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Tryptophan 95, an Amino Acid Residue of the Caprine Arthritis Encephalitis Virus Vif Protein Which Is Essential for Virus Replication

Virginie Seroude,[1] Gilles Audoly,[1] Pablo Gluschankof,[1] and Marie Suzan[2]

INSERM U372, BP 176, 13178 Marseille Cedex 09, France

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The Caprine arthritis encephalitis virus (CAEV) *vif* gene was demonstrated to be essential for efficient virus replication. CAEV Vif deletion mutants demonstrated an attenuated replication phenotype in primary goat cell cultures and resulted in abortive infection when inoculated into goats. In this study, we determined the *in vitro* replication phenotype of five CAEV Vif point mutant infectious molecular clones and the ability of the corresponding *in vitro* translated Vif proteins to interact with the CAEV $Pr55^{gag}$ in the glutathione S–transferase (GST) binding assay. Here we show that (i) three of the mutants (S170E, S170G, S197G) behaved as the wild-type CAEV according to virus replication and Vif–Gag interactions; (ii) one mutant (Vif 6mut) was replication incompetent and bound weakly to GST–Gag fusion proteins; and (iii) one mutant (Vif RG) was impaired for replication while retaining its interaction properties. This mutant points out the critical importance of the CAEV Vif tryptophan residue at position 95 for efficient virus replication, defining for this lentivirus a functional domain unrelated to the Gag binding region.

Key Words: lentivirus; CAEV; replication; Vif mutants; interaction; functional domain.

INTRODUCTION

Caprine arthritis encephalitis virus (CAEV) is a lentivirus that infects goats worldwide and induces progressive degenerative inflammatory diseases in infected animals. The CAEV genome is related to other lentiviruses, and it harbors a *vif* gene in addition to the structural (*gag, pol, env*) and regulatory (*tat, rev*) genes (Saltarelli *et al.,* 1990). The *vif* gene is present in the genome of all known lentiviruses, except the equine infectious anemia virus (EIAV) (Oberste and Gonda, 1992), and is required *in vivo* to establish persistent infection and pathogenesis of CAEV, simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV) (Desrosiers *et al.,* 1998; Harmache *et al.,* 1996; Inoshima *et al.,* 1996). Our previous results clearly show that *in vitro* infection of primary goat synovial membrane cells or blood-derived macrophages with different CAEV Vif deletion mutants resulted in strongly attenuated virus replication due to a defect in the late stage of virus production (Harmache *et al.,* 1995). *In vitro* studies on *vif* genes mutants of HIV-1, FIV, and SIV revealed that the phenotype is dependent on the host cell type from which the virus is produced (Courcoul *et al.,* 1995; Fan and Peden, 1992; Fisher *et al.,* 1987; Gabuzda *et al.,* 1992; Gibbs *et al.,* 1994; Lockridge *et al.,* 1999; Sakai *et al.,* 1993; Shacklett and Luciw, 1994; Sova and Volsky, 1993; Tomonaga *et al.,* 1992; von Schwedler *et al.,* 1993). These results suggested crucial interaction(s) between Vif and host cell factor(s) for production of infectious virus. Altogether these findings are consistent with the idea that Vif functions at a late stage of the viral cycle such as assembly, budding, release, or maturation. However studies on HIV-1 Vif^- virions reported contradictory results in core protein composition (Bouyac *et al.,* 1997b; Fouchier *et al.,* 1996; Ochsenbauer *et al.,* 1997; Simm *et al.,* 1995) and core morphology (Borman *et al.,* 1995; Hoglund *et al.,* 1994). In the same way, analyses of HIV-1-infected cells produced conflicting data in regard to the co-localization of Vif and Gag and their association to cellular membranes (Goncalves *et al.,* 1995; Michaels *et al.,* 1993; Simon *et al.,* 1999a, 1997), while others demonstrated HIV-1 Vif and $Pr55^{gag}$ interactions (Bouyac *et al.,* 1997a; Huvent *et al.,* 1998). From all these studies, it comes out that a comprehensive knowledge of the Vif function(s) is still lacking.

Understanding the function of a protein at the molecular level needs the determination of functional motifs or domains. Although the different Vif proteins share little sequence homology, they all are highly basic proteins with an unusually high content in tryptophan residues. A conserved motif, SLQXLA, was identified among all the known Vif sequences (Oberste and Gonda, 1992) and demonstrated to be important for function (Harmache *et al.,* 1995; Simon *et al.,* 1999b; Yang *et al.,* 1996). Another

[1] Present permanent address: Unité des Rickettsies et Pathogènes Emergents, UPRES A6020 CNRS, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille cedex 5, France.

[2] To whom reprint requests should be addressed at Unité des Rickettsies et Pathogènes Emergents, UPRES A6020 CNRS, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille cedex 5, France. Fax: (33) 4 91 38 77 72. E-mail: marie.suzan@medecine.univ-mrs.fr.

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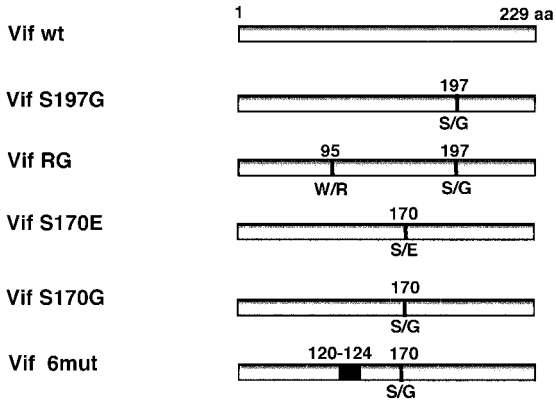

FIG. 1. Schematic representation of the five Vif mutant proteins used in this study. The *vif* gene mutants were obtained by PCR as described under Materials and Methods; they were either used for *in vitro* protein translation for the GST binding assay or subcloned into the CAEV molecular clone for replication phenotype determination.

common characteristic is the presence of potentially phosphorylated serine threonine residues. In the case of HIV-1, the phosphorylation of Ser^{144}, contained within the conserved SLQXLA motif, was demonstrated to be important for virus replication (Yang *et al.,* 1996). The CAEV Vif protein also contains several serine/threonine residues that could potentially be phosphorylated, especially Ser^{170}, within the SLQXLA motif, and Ser^{197}. Both of them are in the context of recognition motifs (R/KXXS*/T*) used by serine/threonine protein kinase such as cAMP- and cGMP-dependent protein kinase.

