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Human Immunodeficiency Virus Type 1 Vpr Protein Binds to the Uracil DNA Glycosylase DNA Repair Enzyme

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The role of the accessory gene product Vpr during human immunodeficiency virus type 1 infection remains unclear. We have used the yeast two-hybrid system to identify cellular proteins that interact with Vpr and could be involved in its function. A cDNA clone which encodes the human uracil DNA glycosylase (UNG), a DNA repair enzyme involved in removal of uracil in DNA, has been isolated. Interaction between Vpr and UNG has been demonstrated by in vitro protein-protein binding assays using translated, radiolabeled Vpr and UNG recombinant proteins expressed as a glutathione *S*-transferase fusion protein. Conversely, purified UNG has been demonstrated to interact with Vpr recombinant protein expressed as a glutathione *S*-transferase fusion protein. Coimmunoprecipitation experiments confirmed that Vpr and UNG are associated within cells expressing Vpr. By using a panel of C- and N-terminally deleted Vpr mutants, we have determined that the core protein of Vpr, spanning amino acids 15 to 77, is involved in the interaction with UNG. We also demonstrate by in vitro experiments that the enzymatic activity of UNG is retained upon interaction with Vpr.

The human immunodeficiency virus type 1 (HIV-1) genome contains, in addition to the *gag*, *pol* and *env* structural genes, at least six auxiliary genes (*vif*, *vpr*, *tat*, *rev*, *vpu*, and *nef*) that are involved in the regulation of virus production and infectivity. Two of them, *tat* and *rev*, are indispensable for viral gene expression. The others, so-called nonessential genes, can be deleted without alteration of virus propagation in cultured cell lines (13). However, the conservation of these accessory genes in natural isolates of all primate lentiviruses argues for an important role in viral replication in vivo and in the induction of associated pathologies. The *vpr* open reading frame encodes a 96-amino-acid protein with an apparent molecular mass of 15 kDa (36, 53). It is conserved within the genomes of HIV-1 and HIV-2 as well as within most of the simian immunodeficiency virus genomes. Amino acid comparison between *vpr* and *vpx* gene products in the HIV-2 group suggests that *vpx* may have arisen by duplication of the *vpr* gene (49). Both Vpr and Vpx seem to play an important role in vivo, since rhesus monkeys infected with a *vpr-vpx* double-defective simian immunodeficiency virus maintained a low virus burden and did not exhibit clinical symptoms (21).

Distinct biological roles for Vpr in viral replication in cell culture have been reported. Defective-Vpr virions are able to replicate in established cell lines and primary peripheral blood mononuclear cells but replicate poorly in monocyte-derived macrophages (MDM) (1a, 2, 12, 14, 37, 52). This apparent phenotype in MDM could be, however, attributed to different susceptibilities to HIV-1 infection of MDM from different donors (18). Vpr, in association with the matrix (MA) protein, could play a crucial role in the translocation of viral preintegration complex to the nucleus in infected cells which were maintained in a nonproliferative status (23). However, these data are in contradiction with recent results (18), which showed that infectivity in MDM was retained even when combined MA and Vpr mutations were introduced. Vpr is a weak transactivator of the HIV long terminal repeat and several other heterologous viral promoters (11), and it could play a role in the reactivation of viral gene expression, as demonstrated by addition of exogenous Vpr to cultures of latently infected T-cell lines (27, 28). Vpr is selectively incorporated into the viral particle, and this incorporation required the $Pr55^{gag}$ precursor, and in particular its carboxy-terminal region, P6 (10, 24, 30, 39). This unusual packaging for an accessory gene product could be related to the role played by this protein in the early events during viral replication.

A role for Vpr in the regulation of some cellular functions was also reported. Indeed, Vpr was involved in the prevention of T-cell proliferation during chronic infection in cell culture (35, 42). This may result from a cytostatic activity of Vpr leading to an arrest of cell cycle progression in G_2 (42). Results from Levy et al. (26) demonstrated that Vpr could cause terminal differentiation of rhabdomyosarcoma cells. Finally, expression of Vpr in the yeast *Saccharomyces cerevisiae* resulted in cell growth arrest and gross cell enlargement (31). All of these effects of Vpr are probably mediated by interactions with cellular proteins. In an attempt to identify cellular proteins that interact with Vpr and could be implicated in its functions, we used the yeast two-hybrid system (16). We isolated a cDNA encoding uracil DNA glycosylase (UNG), a DNA repair enzyme, and confirmed the interaction between Vpr and UNG both in vitro and ex vivo in Vpr-expressing cells. Furthermore, we demonstrated that the enzymatic activity of UNG could still be detected, in in vitro experiments, after interaction between UNG and Vpr.

