) and furthermore that, in a normal HIV infection, establishment of a functional reverse transcription complex involves the cytoskeleton (Bukrinskaya et al., 1998). It is conceivable that the nature of the virus-producing cell (permissive/non-permissive) may influence the ability, in the target cell, of the preintegration complex to attach to cytoskeletal components and be able to complete reverse transcription and nuclear transport. It is theoretically possible that a cytoskeletal protein itself, as a component of the virus particle, may mediate an essential interaction with the cytoskeleton in the infected target cell. Thus, a theory combining the observations made here with published data on the time-point of the infectivity block of vif-defective virions would be that the incorporation of this essential cytoskeletal component occurs only when virus is produced in permissive cells or in Vif-expressing non-permissive cells. Thus the function of Vif could be to influence the cytoskeleton in the producer cell such that the required cytoskeletal component becomes available for incorporation. While such speculations have no experimental support, they do at least provide a framework for future studies.

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