Defining functional domains of the CAEV Vif protein would help to understand the molecular mechanisms that underlie the function of this essential gene in CAEV replication and pathogenesis. In this study, we determined the replication phenotype of five CAEV Vif point mutants, and we analyzed the ability of the corresponding Vif proteins to interact with the CAEV $Pr55^{gag}$ in the *in vitro* glutathione S–transferase (GST) binding assay. Besides the fact that this work demonstrates that the Vif/Gag interaction seems to be conserved among distantly related lentiviruses such as CAEV and HIV-1, it identifies a functional domain of CAEV Vif, essential for virus replication, but distinct from the Gag binding region.

RESULTS

Mutational analysis of the CAEV Vif protein

To define amino acid residues important for Vif function, several Vif point mutants were created by PCR (Fig. 1). We focused on the potentially phosphorylated Ser residues at positions 170 and 197 since many protein–protein interactions are governed by phosphorylation. Moreover, Ser170 belongs to the Vif-conserved SLQXLA motif. Serine was replaced by a glycin (mutants S170G and S197G) or by a glutamic residue (mutant S170E) to mimic phosphorylation by replacing the potential phospho-Ser by a negatively charged amino acid residue in this latter case (Alessi *et al.,* 1994; Zhao *et al.,* 1994). During the selection of these mutants, two additional mutants were randomly obtained: the Vif RG mutant containing a W95R substitution in addition to S197G and the Vif 6mut where the 120–124 DRHFW sequence was replaced by SIVSR in addition to the S170G substitution. Interestingly, Trp at position 95 as well as Phe-Trp at position 123–124 are strictly conserved among the different sequenced maedi-visna and CAEV isolates (Quérat *et al.,* 1990; Saltarelli *et al.,* 1990; Sargan *et al.,* 1991; Sonigo *et al.,* 1985). Each of the *vif* mutants described above was then subcloned into the PK-9kb plasmid, containing most of the CAEV proviral DNA (Turelli *et al.,* 1996), using the overall strategy described under Materials and Methods. Viral supernatants were obtained by co-transfection of CFSM cells with each of PK-9kb plasmid, containing wt or mutant *vif* genes, with the overlapping BS-3′LTR plasmid (Turelli *et al.,* 1996), coding for the remaining sequence of CAEV proviral DNA. Viral replication was monitored by measuring the reverse transcriptase (RT) activity contained within cell-free supernatants collected after transfection. Infection experiments were performed with equal amounts of RT activity, and representative replication curves of the different CAEV Vif wt or mutants are shown in Fig. 2. As can be seen in Fig. 2A, the introduction of new *Bam*HI and *Mlu*I sites in the *vif* gene (Vif wt B/M) had no effect on the virus phenotype since both CAEV Vif wt or Vif wt B/M had similar replication profiles, with a peak in virus production at 12–16 days p.i. In the same way, infection of CFSM cells with CAEV Vif S170E or Vif S170G resulted in a Vif phenotype comparable to the CAEV Vif wt B/M (Fig. 2A). Sequencing of late recovered viruses demonstrates no reversion of these mutants to the Vif wt sequence (data not shown). On the contrary, when infection was performed with CAEV Vif 6mut, only background level of RT activity could be detected (Fig. 2A), characteristic of the Vif^- phenotype. These results indicated that the serine residue at position 170 was not involved in the Vif replication phenotype. However, changing the amino acid sequence at positions 120–124 had serious consequences on virus replication.

When compared to CAEV Vif wt B/M, CAEV Vif S197G induced a similar productive infection in CFSM cells (Fig. 2B). This result demonstrated the irrelevant role of Ser197 in virus replication as also shown above for Ser170. The wt replication phenotype of CAEV Vif S197G could not be due to a reversion to the Vif wt since no modification was observed in the virus sequences obtained at late time points (data not shown). In contrast, infection with CAEV Vif RG resulted in a strongly attenuated Vif^- phenotype (CAEV Vif RG-1, Fig. 2B). CAEV Vif RG-1 cell-free supernatants were taken at various time p.i., and the RT–PCR products of the viral RNA were submitted to nucleotide sequencing. The deduced amino

