The Tyrosine Kinase Hck Is an Inhibitor of HIV-1 Replication Counteracted by the Viral Vif Protein*

Received for publication, October 4, 2000, and in revised form, February 8, 2001

Ghérici Hassaïne‡§, Marianne Courcoul†‡, Gilles Bessou‡, Yves Barthalay‡,
Christophe Picard**, Daniel Olive†, Yves Collette‡, Robert Vigne‡ ¤, and Etienne Decroly‡ §§ ¶¶

§INSERM Unit 372, Université de la Méditerranée, 163 Avenue de Luminy, 13276 Marseille Cedex 9, France.
§‡INSERM Unit 119, Institut Pouliti-Calmettes, Université de la Méditerranée, 27 Boulevard Lei Rue, 13009 Marseilles, France, and §§LCPMI, Free University of Brussels, Boulevard du Triomph, 1050 Brussels, Belgium

The virus infectivity factor (Vif) protein facilitates the replication of human immunodeficiency virus type 1 (HIV-1) in primary lymphocytes and macrophages. Its action is strongly dependent on the cellular environment, and it has been proposed that the Vif protein counteracts cellular activities that would otherwise limit HIV-1 replication. Using a glutathione S-transferase pull-down assay, we identified that Vif binds specifically to the Src homology 3 domain of Hck, a tyrosine kinase from the Src family. The interaction between Vif and the full-length Hck was further assessed by co-precipitation assays in vitro and in human cells. The Vif protein repressed the kinase activity of Hck and was not itself a substrate for Hck phosphorylation. Within one single replication cycle of HIV-1, Hck was able to inhibit the production and the infectivity of vif-deleted virus but not that of wild-type virus. Accordingly, HIV-1 vif − replication was delayed in Jurkat T cell clones stably expressing Hck. Our data demonstrate that Hck controls negatively HIV-1 replication and that the restricted phenotype is dominant over the permissive expression of Vif. Hck, which is present in monocyte-macrophage cells, represents the first identified cellular inhibitor of HIV-1 replication over- come by Vif.

The Vif protein of human immunodeficiency virus type 1 (HIV-1) is required for efficient virus replication in peripheral blood T lymphocytes in vitro (1–3), since vif-deleted (vif−) mutants are several orders of magnitude less infectious than wild-type (WT) HIV-1 virions (1, 3–5). This critical role of Vif was also observed in primary macrophages, where both production of viral particles and infectivity of vif− viruses are reduced (6). The importance of Vif has been confirmed in animal studies where vif-deleted simian immunodeficiency viruses were drastically impaired in their propagation and highly attenuated in their pathological properties (7, 8).

Human cell lines are restrictive or permissive based on their ability to support the replication of HIV-1 in the absence of Vif. Restrictive human T cell lines H9 and HUT78 are resistant to infection by HIV-1 in the absence of Vif (1, 9, 10). In contrast, permissive cell lines such as Jurkat, C8166, HeLa, and 293 produce high amounts of vif− viruses in a single replication cycle, whose properties are indistinguishable from those of WT viruses. Intermediate phenotypes were also reported in U937 cells and primary macrophages. Those semipermissive cells support the replication of vif− HIV-1 at reduced levels. Using a fusion technique between permissive and restrictive cells, Simon et al. (11) and Madani and Kabat (12) demonstrated that the restrictive phenotype is dominant over the permissive phenotype. These findings suggest that restrictive cells contain a potent activity inhibiting HIV-1 replication, which can be counteracted by the expression of Vif.

The Vif protein acts late in the virus life cycle, and, in its absence, virus particles exhibit an abnormal condensed core as seen by electron microscopy (13–15). The co-localization of Vif with the major component of the viral core, i.e. Gag (16), and the direct association of Vif with Gag in vitro and in infected cells (17) support the hypothesis that Vif acts as a scaffold protein regulating the virion morphogenesis. Interestingly, it was recently shown using highly purified HIV-1 virus that Vif is not significantly incorporated into the HIV-1 budding virions (18). This finding implicated that, during virus budding, Gag and Gag-Pro-Pol precursors are incorporated into newly synthesized virus particles where maturation process of structural proteins may occur, whereas Vif remains associated to the cell membrane. Consequently, cellular factors have been suggested to play a crucial role in the retention of Vif at the plasma membrane of the infected cells (17, 18).