MATERIALS AND METHODS

Plasmid constructions. All recombinant DNA methods were performed according to standard protocols (45). The construction of plasmids pGBT10 and pGAD1318 has been described elsewhere (9). The *vpr* gene from the HIV-1 LAI isolate (40) was amplified by PCR, using the 5′ primer Vpr1 and 3′ primer Vpr2, containing *Bam*HI and *Pst*I restriction sites, respectively, and cloned in frame

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into the same sites of pGBT10 to obtain the bait plasmid (pGB-Vpr) used in this study. The protein expressed from pGB-Vpr was a chimeric protein containing at the N terminus the GAL4 DNA-binding domain (GAL4BD) and at the C terminus the Vpr protein. pGAD1318-cDNA prey plasmids contained the GAL4 activation domain (GAL4AD) fused to a cDNA expression library made from the Jurkat T-cell line (5). N-terminal deletion mutants (called N15, N27, N42, and N64) were constructed by PCR amplification of the *vpr* open reading frame, using the 3′ primer Vpr2 and a set of 5′ primers (N15, N27, N42, and N64) containing a *Bam*HI restriction site. The PCR products were then cloned back into the *Bam*HI and *Pst*I restriction sites of pGBT10. The resulting N-terminal Vpr mutants encoded a Vpr protein lacking the first 14, 28, 41, and 63 amino acids respectively. C-terminal deletion mutants (called C41, C63, and C77) were constructed by PCR amplification, using the 5′ primer Vpr1 and 3′ primer Vpr2 of the *vpr* open reading frame containing either *Nco*I (nucleotide [nt] 5257), *Eco*RI (nt 5325), or *Sal*I (nt 5367) restriction sites previously filled in with Klenow enzyme. The PCR products were then cloned back into the *Bam*HI and *Pst*I restriction sites of pGBT10. The resulting C-terminal Vpr mutants encoded a Vpr protein containing the first 42, 63, and 77 amino acids, respectively. All sequence junctions were checked by DNA sequencing.

For in vitro protein-protein interaction experiments, the *Eco*RI-*Not*I fragment containing the cDNA UNG insert from clone 16 was subcloned into the pGEX-5X-2 plasmid vector (Pharmacia) cleaved with the same enzymes, and the resulting plasmid produced a glutathione *S*-transferase (GST)-UNG fusion protein. The GST-Vpr fusion protein expression plasmid used in this study was kindly provided by F. Bachelerie (Pasteur Institute, Paris, France). It contained the HIV-1 LAI *vpr* open reading frame subcloned into the *Bam*HI site of the pGEX-2T plasmid vector (Pharmacia).

To obtain the mammalian Vpr expression vector, the *vpr* gene from the HIV-1 LAI isolate was amplified by PCR with a 5′ primer (pTEJ5′) corresponding to nt 5141 to 5152 and containing an *Eco*RV restriction site and a 3′ primer (pTEJ3′) corresponding to nt 5363 to 5387 and overlapping the *Sal*I restriction site located in the *vpr* gene. The resulting amplified fragment was then cleaved with *Eco*RV and *Sal*I, end blunted, and subcloned in frame at the *Eco*RV site of plasmid pTEJ-8-HA-Tag (kindly provided by D. Marguet, CIML, Marseille, France), containing the hemagglutinin leader sequence and the flag M2 sequence. The resulting pTEJ-Vpr plasmid encoded a chimeric tagged Vpr protein containing the first 77 amino acids of Vpr recognized by the flag M2 antibody (IBI, Inc.). Expression of the tagged Vpr protein was driven by the ubiquitin promoter.

Yeast two-hybrid system. Yeast reporter strain HF7c (4), containing two GAL4-inducible reporter genes, *HIS3* and *lacZ*, was transformed with the pGB-Vpr bait plasmid and selected on tryptophan-deficient synthetic medium. Yeast cells carrying pGB-Vpr were then transformed with the human cDNA library in pGAD1318 and were plated on tryptophan-, leucine-, and histidine-deficient synthetic medium. After 3 days of growth, His^+ colonies were patched on selective medium, lifted onto Whatman 40 filters, and transferred to nitrocellulose filters for the β-galactosidase filter assay (8).