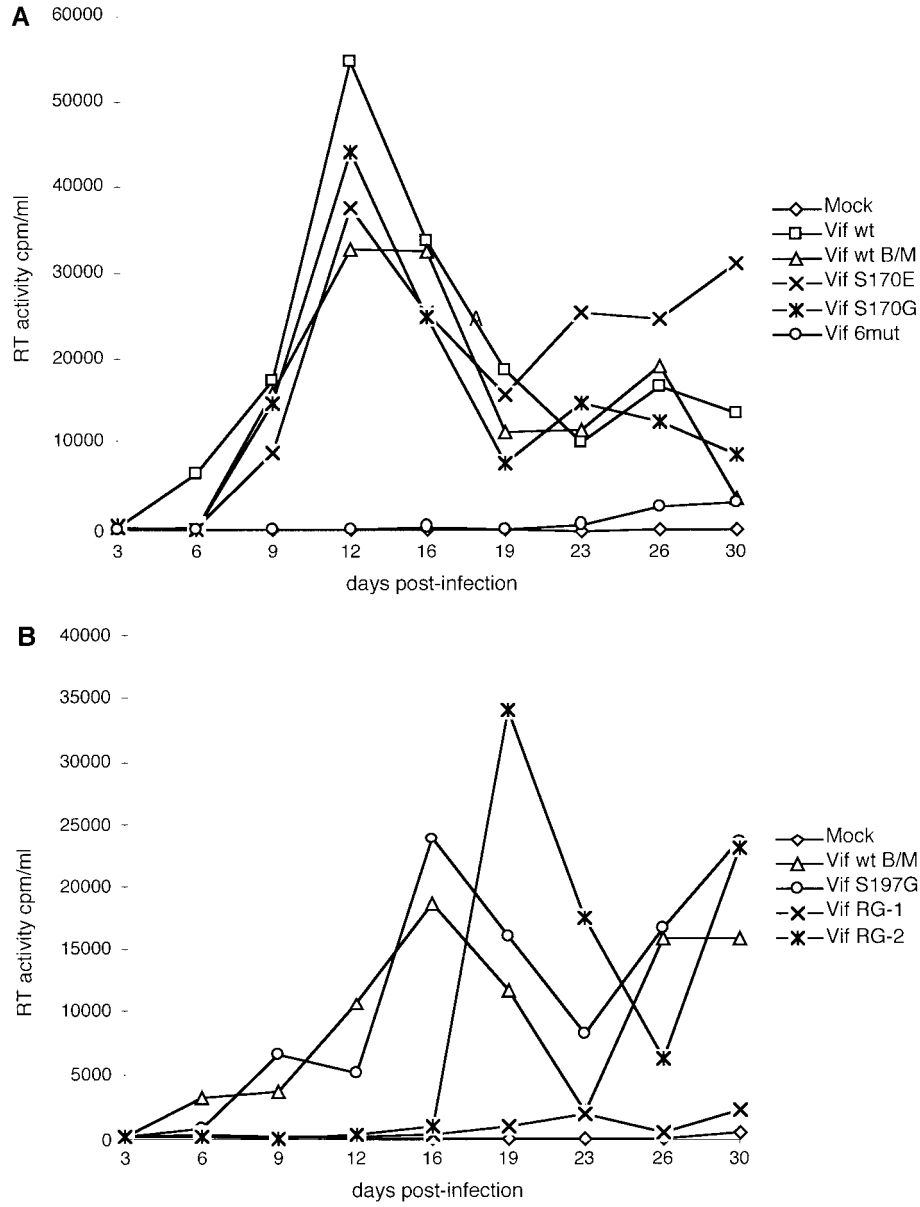

FIG. 2. Replication phenotype of the different CAEV Vif mutants. Viral stock supernatants were obtained by co-transfection of primary goat synovial membrane cells (CFSM) with the PK-9kb plasmids containing the different *vif* mutants and the BS-3′LTR clone to allow reconstitution of complete proviral DNA. The replication phenotype of the resulting viruses was determined by infection of CFSM cells with equal amounts of RT activity of each viral stock. The virus production was monitored by measuring the RT activity present in the cell-free supernatant.

acid sequences, between positions 26–216, are summarized in Fig. 3. When compared to the Vif wt primary sequence, the original Vif RG sequence deduced from the CAEV molecular clone harbored the two amino acid substitutions introduced by PCR, i.e., W95R and S197G. Among the five amino acid sequences shown, deduced from CAEV Vif RG-1 virions obtained after three different infection experiments, one had a stop codon at position 49 (Vif RG-1a), resulting in a truncated Vif protein. The four other clones (Vif RG-1b, -1c, -1d, and -1e) had full-length Vif sequences still containing the original W95R and S197G mutations. Three of them also harbored one or two additional amino acid changes introduced at different non-conserved positions of the known maedi-visna/CAEV Vif sequences, except the G53R substitution in the Vif RG-1d sequence. Since all these replication attenuated clones harbored the original W95R S197G mutations, it could be assumed that the additional random mutations detected are unrelated to the modification of the virus phenotype. Surprisingly, in some other infection experiments, using different viral stocks, CAEV Vif RG was able to establish a productive infection showing a Vif wt phenotype (CAEV Vif RG-2, Fig. 2B). Analysis of the Vif protein sequences deduced from three CAEV Vif RG-2 clones revealed that the arginine residue introduced at position 95 was reverted to a tryptophan, like in

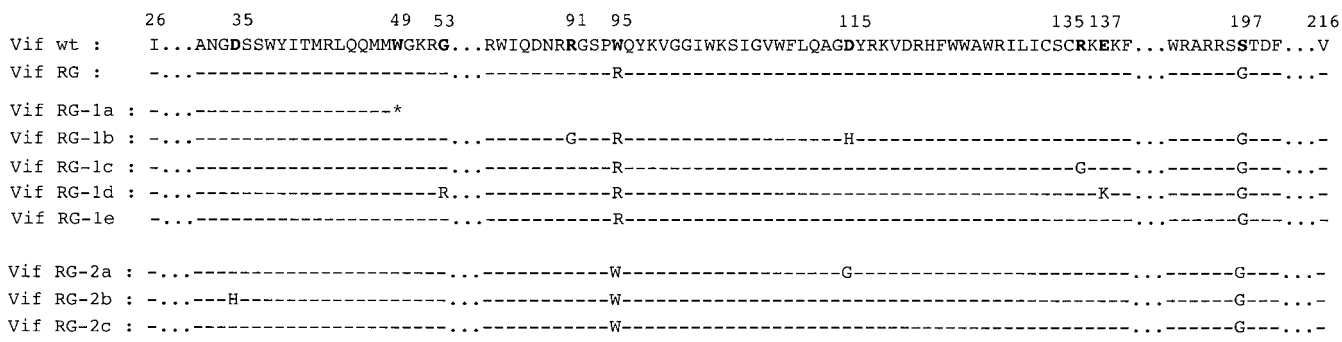

FIG. 3. Alignment of the Vif amino acid sequences. The *vif* genes were amplified by RT–PCR on RNA extracted from virions pelleted by ultra-centrifugation of cell-free supernatants. The Vif amino acid sequences, between positions 26 and 216, were deduced from the nucleotidic sequences and are shown in comparison to the Vif wt sequence. Gaps were introduced between positions 27 and 31, 54 and 83, 140 and 190 and 201 and 215 since no modifications were observed in these regions compared to the wt sequence. In the Vif wt sequence, the amino acid residues in bold are those which were found mutated in the different Vif isolates. In the original Vif RG mutant only the W95R and S197G substitutions were introduced. *, stop codon.

the Vif wt clone, while the original S197G mutation remained. As for the Vif RG-1 clones, some additional mutations were detected at non-conserved positions in two of three clones. From these results, it appeared that reversion from Arg to Trp at position 95 was clearly associated to restoration of the Vif wt phenotype in the corresponding virus population (Fig. 2B). Since Vif/Gag interaction was described to be related to the biological activity of HIV-1 Vif, it appeared interesting to test whether this might also be the case for the CAEV Vif protein, and whether this interaction would take place with the replication defective CAEV Vif mutants.