Vif of HIV-1 contains a proline-rich motif (PPLP) localized within its C-terminal domain, which is highly conserved among the different subtypes of HIV-1 (Fig. 1). Src homology 3 (SH3) domains correspond to intracellular modules, which mediate interaction with proteins containing proline-rich motifs. The presence of SH3 domains was reported in a wide variety of proteins participating to signal transduction pathways, such as tyrosine kinases and adaptor proteins. In this work, we hypothesize that a direct interaction may occur between Vif and SH3 domains. We show that Vif of HIV-1 binds preferentially to the SH3 domain of the tyrosine kinase Hck and not significantly to other SH3 domains representative of the tyrosine kinases families and of adaptor proteins. The interaction of Vif with the full-length Hck being confirmed, we have analyzed the consequence of this interaction on vif− HIV-1 replication. In the
absence of Vif, constitutive expression of Hck rendered T cells less permissive for vif- HIV-1 replication. Moreover, in cells expressing Hck, release of HIV-1 viral particles was strongly inhibited and virion infectivity was reduced, when Vif was lacking. Therefore, Hck represents the first identified cellular factor that inhibits the replication of HIV-1 and that is overcome by Vif.

**EXPERIMENTAL PROCEDURES**

**Cells**

Human kidney 293 cells were maintained in Dulbecco’s modified Eagle’s medium (supplemented with 10% fetal calf serum, antibiotics (penicillin/streptomycin, 100 μg/ml), and 2 mM glutamine. U937 promonocytes and SupT1, Jurkat JH6.2, and H9 CD4+ T lymphocytes were grown in 1640 RPMI medium supplemented with 10% fetal calf serum, antibiotics, and 2 mM glutamine. For Jurkat cells the virus expression vector by PCR to generate Pos7-Vif. The GST-Vif expression vector was a generous gift of M. Malim (20).

**DNA Constructions**

Vit Vectors—pGST-Vif and Pos7-Vif, strain NL4.3 of HIV-1, were described previously (17). GST-Vif was also cloned in the Pos7 vaccinia virus expression vector by PCR to generate Pos7-GST-Vif. The GST-Vif DNA was amplified by PCR using the primers GST-Vif-NoI (s), and GST-Vif-XhoI (a), and inserted in the Pos7 vector by using the Neol and XhoI restriction sites, as described previously (17). The pg-Vif expression vector was a generous gift of M. Malim (20).

**In Vitro Protein-Protein Interactions**

For in vitro transcription, appropriate genes were amplified by PCR using 5’–oligonucleotides that contain the T3 RNA polymerase promoter upstream of the initiation position and 3’–oligonucleotides that contain a stop codon. PALLA and AALAA mutant vif genes were obtained by a two-step PCR on pNDK using, respectively, Vif-PALLA and Vif-AALAA (s) and (as) primers. Amplified DNAs were subjected to in vitro transcription-translation using the TNT coupled wheat germ extract system (Promega) as recommended (19). Proteins were translated in the presence of 125I (1 Ci/mmol; Amersham Pharmacia Biotech), and resolved on 12% SDS-polyacrylamide gels, and autoradiography and phosphorimager analysis.

**Preparation of Cell Lysates for GST Pull-down**

U937 promonocytic cells were washed twice in PBS and lysed in Hepes buffer (1 mM HEPES) containing 10 mM Hepes, pH 7.0, supplemented with the protease inhibitor mixture described above. Nucleic acids and insoluble materials were removed by centrifugation at 15,000 × g for 15 min at 4 °C.