The cDNA library plasmids were recovered from positive colonies and tested for specificity by retransformation into HF7c either with pGB-Vpr or with the extraneous target yeast SNF1 protein fused to GAL4BD. Liquid culture assay for β-galactosidase activity was performed as described previously (4), using SFY526 as the yeast reporter strain (3) and *o*-nitrophenyl-β-D-galactopyranoside as the substrate. Each assay was performed in triplicate.

In vitro protein-protein interaction studies. GST-UNG or GST-Vpr fusion protein expression plasmids were grown in *Escherichia coli* JM105 or TG1, respectively. The transformed bacteria were grown overnight at 30°C and were induced with isopropyl-β-D-thiogalactopyranoside for 3 to 4 h. The induced bacteria were resuspended in phosphate-buffered saline buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 100 mM EDTA [pH 7.3]) containing 1% Triton X-100 and lysed by sonication. Insoluble material was pelleted, and supernatants were adjusted to 10% glycerol and stocked at −70°C. The bacterial lysate (1 ml) expressing either GST-Vpr or GST-UNG was incubated with 200 μl of 50% (vol/vol) glutathione (GSH)-agarose beads (Sigma) at 4°C for 30 min. After being washed in binding buffer, GST-Vpr or GST-UNG immobilized on GSH-agarose beads was mixed with either purified UNGΔ84 or in vitro-translated ^{35}S-Vpr protein, respectively. UNGΔ84 was a bacterially expressed protein lacking the N-terminal 84 amino acids. The mature nuclear form of UNG begins at residue 77, whereas UNGΔ84 starts at residue 85. UNGΔ84 corresponds in fact to the nuclear form of UNG deleted of eight amino acids residues at the N terminus (48). Purified recombinant HIV-1 Nef protein (5) was used as an irrelevant protein in the binding assays. Binding reactions were performed for 2 h at 4°C in a buffer containing 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, and 0.02% Tween 20 in the presence of bovine serum albumin (200 μg/ml) in a total volume of 300 μl. Beads were then extensively washed in 50 mM Tris-HCl (pH 7.6)–150 mM NaCl–0.02%. Tween 20 and resuspended in 2× sample buffer (45) before being subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Where indicated, uracil DNA glycosylase inhibitor (UGI; New England Biolabs) was incubated for 10 min with 2.6 μg of purified UNG (48) prior to the addition of GST-Vpr.

DNA transfection and coimmunoprecipitation assay. Transfections were performed by the Lipofectamine method (22) as recommended by the manufacturer (GIBCO). Briefly, HeLa cells were plated at 5×10^5/35-mm-diameter plate and grown overnight in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Cells were washed with optiMEM medium (GIBCO) and incubated for 6 h with 10 μl of Lipofectamine and 1 μg of plasmid pTEJ-Vpr before addition of complete medium culture. At 48 h after transfection, cells were harvested and resuspended in 150 μl of Tris-EDTA buffer (0.5 M Tris-HCl [pH 7.0], 5 mM EDTA). Cell extracts were prepared by freeze-thaw cycles and used for immunoprecipitation experiments. Extracts from transfected HeLa cells were incubated overnight at 4°C with flag M2 antibody (100 μg/ml) in a binding buffer containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid (HEPES; pH 7.9), 150 mM KCl, 0.1% Nonidet P-40, 5% glycerol, 0.5 mM dithiothreitol, and 0.4 mM phenylmethylsulfonyl fluoride in a total volume of 100 μl. Protein A-Sepharose beads (Pharmacia) were added for 2 h, and after extensive washes with the binding buffer, bound proteins were eluted and then loaded onto an SDS–15% polyacrylamide gel. The gel was processed for Western blotting (immunoblotting) with either an rabbit anti-Vpr or rabbit anti-UNG antibody and peroxidase-linked donkey anti-rabbit immunoglobulin.

Western blotting analysis and antibodies. Proteins were electrotransferred to polyvinylidene difluoride membranes (Amersham) with a Bio-Rad apparatus. Blots were incubated with either a rabbit anti-UNG antibody diluted 1:2,000 (48) or a rabbit anti-Vpr antibody diluted 1:5,000 (12) and horseradish peroxidase-linked donkey anti-rabbit immunoglobulin (Amersham). The anti-Nef monoclonal antibody FM11-BF7 was a gift from J. C. Ameisen (Pasteur Institute Lille); incubation with FM11-BF7 (diluted 1:500) was followed by incubation with horseradish peroxidase-linked sheep anti-rabbit immunoglobulin (Amersham). Antibody binding was detected with ECL (enhanced chemiluminescence) Western blotting detection reagents (Amersham).