CAEV Vif protein interacts *in vitro* with the NC domain of the Pr55^{gag}

The CAEV *vif* gene was cloned in frame with the GST gene to produce the GST–Vif fusion protein. After binding to glutathione beads, GST or GST–Vif proteins were allowed to react with *in vitro* translated ^{35}S-methionine-labelled CAEV Pr55^{gag}. As shown in Fig. 4A, radiolabelled Gag protein was revealed by autoradiography after binding to GST–Vif, but not to GST, in the presence of 0.15 M NaCl. Conversely, the GST–Gag fusion protein showed a strong interaction with ^{35}S-labelled Vif protein, even in the presence of 0.5 M NaCl (Fig. 4B). No binding was observed between GST–Gag and *in vitro* translated ^{35}S-labelled luciferase (data not shown), suggesting the specificity of the interaction. These results demonstrated an *in vitro* interaction between CAEV Vif and Gag proteins. However, the difference in maximal ionic strength allowing binding between GST-Vif/Gag and GST-Gag/Vif might reflect a particular conformation of the GST–Vif fusion protein resulting in minimal fixation of Gag.

In the next step, we characterized which domain of the Pr55^{gag} was involved in this interaction using GST fusion proteins expressing the different mature domains of Gag. *In vitro* translated ^{35}S-labelled Vif protein was tested for binding with equal amounts of GST–CA, GST–MA, or GST–NC, in the presence of NaCl concentration ranging from 0.1 to 0.5 M. As shown in Fig. 4B, the CAEV Vif protein was unable to interact with GST–CA and bound weakly to GST–MA at 0.1 M NaCl. On the contrary, interaction with GST–NC was as efficient as with GST–Gag. The CAEV NC harbors two zinc (Zn) finger motifs of the **C**X2**C**X4**H**X4**C** type (Saltarelli *et al.,* 1990). To determine if the Zn finger motifs were involved in the GST–NC/Vif interaction, mutations were introduced to disturb the overall folding of one or the other of these motifs. The amino acid sequence of the first Zn finger motif (Z1) in the CAEV NC is located at positions 20–33 and the second one (Z2) at positions 39–52. PCR mutagenesis was used to replace cystein by glycin residues at positions 20 and 23 in Z1 and 39 and 42 in Z2. The corresponding *NC* mutant genes were cloned in frame with the *GST* gene to produce the GST–NC Z1* and GST–NC Z2* fusion proteins. These proteins were compared to the wild-type (wt) GST–NC for their ability to bind *in vitro* translated ^{35}S-labelled Vif protein (Fig. 4C). Mutations in either the Z1 or Z2 motifs of the CAEV NC resulted in 8- to 10-fold less interaction with Vif compared to GST–NC, as quantified by phosphorimager analysis (data not shown). Background level of binding could be detected on GST. As a control for the specificity of Vif/NC interaction through the Zn finger motifs, we measured the binding between Vif and an irrelevant Zn finger containing protein. The chosen protein was teashirt, a DNA binding protein with three Zn finger motifs, involved in the development of *Drosophila* (Alexandre *et al.,* 1996; Fasano *et al.,* 1991). No binding was observed between Vif and the GST–teashirt fusion protein (data not shown). These results demonstrated that the interaction of CAEV Vif with the viral NC domain of the Pr55^{gag} was specific and was dependent on the presence of the two NC Zn finger motifs.