**In Vitro Protein-Protein Interactions**

Binding reactions were performed overnight at 4 °C in TBST binding buffer containing 50 mM Tris-HCl, pH 7.0, 0.2% Tween 20, and appropriate concentrations of NAD (150–350 μM) in the presence of bovine serum albumin (200 μg/ml) in a total volume of 300 μl. For the interaction with cytoplasmic extract, the incubation buffer was the same as used for preparation of cell lysate. Agarose beads coupled to the GST fusion protein were incubated overnight in 300 μl with either 8 μl of vif translated 35S-labeled proteins or 200 μl of cytoplasmic extract. Bound proteins were analyzed by SDS-PAGE, followed by autoradiography or by Western blotting.

**Vaccinia Virus Expression**

Human U937 cells (1 × 10⁷) were infected for 1 h with 1 plaque-forming unit of recombinant vaccinia virus/cell to express T7 polymerase and then transfected with 20 μg of Pos7, pCDNA3-Hck, or Pos7-Vif, 5'-ATACGACTGAGCTAAGTCATTCATGTATGGCC-3'.
Using the electroporation technique, as recommended by the manufacturer (Bio-Rad). Cells were harvested 24 h after transfection.

**Antibodies**

Mouse monoclonal antibodies used were anti-phosphotyrosine 4G10 (Upstate Biotechnology), anti-Lck 3A5 (Santa Cruz Biotechnology), anti-Hck H28520 (Transduction Laboratory), and anti-Fyn sc-434 (Santa Cruz Biotechnology). Rabbit polyclonal antibodies used were anti-Vif (gift of D. Gabuzda; Ref. 24), anti-Hck N30 (Santa Cruz Biotechnology), and anti-GST 459 (Santa Cruz Biotechnology). The human anti-HIV-1 serum was a gift from J. Conaux (Pasteur Institute, Belgium).

**Western Blot Analyses**

Following SDS-PAGE, proteins were electrotransferred to polyvinylidene difluoride membranes (PerkinElmer Life Sciences). Blots were saturated with 1% bovine serum albumin in TBST or in PBS (for 4G10 antibody) and incubated with appropriate primary antibodies (rabbit anti-Vif (1:1000), mouse anti-Hck (1:1000), mouse anti-phosphotyrosine 4G10 (1:1000), mouse anti-Lck (1:1000), or rabbit anti-GST (1:400) antibodies). The secondary immunoreactions were performed by using horseradish peroxidase-linked anti-rabbit, anti-human, or anti-mouse immunoglobulins (Dako, 1:5,000), and followed by ECL detection (Amersham Pharmacia Biotech).

**Infection and Virus Propagation**

Recombinant viruses were harvested 48 h after transfection of HeLa cells with appropriate pNDK WT or vif− molecular clones. Virus stocks were then amplified by acute infection of SupT1 cells. Virus present in cell-free supernatants was quantified in CS166 indicator cells as described previously (15). Cells were infected by incubating 2 × 10^6 cells in 1 ml of virus supernatant at 37 °C with gentle shaking. After centrifugation at 800g for 5 min, cells were resuspended at 5 × 10^5 cells/ml in culture medium. Virus replication was assayed twice a week by determining the RT activity in the cell-free supernatant as described (15). Virus infectivity was titrated on the HeLa-derived p4 indicator cell line as described previously (15).

**In Vitro Kinase Assay**

Human U937 (1 × 10^5) cells were lysed in 25 mM Hepes, pH 7.0, containing 1% Nonidet P-40, 100 mM NaCl, and 1 mM orthovanadate before clarification. Hck was then immunoprecipitated by using rabbit anti-Hck antibodies and protein G-Sepharose. After several washes in lysis buffer containing 0.1% SDS, similar amounts of immunoprecipitated Hck were incubated in kinase buffer (25 mM Hepes, pH 7.0, 100 mM NaCl, 10 mM MgCl2, 5 mM MnCl2) in the presence of 5 μg of denatured enolase (Sigma), [γ-32P]ATP (150 Ci/mmol), and increasing concentrations of either GST or GST-Vif. After 30 min of kinase reaction, the samples were separated by SDS-PAGE and analyzed by autoradiography. The autophosphorylation activity of Hck and the enolase phosphorylation were then quantified by phosphorimager analysis.