In vitro transcription and translation. For in vitro transcription, the *vpr* open reading frame was amplified by PCR, using as the 5′ primer an oligonucleotide (T3wt) containing the T3 RNA polymerase recognition site (20) upstream of the ATG initiation codon of the *vpr* open reading frame and as the 3′ primer the Vpr2 oligonucleotide. The PCR product was then treated with proteinase K (40 μg/ml) for 30 min at 37°C, extracted with phenol, precipitated with ethanol, and resuspended in diethyl pyrocarbonate-treated water. DNA template (1 μg) was then subjected to in vitro transcription-translation. To obtain DNA templates containing 3′ deletions in the *vpr* gene, the *vpr* open reading frame was amplified with the T3wt 5′ primer and a panel of different 3′ primers (C77, C63, and C41). PCR products were treated as described above. Translation of the resulting deletion *vpr* transcripts led to Vpr proteins (C77, C63, and C41) containing the first 77, 63, and 41 amino acids, respectively.

The TNT coupled wheat germ extract system (Promega) was used as recommended by the manufacturer. Proteins were translated in a final volume of 50 μl in the presence of [^{35}S]methionine (>1,000 Ci/mmol; Amersham), and 5 μl was assayed for the ability to bind GST-UNG immobilized on GSH-agarose beads. Bound proteins were analyzed by SDS-PAGE followed by autoradiography.

Detection of UNG activity. To detect UNG enzymatic activity, we used an indirect assay based on a PCR approach as depicted in Fig. 5A. When a deoxynucleoside triphosphate substrate in PCR amplification contained dUTP instead of dTTP, addition of the UNG enzyme to the PCR resulted in the excision of uracil residues from a dU-containing PCR product. Subsequent heat treatment and alkaline pH resulted in the degradation of the abasic polynucleotide of the dU-containing PCR product followed by inhibition of subsequent PCR reamplification. Inability to reamplify the (dU)+ DNA template (see below) was the hallmark of the presence of the enzymatic activity of UNG.

The purpose of the first amplification was to incorporate dUTP in the DNA template, and any fragment could be used regardless of its origin. The HIV-1 *vif* gene was used as the DNA template and amplified with primers 5′Vif and 3′Vif either with dTTP in the mix of deoxynucleoside triphosphates or with dUTP instead of dTTP in the mix of deoxynucleoside triphosphates, leading to a PCR product of 637 bp, designated the (dT)+ DNA template or (dU)+ DNA template, respectively. Subsequent PCR amplification of the DNA templates with the same primers was then performed as described previously (44) and was done in the linear range of amplification extended up to 30 amplification cycles. Briefly, 1 ng of DNA templates was assayed in a final volume of 50 μl in the presence of 1.5 mM $MgCl_2$, 100 ng of each amplification primer, 0.2 mM each deoxynucleoside triphosphate, and 2.5 U of *Taq* polymerase (Promega). Samples were run in a Hybaid thermocycler for 30 cycles of 30 s at 94°C, 60 s at the annealing temperature, and 2 min at 72°C. PCR products were resolved on a 2% agarose gel, transferred onto a Hybond N+ membrane (Amersham), and hybridized with the ^{32}P-labeled *vif* internal oligonucleotide (Vif3). DNA templates were used at two dilutions (10^{-7} and 10^{-8}), and only the 10^{-8} dilution was used for PCR analysis in the presence of anti-UNG antibodies.

HeLa cell extracts were prepared from HeLa cells (5×10^5/ml) resuspended in Tris-EDTA buffer and lysed by freeze-thaw cycles. HeLa cell extracts were incubated with GST or GST-Vpr immobilized on GSH-agarose beads, as described above, to allow binding of cellular UNG. Beads were then extensively washed and incubated for 10 min at 45°C with each dilution of the (dU)+ or (dT)+ template and heat treated at 95°C for 10 min. Where indicated, rabbit anti-UNG antibody PU101 (0.5 μg of purified immunoglobulin G per ml) or nonimmune rabbit serum was added for 10 min at 4°C to the GST-Vpr beads preincubated with HeLa cell extracts, before PCR reamplification. As a positive control, we used the *E. coli*-derived UNG (Perkin-Elmer).