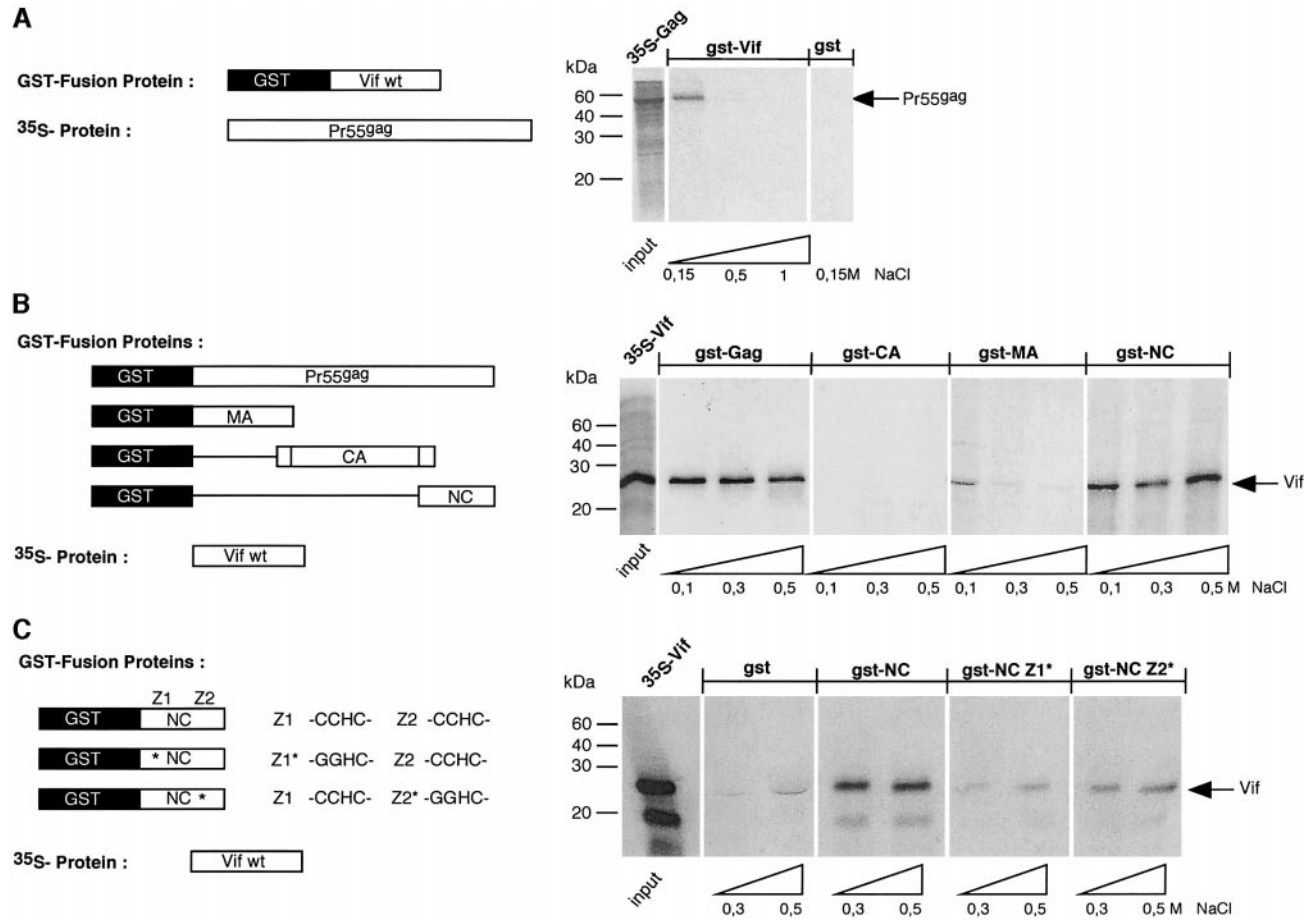

FIG. 4. *In vitro* interaction between CAEV Vif and Gag proteins. The different proteins tested in the GST binding assay are schematized on the left part of the figure. Pictures of representative interaction experiments are shown on the right part of the figure. (A) Binding of ^{35}S-Pr55^{gag} to the recombinant GST–Vif protein in the presence of increasing NaCl concentrations. (B) Binding of ^{35}S-Vif to the fusion proteins GST–Gag, GST–MA, GST–CA, or GST–NC at various NaCl concentrations. (C) Interaction of ^{35}S-Vif with GST–NC or with the mutant proteins modified in the first or second Zn finger motif (GST–NC Z1* and GST–NC Z2*, respectively). Equal amounts of GST or GST–fusion proteins were bound to glutathione beads and incubated with *in vitro* translated ^{35}S-labelled proteins. The amount of radioactive material used for each assay is shown in the left lane (input). Bound proteins were eluted, resolved on 12% SDS–PAGE and revealed by autoradiography.

Analysis of Vif mutants interaction with GST–Pr55^{gag} or GST–NC

Having demonstrated that interaction of CAEV Vif with the Pr55^{gag} occurred at the NC domain, we then tested whether this interaction could represent one of the molecular mechanisms involved in the Vif function on viral replication. The five-point mutant Vif proteins were *in vitro* translated in the presence of [^{35}S]methionine and allowed to react with equal amounts of GST, GST–NC, or GST–Gag proteins. Attempts to translate deletion mutants of the CAEV Vif protein were unsuccessful since these mutants proved to be either unstable or very inefficiently synthetised in the *in vitro* translation system (G. Audoly, unpublished results). As shown in Fig. 5, the Vif S197G, Vif RG, Vif S170E, or Vif S170G proteins bound to GST–NC or GST–Gag to the same extent as the wt protein. All but the Vif RG were associated to a wt replication phenotype (Fig. 2B). On the contrary, the Vif 6mut protein demonstrated a 10-fold less interaction capacity compared to the Vif wt, as determined by phosphorimager analysis (data not shown), while being impaired for replication (Fig. 2A). No interaction could be detected between all these proteins and GST. These results suggested that serine residues at positions 170 and 197 were neither implicated in virus replication nor in Vif/Gag interaction. The five amino acid sequence at position 120–124 defined a Vif region either directly involved in interaction and replication, or important to maintain the native protein conformation. These results suggested that the interaction, in an *in vitro* GST fusion-based assay, between CAEV Vif and the NC domain of the Pr55^{gag}, might be through conformational domains of both proteins. Interestingly, the Trp95 amino acid residue, essential for virus replication, seems to be unrelated to the Gag binding domain.

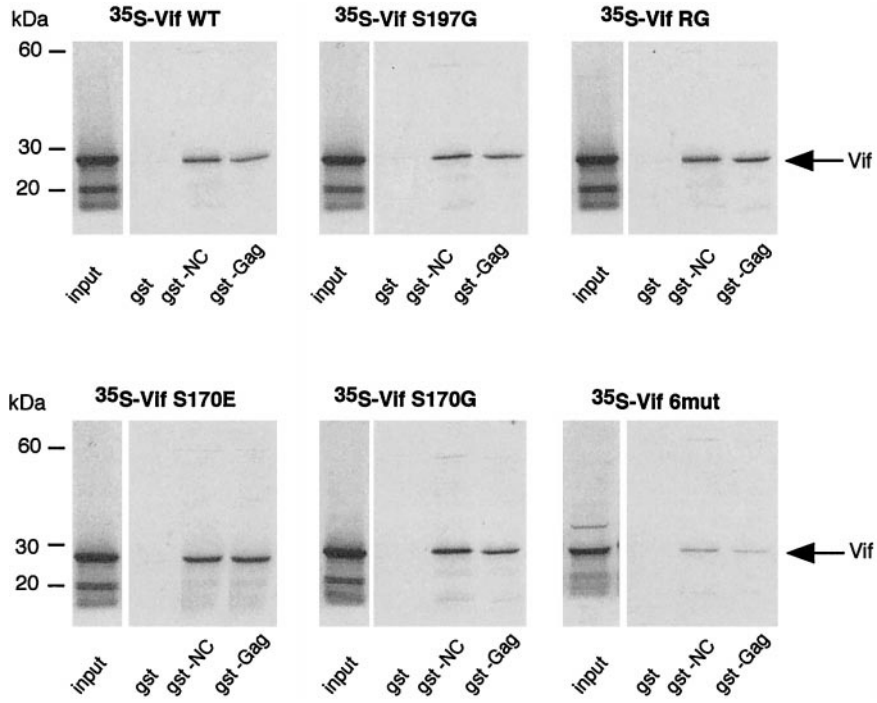

FIG. 5. *In vitro* interactions of CAEV Vif mutants with GST–Gag or GST–NC. The different ^{35}S-labelled Vif wt or mutant proteins were incubated with equal amounts of GST, GST–NC, or GST–Gag fixed to glutathione beads, in the presence of 0.15 M MaCl and analyzed as described in the legend to Fig. 4.