**RESULTS**

Vif of HIV-1 Interacts Specifically with the SH3 Domain of Hck—SH3 domains recognize especially proline-rich motifs. Since the Vif protein of HIV-1 contains a conserved PPLP motif in its C-terminal domain (Fig. 1), we addressed the question whether Vif could bind SH3 domains from different proteins. Eight different SH3 domains of tyrosine kinases and adaptor proteins were expressed as fusion GST proteins in bacteria. Following purification, by affinity chromatography on GSH-agarose beads, GST fusion proteins were incubated with in vitro translated [35S]Vif. After pull-down, bound [35S]labeled Vif protein was resolved by SDS-PAGE and revealed by autoradiography. As shown in Fig. 2A, [35S]-labeled Vif protein binds to GST-SH3(Hck) but not to the control GST. In contrast, the GST-linked SH3 domains of two members of the Src tyrosine kinase family (Lck and Yes), or the Tec kinase family members (Itk and Tec) or two adaptor proteins (Grb2, CrkII) failed to pull-down Vif. To assess the functional activity of all GST-SH3 constructs, we measured the binding of Sam68, a proline-rich protein, previously reported to interact with most SH3 domains of different tyrosine kinases and adaptor proteins. Sam68 bound strongly to the SH3 domains of Hck and Lck tyrosine kinases and Crk II and Grb2 adaptor proteins (Fig. 2A). A weaker interaction between Sam68 and the SH3 domains of Itk and Tec SH3 was also observed. Finally, Sam68 interacted very faintly with the Yes SH3. Coomassie Blue staining of individual GST chimera showed that the binding capacities of Vif and Sam68 were not dependent of different stabilities and quantities of individual GST fusions (Fig. 2A). The strength of the interaction between Vif and Hck SH3 domain was further assessed using increasing NaCl concentrations during the binding and washing steps. Interestingly, Vif binding to the GST-SH3(Hck) of Hck was enhanced at 350 mM NaCl compared with 150 mM NaCl concentration (Fig. 2B), suggesting that hydrophobic residues participated to the interaction. These data indicate that Vif is recognized preferentially by the SH3 domain of the tyrosine kinase Hck.

To exclude a possibility that the recognition of Vif by GST-SH3(Hck) was due to a particular conformation and accessibility of the SH3 domain in the context of the GST fusion protein, we performed the reverse experiment in which Vif was expressed as a GST-fusion protein. U937 promonocyte cells lysate, endogenously expressing Hck, was incubated with GST-Vif immobilized on GSH-agarose beads for pull down assay. The presence of Hck was analyzed by Western blotting with anti-Hck antibody. As shown in Fig. 3A, full-length Hck retains its ability to bind GST-Vif but not GST, confirming the specificity of the interaction. The Vif/Hck interaction was then analyzed when both Hck and GST-Vif have been expressed in human cells. GST-Vif and Hck were subcloned in plasmids under the control of T7 polymerase promoter. GST-Vif and Hck were then co-expressed in human promonocytic cells previously infected with recombinant vaccinia virus expressing T7 polymerase. Twenty hours after transfection, cell lysates containing GST fusion proteins were precipitated by addition of GSH-agarose beads and bound Hck protein was detected by Western blotting with anti-Hck antibody (Fig. 3B). Co-precipitation of Vif with GST-Vif but
not with the control GST protein confirmed that Vif and Hck interact in a specific manner in human cells.