Oligonucleotides primers. Sequences of oligonucleotides (all are shown in the sense orientation; restriction sites are underlined; the ATG initiation codon of

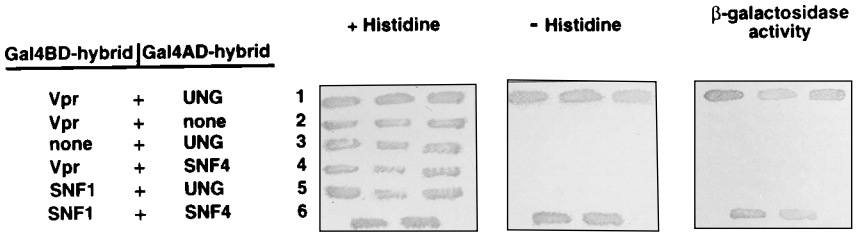

FIG. 1. Specific interaction of Vpr with human UNG in the two-hybrid system. The HF7c reporter strain expressing pairs of hybrid proteins fused to GAL4BD and to GAL4AD was analyzed for histidine auxotrophy and β-galactosidase activity. Double transformants were patched on selective medium with or without histidine and on Whatman filters for β-galactosidase assay. Growth in the absence of His and a high level of β-galactosidase activity are indications of the interaction between hybrid proteins. Interaction between SNF1 and SNF4 yeast proteins was used as a positive control.

T3wt is underlined) are as follows: Vpr1, AGTGGGATCCAGGGAACAAGC CCCAGAA; Vpr2, GGAGCCAGTAGATCCTAGCTGCAGATTCA; N15, GA CTGGATCCCACAATGAATGGACA; N27, GACTGGATCCAAGAATGAA GCTGTTA; N41, AGTGGATCCAGGCATGGCTTAGGGCAACATATC; N63, AGTGGATCCAGGATTCTGCAACAACTGCTG; pTEJ5′, AAAGATAGAT ATCACATGGAACAAGCC; pTEJ3′, GGGTGTCGACATAGCAGAATAGG CG; T3wt, CGAATTACCCTCACTAAAGAAAGAAGGATGGAACAAGCC CCAGAAGAC; C77, CGCCTATTCTGCTATGTCGACACCC; C63, AGTGG AAGCCATAATAAGATAG; C41, TTTCCTAGGATTTGGCTCTAG; 5′Vif, GTAGACCCTGGCCTGGCAGACC; 3′Vif, ATGGCAGGAAGAAGCGGA GAC; and Vif3, GTCCATTCATTGTATGGCTCC.

RESULTS

Screening for cellular proteins interacting with Vpr. We used the two-hybrid system to identify host proteins that bind to the HIV-1 Vpr protein. The yeast strain HF7c was cotransformed with a plasmid expressing the Vpr protein fused to GAL4DB and a Jurkat cell cDNA library fused to GAL4AD. About 10^6 double transformants were screened for the ability to grow on His-deficient medium, and His^+ colonies were then tested for the ability to induce β-galactosidase expression. Plasmids from His^+ β-galactosidase-positive colonies were isolated and used to retransform HF7c in combination with either a Vpr hybrid or an extraneous target, a yeast SNF1 hybrid. Four independent clones remained specifically positive with Vpr only. DNA sequence analysis of these four clones revealed that three of them corresponded to new sequences for which no homology was found in the databases. The fourth one (clone 16) was completely homologous to the human UNG cDNA, described by Olsen et al. (38) (GenBank accession number X15653), and was selected for this study. cDNA clone 16 was not full length at its 5′ end, and the resulting open reading frame encoded a UNG protein of 253 amino acid residues, lacking 52 amino acids at its N terminus. This clone conferred on HF7c the ability to grow in the absence of histidine and to produce β-galactosidase activity in the presence of the GAL4BD-Vpr hybrid but not with a GAL4BD-SNF1 hybrid or with GAL4BD alone (Fig. 1).

To localize the portion of Vpr that mediated binding to UNG, a panel of N- and C-terminal deletions in the *vpr* coding sequence fused to GAL4BD was then assayed in yeast cells for interaction with the GAL4AD-UNG hybrid. The levels of expression reached in yeast cells for all of the Vpr-deleted mutants was checked by Western blotting on yeast extracts with an anti-GAL4BD monoclonal antibody (Santa Cruz Biotechnology) and found to be comparable (data not shown). As shown in Fig. 2, deletions in the Vpr protein of either the 19 C-terminal amino acids (mutant C77) or the 14 N-terminal amino acids (mutant N15) did not disrupt binding to UNG. More extended deletions in the Vpr protein of either 33 residues at the C terminus (mutant C63) or 26 residues at the N-terminus (mutant N27) impaired binding to UNG.