DISCUSSION

In this study, we analyzed the replication phenotype as well as the Gag interaction of several CAEV Vif point mutants in order to define functional motifs or domains. The results are summarized in Table 1. The Vif mutants harboring mutations on Ser 170 or Ser197 (Vif S170E, S170G or S197G) were indistinguishable from Vif wt concerning their interaction with Gag and their replication phenotype. These serine residues were targeted for mutagenesis for several reasons, (i) both are in the C-terminal region of Vif, and Ser170 is contained within the highly conserved SLQXLA motif in Vif proteins from all lentiviruses. Our previous work had shown that deletion of this motif resulted in CAEV impaired replication (Harmache *et al.,* 1995), whereas studies on HIV-1 and SIVmac revealed the importance of Ser144 phosphorylation in this motif for Vif function (Simon *et al.,* 1999b; Yang *et al.,* 1996). (ii) Both are thought to be potentially phosphorylated since they are localized in the context of recognition motifs used by serine/threonine protein kinases, cAMP- and cGMP-dependent protein kinase for Ser170 and Ser197, but also casein kinase II for Ser197. The present results showed that substitution of serine by either glycin or glutamic acid residues did not modify CAEV Vif activity, suggesting that phosphorylation of Ser170 or Ser197, if any, has no effect on CAEV Vif function. Computer data-base searches identified other potentially phosphorylated sites in the CAEV Vif sequence. Whether phosphorylation of these different residues plays a role in regulation of CAEV Vif function needs further analysis. Among the Vif mutants tested in this study, only the Vif 6mut protein exhibited both a Vif$^-$ phenotype when replaced into the CAEV molecular clone while being largely impaired in its interaction with Gag. In this mutant, the original DRHFW (120–124) amino acid sequence was replaced by SIVSR. The Phe-Trp residues are highly conserved among all known visna/CAEV Vif

TABLE 1

Results Summary

Vif sequence	Interaction with the NC domain of the Pr55^{gaga}	Replication in CFSM cells[b]
wt	+	+
S170E	+	+
S170G	+	+
6mut	−	−
S197G	+	+
RG	+	−
RG-1	+	−
RG-2	+	+

Note. Summary of the results obtained with the different CAEV Vif mutants as to their interaction with the NC domain of the Pr55^{gag} (a) and their replication phenotype in target cells (b).

[a] + indicates the detection of Vif/Gag interaction in the GST binding assay; − strongly diminished or lack of interaction.

[b] + indicates a CAEV Vif wt replication phenotype; − is for a strongly attenuated replication (Vif− phenotype).

sequences, and as non-polar amino acid residues, they are thought to be inside the protein. Their replacement by two polar amino acid residues, Ser-Arg, which are rather exposed on the outside of the protein, might have imposed a conformational change of the secondary structure of the CAEV Vif protein, thus having a strong impact on the replication phenotype and resulting in a 10-fold less interaction capacity with Gag than the Vif wt. This result strengthens the current idea that structure-function studies on the lentiviral Vif proteins are hindered by their unusual biochemical properties (Gabuzda *et al.,* 1992; Harmache *et al.,* 1995; Oberste and Gonda, 1992; Simon *et al.,* 1999b; Yang *et al.,* 1996).

Interestingly, the Vif RG mutant allowed us to demonstrate that the *in vitro* Vif/Gag interaction per se did not correlate with the Vif function on the viral replication cycle. Indeed the Vif RG protein bound to the GST–Gag or GST–NC as efficiently as the Vif wt, while showing a Vif$^-$ phenotype when introduced into the CAEV molecular clone. The Vif RG mutant protein contains the S197G and W95R substitutions. As the S197G modification was demonstrated to have no influence on Vif activity, it can be argued that the phenotype of the Vif RG mutant was due to the W95R substitution. Tryptophan at position 95 is localized in a strictly conserved WQYK sequence of Vif visna/CAEV isolates and its replacement by the polar arginine amino acid residue might result in the disruption of a functional domain of the protein. However, this change had only consequence on viral infectivity, suggesting that the two Vif properties examined in this study are independent and might be mediated by different functional domains of the protein. The critical role of Trp95 for Vif function in viral infectivity was assessed by the isolation of viruses that reverted from the Arg95 mutant to the wt sequence during the time course of infection. This result confirms our previous studies on a CAEV Vif *NcoI*$^-$ mutant (Harmache *et al.,* 1995), in which a frameshift insertion of 4 bp at the *Nco*I site resulted in a truncated 140 amino acids protein, showing an unrelated sequence starting at Trp95. Analysis of the *vif* gene sequences, contained within virions released during infection, revealed that reversions occurred in the variable 83–94 amino acid region, that resulted in restoration of full-length wt Vif sequence, starting at Trp95. These results suggest that this position is submitted to a high selection pressure and might represent an important functional region. In the case of HIV-1, the amino-terminal Trp21 residue was recently shown to be functionally important for Vif function in non-permissive cells (Boyce *et al.,* 1999). Altogether, these results led us to think that the many tryptophan residues conserved among visna/CAEV or HIV/SIV isolates might be essential in interactions with host cellular factor(s) important for the viral phenotype.

We demonstrated *in vitro* CAEV Vif/Gag interactions using the GST-binding assay, at the NC domain of the Pr55^{gag}. This interaction seems to occur through recognition of the native conformation of the NC domain since mutations resulting in the misfolding of the first or second Zn finger motif largely diminished the interaction with Vif. Since such retroviral NC mutants are generally known to be impaired in their binding to nucleic acid molecules, it remains to be determined whether the Vif fixation to NC is the result of direct interaction between these proteins or is mediated via the nucleic acid-NC complex. Recent reports described the HIV-1 Vif protein as an RNA binding protein (Dettenhofer *et al.,* 2000; Zhang *et al.,* 2000). In the case of CAEV, we performed RNase treatments of each GST–NC and Vif protein before or after interaction, but we could not observe any modification in the binding capacity of the treated. However, we could not exclude that short RNA sequences bound to the GST–NC and/or Vif proteins could be protected from the RNase action due to this binding (data not shown). Since it seems that *in vitro* Vif/Gag interaction is not directly related to Vif activity, the biological significance of these interactions is still an open question. Lentiviral Vif proteins are commonly recognized to be multi-functional proteins. In the case of HIV-1 Vif, the N-terminal domain is associated to the inhibition of viral protease activity (Kotler *et al.,* 1997; Potash *et al.,* 1998), while the C-terminal part is required for Gag and cell membrane binding (Bouyac *et al.,* 1997a; Goncalves *et al.,* 1994; Simon *et al.,* 1997). Co-localization, interaction, and membrane association of HIV-1 Vif and Gag are subject to controversial results (Simon *et al.,* 1999a) as well as the importance of the Vif C-terminal domain for interaction and function. Indeed, whereas some studies reported a 30% loss of Vif/Gag interaction using an HIV-1 Vif protein deleted of its 22 C-terminal amino acid residues (Bouyac *et al.,* 1997a) or the characterization of amino acid residues or domain in this region essential for Vif activity (Bouyac *et al.,* 1997b; Goncalves *et al.,* 1994, 1995; Simon *et al.,* 1999b), another report described the unimpaired function of a natural C-terminal truncated Vif protein derived from a primary HIV-1 isolate (Ochsenbauer *et al.,* 1996). In the caprine lentivirus, deletion of amino acids 189–205 from the Vif sequence was associated with an attenuated replication phenotype (Harmache *et al.,* 1995), whereas deletion of the last 24 C-terminal amino acid residues had no effect on the capacity to interact with the Pr55^{gag} (G. Audoly, unpublished results).