**Mapping of the Hck/Vif Interaction Domains**—The binding affinity of proline motifs, including those present in HIV-1 Nef protein, depends on hydrophobic interactions between residues of the SH3 domain (such as Tyr-66, Tyr-68, and Trp-93 in Hck), but also on ionic interactions between a basic residue found before or after the proline motif and a highly conserved acidic residue in the SH3 domain (Asp-75, in Hck) (25–27). Mutation of various residues of Hck SH3, known to be important for the interaction between SH3 domains and their specific ligands, were generated within GST fusion proteins (Y66A, Y68A, and W93F or W93A, and D75A) and analyzed by pull-down assays (Fig. 4). Most single mutations in the SH3 domain of Hck, except the most conservative one (W93F), resulted in the decrease of the interaction between Vif and Hck SH3 domain. Coomassie Blue staining of Hck GST-SH3 mutants indicated that the binding capacity of Vif was not dependent of different stabilities and quantities of individual GST fusions (Fig. 4). Altogether, these observations confirm that both hydrophobic and ionic residues of the SH3 domain participate in the interaction with Vif protein.

We then mapped the domain of Vif interacting with the SH3 domain of Hck. For this purpose we analyzed the binding of deleted mutants of Vif protein with the SH3 domain of Hck. Vif protein was truncated in three domains and expressed by in vitro translation. After incubation with the GST or GST-SH3(Hck) constructs, bound proteins were separated on SDS-PAGE and analyzed by phosphorimager. Fig. 5 reveals that the three thirds of Vif may contribute to the interaction with the Hck SH3 domain, with a weaker participation of the N-termini.
nal third. We tested whether the PPLP conserved proline-rich motif located in the C-terminal third of Vif participates in the interaction with Hck SH3 domain. We produced in vitro \(^{35}\)S-labeled Vif proteins in which the PPLP motif was substituted by PPLA or AALA (Fig. 5). Pull-down analysis, using GST-SH3(Hck), reveals that mutations of proline in the C-terminal third domain of Vif decreased significantly the binding of Vif to the SH3 domain of Hck. In contrast when PPLP was substituted to AALA in the context of full-length Vif protein, the SH3 domain of Hck was still able to interact. This observation confirms that other domains of Vif participate in the interaction with the Hck SH3 module. Moreover, we were unable to identify any point mutants of the full-length Vif strongly affected in their binding to the SH3 domain of Hck. In contrast when PPLP was substituted to AALA in the context of full-length Vif protein, the SH3 domain of Hck was still able to interact. This observation confirms that other domains of Vif participate in the interaction with the Hck SH3 module. Moreover, we were unable to identify any point mutants of the full-length Vif strongly affected in their binding to the SH3 domain of Hck. Altogether, our data indicate several domains distributed all along the Vif protein participate in the interaction of Vif with the SH3 domain of Hck, including the PPLP motif contained in the C-terminal third of Vif.

**Vif Inhibits Hck Activity and Is Not Itself a Substrate for Hck**—To determine whether the interaction between Vif and Hck contributes to the control of the Hck kinase activity, we performed an in vitro kinase assay. Cell-derived Hck immunoprecipitated from U937 cell lysate was incubated with increasing concentrations of GST-Vif or GST. The catalytic activity of Hck was followed both by autophosphorylation of Hck and by transphosphorylation of exogenously added enolase (Fig. 6B). The kinase activity of Hck was unchanged upon incubation with increasing amounts of GST. By contrast, GST-Vif down-regulated both Hck auto- and transphosphorylation activities in a dose-dependent manner. In these experiments, no signal corresponding to the phosphorylation of GST-Vif could be detected, consistent with the idea that Vif is not a substrate for Hck. Together, these observations suggest that Vif represses the tyrosine phosphorylation activity of Hck in human cells.