The results of this analysis with the Vpr deletion mutants indicate that the part of Vpr necessary for binding to UNG corresponds to a core Vpr protein containing amino acids 15 to 77. Within this region, two domains spanning residues 15 to 27 at the N terminus and residues 63 to 77 at the C terminus seem to be essential for Vpr-UNG interaction.

In vitro-translated ^{35}S-Vpr binds to GST-UNG. In vitro experiments using GST-UNG fusion protein and in vitro-translated ^{35}S-Vpr were performed to demonstrate the direct interaction between Vpr and UNG. UNG cDNA clone 16 was subcloned into the pGEX-5X-2 vector, and the recombinant plasmid was transformed into *E. coli* and expressed as a GST-UNG fusion protein. The bacterially expressed GST or GST-UNG protein was incubated with GSH-agarose beads, and bound proteins were separated by SDS-PAGE and revealed by Coomassie blue staining (Fig. 3A, lanes 1 and 2) or by immunoblotting with anti-UNG antibody PU101 (lanes 3 and 4). We examined the binding of in vitro-translated, radiolabeled wild-type or C-terminally deleted Vpr to GST-UNG proteins. GST or GST-UNG beads were incubated with either ^{35}S-labeled wild-type Vpr or ^{35}S-labeled C-terminally deleted Vpr (C77, C63, and C41). Bound labeled proteins were resolved by SDS-PAGE and revealed by autoradiography. As shown in Fig. 3B,

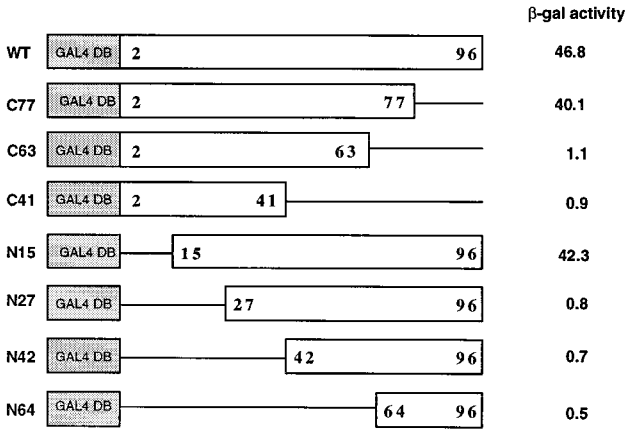

FIG. 2. In vivo interaction of Vpr mutants with UNG in the two-hybrid system. Mutant forms of Vpr were subcloned into pGBT10. Coding sequences retained by the mutants are shown by boxes. Yeast strain SFY526 was cotransformed with each GAL4BD-Vpr mutant form and GAL4AD-UNG fusion expression plasmid (clone 16) and assayed for β-galactosidase (β-gal) activity in a liquid culture assay. Values are means of triplicate determinations made on three independent transformants. The background level is around 1 U and corresponds to yeast cotransformed with the GAL4AD-UNG (clone 16) expression plasmid and GAL4BD-SNF1 expression plasmid. Interaction between SNF1 and SNF4 was used as a positive control and gave a value of around 12 U in this assay. WT, wild type.