In vitro Vif/Gag specific interactions, now described for HIV-1 and CAEV, would not necessarily mean a stable association between these two proteins. It could be hypothesized that Vif interacts transiently with the Pr55^{gag} and also with species specific cellular factor(s) (Simon *et al.,* 1995, 1998b) at the site of virus assembly to allow the efficient production of infectious viruses. On the other hand and during earlier steps of the replication cycle, Vif might counteract an inhibitory activity associated to non-

permissive cell lines (Madani and Kabat, 1998; Simon *et al.,* 1998b) as well as regulate the viral protease activity. Definitive confirmation of the different functions and of the multiple viral or cellular partners of Vif still requires further studies to allow a better understanding of the regulation of lentiviruses replication.

MATERIALS AND METHODS

Construction of pGEX plasmids

The open reading frames of CAEV *gag* and *vif* were amplified by PCR from the PK-9kb plasmid (Turelli *et al.,* 1996) derived from the CAEV Cork infectious molecular clone (Saltarelli *et al.,* 1990). The PCR product of *gag* (nt 512–1859), obtained using the GagGEXI/CRT27 primer pair, was cloned into the *Bam*HI site, in frame with the glutathione S–transferase gene into the pGEX-5X-2 vector (Pharmacia). The MA (nt 512–940) and NC (nt 1616–1859) coding sequences were cloned into the *Bam*HI/*Eco*RI sites of the pGEX-5X-2 vector to produce the pGEX-MA and pGEX-NC plasmids, respectively. These plasmids were kindly provided by Dr. P. Turelli. The Z1 and Z2 NC mutants obtained by PCR with the SZ1/RZ1 and SZ2/RZ2 primer pairs, respectively, were inserted in frame into the *Eco*RI site of the pGEX-5X-2 vector. The GST-CA contained the entire coding sequence of the maedi-visna virus CA domain and the 3′-94 bp and 5′-96 bp flanking sequences from MA and NC, respectively. This construct was kindly provided by Dr. Zanoni, Institute of Veterinary Virology, Bern, Switzerland (Zanoni *et al.,* 1992). Amplification products of the CAEV *vif* gene (nt 5006–5692), using the VCECO/VCASECO primer pair, were cloned at the *Eco*RI site of the pGEX-5X-2 vector to obtain the pGEX-vif plasmid.

Mutagenesis of the CAEV *vif* gene

The *vif* gene of CAEV was amplified by PCR reaction using the high-fidelity thermostable polymerase (Roche) and the T3VC/VCASECO primer pair. The forward primer contains the T3 RNA polymerase recognition site upstream from the ATG initiation codon. The PCR product was directly cloned into the pTA cloning vector (Invitrogen) to generate the pTA-vif plasmid. Site-directed mutagenesis of the CAEV *vif* gene was realized by PCR on the pTA-vif plasmid. Different Vif mutants were created: Vif S170G was mutated at position 170 where serine was substituted by glycine (using the S170G/RS170G primers); Vif S170E where serine was substituted by glutamic acid (using the S170E/RS170E primers); and Vif S197G where the serine was substituted by a glycine (using the S197G/RS197G primers). Vif mutants that present both S197G and W95R mutations or both S170G and the substitution mutations at positions 120–124 (DRHFW/SIVSR) were randomly obtained, and characterized by DNA sequencing (Genome Express, Grenoble, France).

CAEV Vif mutants molecular clones were obtained as follows: the *Bst*EII-*Dra*III fragment (positions 3879–6640) was excised from the PK-9kb plasmid and subcloned into pUC19, to generate the pUC19-vif plasmid. The *Bam*HI and *Mlu*I restriction sites were then created by PCR at positions 5079 and 5636, using the SBHI/RBHI and the 33*Mlu*I/AS*Mlu*I primer pairs, respectively. The different *vif* mutant genes were amplified from the respective pTA-vif plasmids using the SBHI/AS*Mlu*I primers. After *Bam*HI-*Mlu*I digestion, the *vif* genes were cloned at these restriction sites into the pUC19-vif plasmid. The *Bst*EII-*Dra*III fragment was then cloned at these sites into the PK-9kb plasmid.

Nucleotidic sequence determination

Sequences of the *vif* genes, contained within progeny virions produced by cells infected with the different CAEV Vif mutants, were determined from RT–PCR products of the extracted RNA. Virions were concentrated from cell-free supernatants by ultra-centrifugation at 100 000 *g* for 45 min at 4°C. The viral pellets were resuspended in 300 μl RNA[B] (Bioprobe) and processed for RNA extraction according to the manufacturer's recommendations. One microliter of RNA was submitted to RT–PCR to amplify the *vif* gene, using the VCP/R10 primer pair and the SuperScript One Step RT–PCR kit (Life Technologies). Amplification products were purified from a 1% agarose gel, digested with *Bam*HI/*Mlu*I and cloned into the pGEM-T Easy vector (Promega). The pGEM-vif plasmids were then transformed into *Escherichia coli* DH5α, and recombinant colonies were selected. DNA extracted from different clones were then sequenced using the VC5079/AS*Mlu*I primer pair (Genome Express, Grenoble, France) allowing the Vif amino acid sequence deduction from positions 26 to 216.

In vitro protein translation

The Gag and Vif proteins synthesis was performed with the TnT T3 wheat germ extract systems (Promega) following the manufacturer's recommendations. The *gag* gene was amplified from the PK-9kb plasmid with the T3GC/R27II primer pair. The *vif* genes were amplified from the different pTA-vif plasmids using the T3VC/VCASECO primer pair. The PCR products were used to allow proteins transcription/translation in the presence of [^{35}S]methionine (>1000 Ci/mmol; Amersham). The radiolabelled products were resolved on 12% polyacrylamide gel electrophoresis (SDS–PAGE) and revealed by autoradiography using Kodak X-OMat films.