**Cells That Express Hck Display Attenuated Replication of Vif**—Human cells were previously classified as restrictive (H9, T CD4\(^+\) cells), semipermissive (U937 cells, macrophages), or permissive (CEM, SupT1, Jurkat, C8166) for vif\(^{-}\)HIV-1. We investigated whether a correlation may exist between the presence of Hck and the level of replication of HIV-1. In absence of Vif, as shown in Fig. 7, comparative analysis of the expression of Hck within human cells known to be infected by HIV-1 revealed that permissive cell lines lack the expression of both isoforms of Hck, p59\(^{hck}\) and p61\(^{hck}\). In contrast, restrictive and semipermissive cells expressed Hck, except for the restrictive primary T CD4\(^+\) lymphocytes which were negative for Hck. Since all Hck-positive cells displayed an attenuated phenotype of vif\(^{-}\) viruses, this suggests that Hck expression may participate to the control of HIV-1 replication in a Vif-dependent manner.

**Inhibition of HIV-1 Replication by Expression of Hck**—
come by Vif—To address the possibility that the Src tyrosine kinase Hck is involved in the regulation of HIV-1 replication in a Vif-dependent manner, several clones of Jurkat T cells expressing constitutively Hck were established by stable transfection. WT HIV-1 and vif−replications were compared in both cell lines by measurement of the virus-associated RT activity in the supernatant of infected cells (Fig. 8A). The replication of vif−HIV-1 was delayed in Jurkat cells stably expressing Hck, as compared with parental Jurkat cells. WT virus replicates efficiently in both Jurkat and Hck expressing Jurkat cells. A much more significant delay of vif− HIV-1 replication was observed in U937 promonocytes, endogenously expressing Hck (Fig. 8B). Interestingly, in both Jurkat Hck and in U937 cells, persistent infections were established with WT and vif− virus. Altogether, these viral replication data suggest that Hck delays vif− virus replication.

We next investigated virus production and infectivity during one single virus cycle in cells expressing or not Hck. Human 293 cells were co-transfected with plasmids expressing WT and vif− virus together with WT Hck or Hck carrying a mutation in its SH3 domain (W93A Hck) that dramatically reduced the interaction between SH3 domain of Hck and Vif (Fig. 4). Both WT and mutated Hck reduced the vif− virus production, measured either by RT activity (Fig. 9A) or by enzyme-linked immnosorbert assay p24 (data not shown), down to 5% (WT) and 13% (W93A Hck) of the virus production observed in Hck-negative cells. In contrast, WT virus production was not significantly affected by the expression of WT Hck, suggesting that Vif can overcome the negative effect induced by Hck expression. Interestingly, W93A Hck mutant was not counteracted by Vif expressed from WT HIV-1. Therefore, there is a correlation between the alteration of Vif binding to the SH3 domain of Hck and its inability to counteract Hck. Anti-Hck Western blot analysis confirmed that the difference in virus production did not result from variation of WT and W93A Hck expression level (Fig. 9B), and in vitro kinase assays demonstrated that both WT Hck and W93A Hck displayed similar kinase activities (Fig. 9C). Furthermore, to confirm the role of Vif in the control of virus production in presence of Hck, we analyzed the restoration of vif− virus release by expressing in trans HIV-1 Vif (pgVif). Human 293 cells were co-transfected with plasmids expressing Hck and vif− HIV-1 in presence or absence of pgVif. Virus production was increased up to 6-fold when Vif was added in trans, confirming that Vif counteracts the effect of Hck (data not shown). Since the vif− phenotype usually described in T lymphocytes is mainly a defect of infectivity of neosynthesized virus, we next analyzed the infectivity of virus produced in presence or in the absence of Hck. Virus-containing supernatant from transfected cells was harvested and titrated for infectivity by end point dilution assay. As shown in Fig. 9D, we observed that the infectivity of vif− virus was reproducibly 3−4-fold lower than that of WT virus in Hck-expressing cells. We also compared the infectivity of vif− and WT viral particles produced by Jurkat-Hck cells and U937 cells, endogenously expressing Hck. The viruses were collected at the peak of virus production and quantified by RT activity measurement. Titration of virus infectivity, by end point dilution assay, revealed that infectivity of vif− virus is decreased about 15-fold in Jurkat-Hck cells and 11-fold in U937 cells. All together, our results indicate that both the virus production and infectivity of vif− HIV-1 are highly decreased in Hck-expressing cells and that Vif is able to counteract this inhibitory activity.