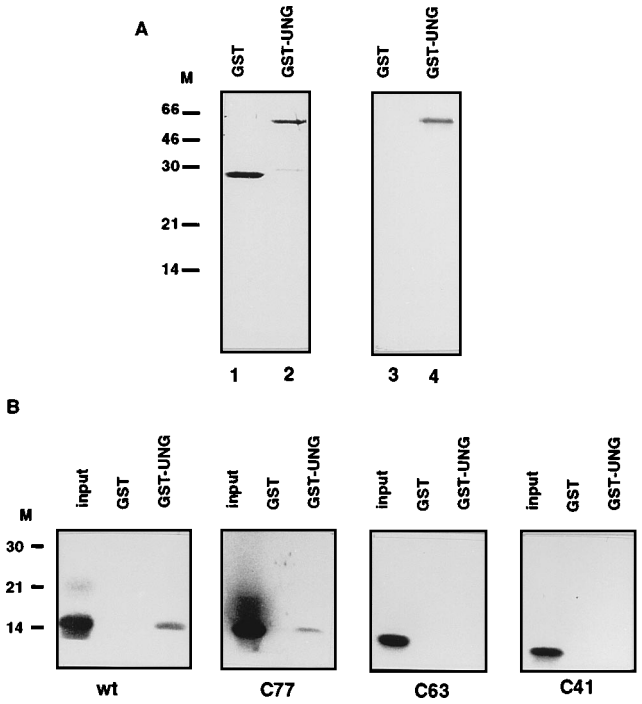

FIG. 3. Binding of Vpr to GST-UNG. (A) GST and GST-UNG fusion proteins were affinity purified on GSH-agarose beads. Bound proteins were separated by SDS-PAGE and analyzed by Coomassie blue staining (lanes 1 and 2). Bound GST and GST-UNG fusion proteins were subjected to immunoblot analysis with rabbit polyclonal anti-UNG antibody PU101 and peroxidase-linked donkey anti-rabbit immunoglobulin (lanes 3 and 4). (B) Analysis of C-terminal deletions of Vpr interacting with UNG. Translated, radiolabeled Vpr mutant proteins (5 μl) were incubated with equivalent amounts of GST and GST-UNG. Bound proteins were separated by SDS-PAGE, and the gel was autoradiographed. Lanes marked input contain 1 μl (1/5) of ^{35}S-proteins before binding to GST or GST-UNG. wt, wild-type Vpr; C77, C63, and C41, C-terminally truncated Vpr proteins, respectively. GST, GST bound to GSH-agarose beads; GST-UNG, GST-UNG bound to GSH-agarose beads. M, molecular mass markers (in kilodaltons).

^{35}S-labeled wild-type Vpr could bind specifically to GST-UNG but not to GST. Deletion of the 19 C-terminal residues of Vpr (mutant C77) did not impair binding to UNG. By contrast, deletions of 33 or 55 C-terminal residues (mutant C63 or C41, respectively) abolished binding to GST-UNG. Scanning analysis showed that the amounts of the C77 protein input bound to GST-UNG are 2.5-fold lower than that of the wild type, indicating a decrease in the affinity of this mutant for UNG. We have to note that only a slightly reduced β-galactosidase activity was observed in in vivo experiments with yeast cells for C77 compared to that for the wild type (Fig. 2), suggesting that the affinity of the C77 protein could be enhanced when association with UNG was allowed in the cellular physiological context.

These results are in agreement with those of the yeast two-hybrid studies and indicate that the interaction between Vpr and UNG is direct and does not need an intermediate bridging protein.

Purified UNG binds to GST-Vpr. We next addressed the question of whether purified UNG could interact with Vpr expressed as a chimeric GST-Vpr protein. The purified bacterially expressed UNGΔ84, containing a deletion of the 84 N-terminal amino acids, was used as a source of UNG (48) and was incubated with either GST or GST-Vpr immobilized on GSH-agarose beads. Bound proteins were eluted, resolved by SDS-PAGE, and analyzed by Western blotting with anti-UNG antibody PU101. In addition to intact UNGΔ84, smaller degradation products were seen on Western blotting with anti-UNG antibodies (Fig. 4A, lane 1). Incubation of UNGΔ84 protein with GST revealed no binding (lane 2), while incubation of UNGΔ84 with GST-Vpr showed a specific interaction (lane 3). As a control, no background could be attributed to GST-Vpr alone (lane 4). To control the specificity of the interaction, we used in binding experiments the recombinant HIV-1 Nef protein (5), and we observed no specific binding of recombinant Nef to GST-Vpr (data not shown).

We next tested whether addition of UGI, a specific uracil DNA glycosylase inhibitor which forms a stable complex with UNG (6, 51), could affect the UNG-Vpr interaction. Purified UNGΔ84 was incubated with various amounts of UGI (from 0 to 20 U) prior to the addition of GST-Vpr. The ability of UNG to bind GST-Vpr, in the presence of UGI, was revealed as described above. Vpr-UNG interaction was still immunodetectable even when 20 U of UGI was added (Fig. 4B). Control experiments showed that 20 U of UGI completely abolished the UNGΔ84 enzymatic activity, indicating that in these conditions, UGI could efficiently bind to UNG (data not shown).

We conclude from these data that (i) purified UNG interacts with GST-Vpr, (ii) the N-terminal part of UNG is not necessary for interaction with Vpr, and (iii) interaction between UNG and Vpr is not disrupted by addition of a specific inhibitor of UNG, suggesting that Vpr does not compete with UGI to interact with UNG and therefore is not a UGI-like inhibitor of UNG.