Oligonucleotides

The oligonucleotides used were as follows (the restriction sites are underlined and the substituted nucleotides are in bold): GagGEX1 5′-CG<u>GGATCC</u>TGGTGAGTCTAG-3′ (nt 514–524); CRT27 5′-CG<u>GGATCC</u>ATAGGTGGTGGTA-

AG-3′ (nt 1876–1859); SZ1 (5′-GCCACAAAGG**G**GTTACAAC**G**GTGGAAAACCGGGACATCAAGC-3′ (nt 1663–1704); RZ1 5′-CCAC**C**GTTGTAAC**C**CCTTTGTGGCTGTCCATTCC-3′ (nt 1686–1653); SZ2 5′-GGAATCATA**G**GTC**T**CAAC**G**GTGGAAAGAGAGGACATATGC-3′ (nt 1721–1760); RZ2 5′-CCACCGTTGAGACCTATGATTCCTTGACTACATTGCC-3′ (nt 1743–1707); VCECO 5′-CGGAATTCAAAATTCATCCCGCCAC-3′ (nt 5009-5026); VCASECO 5′-CGGAATTCTCACTCATCCTCTAA (5695–5681); T3VC 5′-CGAATTACCCTCACTAAAGAAAGAAGGATGCAAAATTCATCCCGC-3′ (nt 5006–5023); S170G 5′-GAACAAAA**G**GTCTGGAAAGACTAGTACTG-3′ (nt 5505–5533); RS170G 5′-TCTTTCCAGAC**C**TTTTGTTCTAGTATGC-3′ (nt 5524–5497); S170E 5′-AGAACAAAA**GAG**CTGGAAAGACTAGTACTGCTAC-3′ (nt 5504–5537); RS170E 5′-TAGTCTTTCCAG**CTC**TTTTGTTCTAGTATGCTTTAC-3′ (nt 5527–5492); S197G 5′-GGAGAAGT**G**GTACAACAGATTTCCCATGG-3′ (nt 5586–5614); RS197G 5′-ATCTGTTGTAC**C**ACTTCTCCTGGCTCTCC-3′ (nt 5605–5577); SBHI5′-GCACTATGGAT**CC**ATATAGCAGAAAGCATTAAT (nt 5072–5104); RBHI 5′-TTCTGCTATATGGAT**CC**ATAGTGCTAAGGGTAA-3′ (nt 5095–5065); 33*Mlu*I 5′-GGATACACGCA**C**GCGTGGTCTGTCCAGGAGTGC-3′ (nt 5630–5662); AS*Mlu*I 5′-GACAGACCACGC**GT**GCGTGTATCCCGTTG-3′ (nt 5653–5625); T3GC 5′-CGAATTACCCTCACTAAAGAAAGAAGGCCGCCATGGTGAGTCTAGATAGAG-3′ (nt 512–530); R27II 5′-GCCAGATCTCCATAGGTGGTGCTGAAG-3′ (nt 1876–1859); VCP 5′-CGGGATTCCCCAAAATTCATCCCGCCACC-3′ (nt 5009–5027); R10 5′-CCCAGTTAAGCGCATGTATC-3′ (nt 5927–5908); VC5079 5′-GGATCCATATAGCAGAAAGC (nt 5079–5098).

Expression and purification of GST fusion proteins

E. coli top 10 colonies transformed with pGEX recombinant plasmids were grown in LB medium containing 100 μg/ml ampicillin at 37°C until an OD_{600} nm of ~1. Expression of the fusion proteins was then induced with 1 mM IPTG for 3 h at 30°C. Bacterial cultures were then pelleted by centrifugation at 5000 *g* for 15 min at 4°C and resuspended in 1/10 vol of MT-PBS (150 mM NaCl, 12.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4, and 100 mM EDTA, pH 7.5). The bacteria were lysed on ice by mild sonication, and the lysate, brought to 1% Triton X-100, was incubated on ice for 30 min before clearing by centrifugation at 15,000 *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, then in PBS-0.1% Triton-X-100 supplemented with protease inhibitor cocktail (aprotinin, leupeptin, pepstatin A, and antipain at 1 μg/ml, PMSF 100 μg/ml, pefabloc 200 μg/ml). Beads were then resuspended in SDS–PAGE sample buffer, and the eluted GST fusion proteins were resolved on 12% SDS–PAGE. Proteins were visualised by Coomassie blue staining and quantified using a Bradford assay (Micro BCA, Pierce).

In vitro binding assays

Equal amounts (2–5 μg) of GST or GST fusion proteins bound to glutathione–agarose beads were incubated with ^{35}S-methionine-labelled Gag or Vif proteins in TNT binding buffer containing 50 mM Tris–HCl pH 7.6, 0.2% Tween 20, 100 μg/ml bovine serum albumin, and different concentrations of NaCl (0.1–1 M) for 1 h at 4°C. Beads were then washed in TNT buffer and resuspended in SDS–PAGE sample buffer. The eluted proteins were resolved on 12% SDS–PAGE, revealed by autoradiography and quantified with a Fuji phosphorimager. The data were analyzed by MacBas V2.5 software (Fuji Photo Film Co, Ltd).

Cells, transfection, and infection

Primary goat synovial membrane cells (CFSM) were derived from the carpal joint of an healthy animal and maintained in culture in Eagle's minimal essential medium (MEM) supplemented with 2 mM glutamin, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (Life Technologies). Transfections of 50×10^4 CFSM cells were performed, using the Lipofectamine reagent (Life Technologies), with 5 μg of a mixture of PK-9kb and BS-3′LTR (Turelli *et al.,* 1996) to allow reconstitution of proviral DNA. The medium was changed every 3–4 days, and the supernatants were harvested for reverse transcriptase (RT) activity measurement. Infections were carried out with CAEV wild-type (wt) or CAEV Vif mutant supernatants containing the same RT activity (10,000 cpm per 25 cm^2 flask), for 2 h at 37°C on subconfluent CFSM cells. Infection was monitored by RT activity measurement in the cell free supernatant. Measurement of RT activity was performed as previously described (Harmache *et al.,* 1995).

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