**DISCUSSION**

In the present work, we have investigated the putative interaction between various SH3 domains and HIV-1 Vif protein. We identified a preferential recognition of Vif by the SH3 domain of Hck and by the full-length Hck tyrosine kinase. The functional consequence of this interaction is the regulation of the HIV-1 replication in a Vif-dependent manner in Hck-expressing cells. Indeed, we demonstrated that HIV-1 vif− replication was inhibited when Hck was stably expressed in permissive Jurkat T cells. Similar effects on the replication kinetics were observed in U937 promonocytes (present work)
and in primary macrophages infected by \textit{vif} \textsuperscript{-} viruses (6). By analyzing HIV-1 production in one viral cycle, we have demonstrated that transient expression of Hck decreased virus release and reduced the infectivity of neo-synthesized viral particles. This last infectivity defect was mostly documented in the context of HIV-1 \textit{vif} \textsuperscript{2} replication in primary T cells and in H9 cells. Vif counteracted this inhibitory effect of Hck, when it was expressed in \textit{cis} and \textit{trans}. We further confirmed this inhibitory effect of Hck, by analyzing the virus production of WT HIV-1 in cells expressing Hck mutated in its SH3 domain, in order to relieve its interaction with Vif of HIV-1. In these conditions, Vif lost its ability to counteract the negative effect of Hck on HIV-1 virus production.

Our data suggest that Vif and Hck interact directly through the SH3 domain of Hck. Mutagenesis analysis revealed that conserved hydrophobic (Tyr-66, Tyr-68, Trp-93) and charged residues (Asp-75) of the SH3 domain participate in the interaction with Vif protein. Those residues were previously shown to play a crucial role for the interaction between SH3 domains and their specific ligands (25–27). Since proline-rich motifs are well known to interact with the SH3 domains, we next attempted to identify the role played by the PPLP motif in the recognition of the SH3 domain of Hck. When PPLP was mutated into PPLA or AALA, the interaction of the C-terminal third of Vif with Hck SH3 was decreased. In contrast, when PPLP was substituted into AALA within the full-length Vif protein, the interaction of Vif with the SH3 domain of Hck was not abrogated. We concluded that the PPLP motif may be necessary but not sufficient for interacting with the SH3 domain of Hck. This is consistent with our observation showing that the central third of Vif binds to Hck SH3; the N-terminal third of Vif binds also with Hck SH3 but at a lower extent. Interestingly, this proline-rich 151–164 region of Vif was also shown to be important for Vif multimerization (28), and the replication of AALA Vif mutated HIV-1 was completely abolished in restrictive cells.\textsuperscript{2} Altogether, these observations indicate that this region of Vif is involved in several functions.

Previous studies revealed also that Vif binds to the NCP7 domain of the Gag protein of HIV-1 (17, 29, 30), and colocalizes with Gag in virus-producing cells (16). However, Vif is not incorporated into viral particles (18). Since the Src tyrosine kinases are located at the inner layer of the plasma membrane in detergent-insoluble glycolipid-enriched microdomains (31–

\textsuperscript{2} G. Bessou, unpublished data.
Hck Is an Inhibitor of HIV-1 Replication Overcome by Vif

Fig. 9. Inhibition of vif- HIV-1 production and infectivity by Hck. A, the production of viral particles is inhibited by Hck. Human 293 cells were cotransfected with a plasmid containing either WT (■) or vif- (□) HIV-1 provirus together with a plasmid expressing Hck or Hck (W93A). The virus production was monitored by RT activity measurement in the supernatants of cell cultures. Data represent means of six independent experiments with standard errors. B, Hck expression in 293 transfected cells. Human 293 cells, transfected as in panel A, were lysed 48 h after transfection, and Hck expression was analyzed by anti-Hck Western blotting. C, Hck and W93A Hck were immunoprecipitated from transfected cells and incubated in kinase buffer in the presence of [γ-32P]ATP for 30 min. After SDS-gel electrophoresis, the autophosphorylation activity of Hck was analyzed by autoradiography. D, Hck expression inhibits vif- HIV-1 infectivity. The HIV-1 stocks for the infectivity assay were collected from transfected 293 cells as in panel A. Following standardization of virus stocks by RT activity, infectivity of WT and vif- viruses was scored on the HeLa-derived P4 indicator cell line. Infectivity values correspond to the mean number of HeLa P4 blue cells counted for the same RT activities of WT and vif- virions. Data represent means of three independent assays with triplicates.

and because HIV-1 buds preferentially from these membrane regions (34), it will be interesting to determine whether Hck controls the retention of Vif within cells during the virus budding.

We have observed that Vif inhibits the kinase activity of Hck both in vitro and in promonocytic U937 cells. Moreover, the expression of Hck in absence of Vif induces a decrease of the viral particle release as well as a significant loss of infectivity. Since Vif has been reported to control functionally the processing of Gag in restrictive T cells and in monocytic cells (6, 14, 35), it is tempting to speculate that Vif controls the tyrosine phosphorylation of the Gag precursor and therefore may also indirectly control the processing of Gag. Further studies will be necessary to determine whether Gag is phosphorylated on tyrosine by Hck in infected cells and to analyze the consequences of these post-translational modifications on the viral production and on the infectivity.

Hck is expressed mainly in promonocytic cells, including monocyte-derived macrophages, in which it mediates relevant functions such as FcγRI receptor signaling, induction of cytokine production triggered by bacterial lipopolysaccharide, phagocytosis, and cell spreading (36–38). Primary macrophages are only weakly permissive for vif- HIV-1 and show a reduction of free virus infectivity and, to a lesser extent, a decrease of viral production (6). These two effects were reproduced in Hck-negative T CD4+ cells transfected with a Hck-expressing plasmid. We observed also that Hck is naturally present in H9 T CD4+ cells (Fig. 7), the only immortalized T CD4+ cells which are restrictive for vif- HIV-1. Therefore, our data suggest that the presence of Hck may contribute to the reduced capacity of vif- HIV-1 to replicate in these cells.

However, the undetectable level of Hck in primary T CD4+ lymphocytes suggests that the vif- restriction phenotype could be dependent upon cellular factors other than Hck in a cell type-dependent manner. Since these cells express Src tyrosine kinases such as Lck, Fyn, Yes, Src, and Fgr, with functions redundant with Hck, it will be important to evaluate carefully the role of these kinases, alone or in combination, in the inhibition of vif- HIV-1 in primary T cells. Moreover, the picture may be further complicated since the expression level of Src kinases has been shown to be modulated in function of the cellular activation state (39, 40).

In conclusion, Hck corresponds to an inhibitor of HIV-1 replication in monocytic cells. It is the first cellular protein identified as interacting with the auxiliary HIV-1 protein Vif. This interaction between Vif and Hck relieves the inhibition of HIV-1 replication and is reminiscent of the antiviral activity described in T cells by others (11, 12). The fact that Hck belongs to the Src family of tyrosine kinases opens new areas of investigation on the role of tyrosine kinases in the replication of HIV-1 and on the development of drugs against HIV-1, possibly through the Vif/Hck binding interface.

Acknowledgments—We thank I. Hirsch, J. Sire, G. Quéré, B. Canard, J.M. Ruyschaert, O. Schwartz, C. Mawas, J. Ewbank, and Q. Sattentau for critical review of the manuscript. We acknowledge M. Malim for the generous gift of pgvif and G. Sutter and B. Moss for the vaccinia virus/po77 constructs.

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doi: 10.1074/jbc.M009076200 originally published online February 27, 2001

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