Cellular UNG associated to Vpr remains active. We then examined whether authentic cellular UNG, associated with Vpr, retains its enzymatic activity. To detect the UNG enzymatic activity, we used an indirect assay based on a PCR approach (29), as described in Materials and Methods and schematized in Fig. 5A. In this assay, when the (dU)+ or (dT)+ DNA template was incubated with *E. coli*-derived

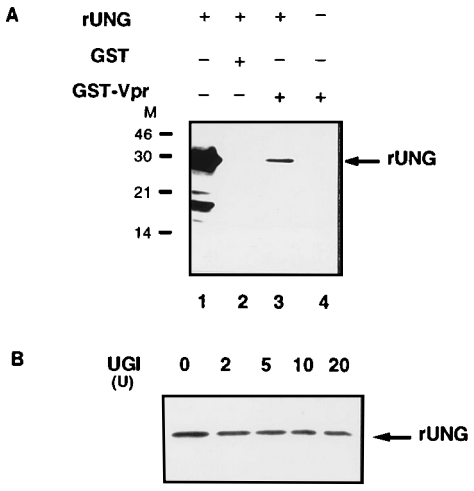

FIG. 4. Binding of purified recombinant UNG to GST-Vpr. (A) Similar amounts of GST and GST-Vpr immobilized on GSH-agarose beads were incubated with 2.6 μg of recombinant UNGΔ84. Samples were subjected to SDS-PAGE followed by Western blotting with anti-UNG antibody PU101 and peroxidase-linked donkey anti-rabbit immunoglobulin. Lane 1, purified recombinant UNG (rUNG) input; lanes 2 and 3, purified rUNG bound to GST (lane 2) or GST-Vpr (lane 3); lane 4, GST-Vpr without addition of purified rUNG. (B) Increasing amounts of UGI (0 to 20 U) were added to purified rUNG before incubation with GST-Vpr. Processing of samples was as for panel A. GST, GST bound to GSH-agarose beads; GST-Vpr, GST-Vpr bound to GSH-agarose beads; M, molecular mass markers (in kilodaltons).

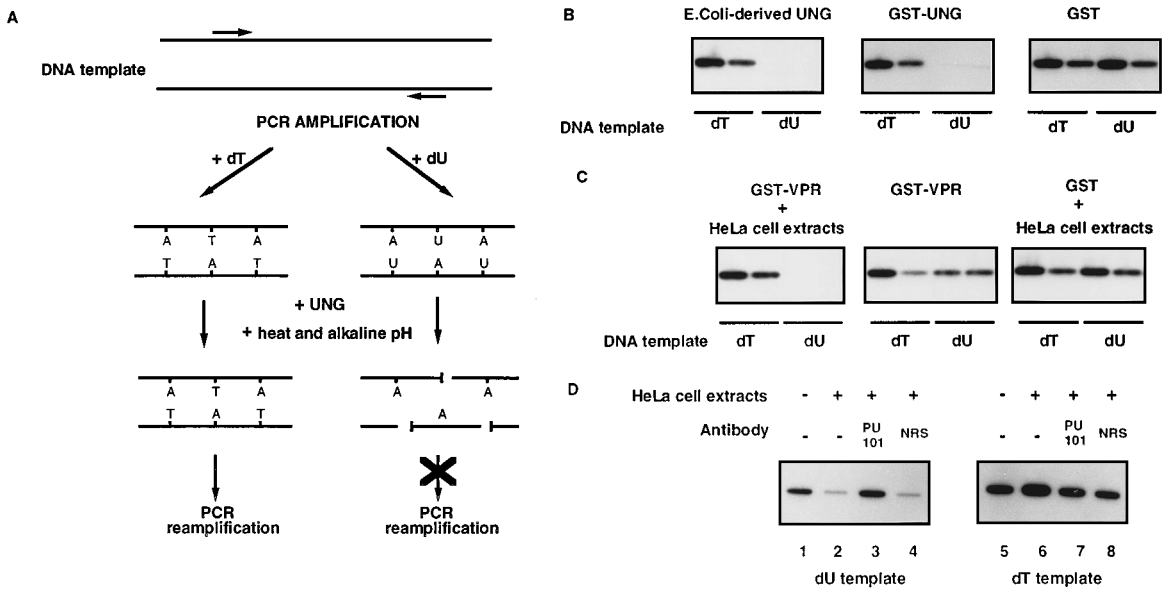

FIG. 5. The enzymatic activity of cellular UNG is retained after binding to Vpr. (A) The PCR protocol used to detect the UNG enzymatic activity is as described by Perkin-Elmer. (B) Two dilutions (10^{-7} and 10^{-8}) of the (dT)+ and (dU)+ templates were used for analysis of the susceptibility of the UNG enzymatic activity. Each template was incubated in the same experimental conditions with 2 μl of *E. coli*-derived UNG (left panel), with GST-UNG beads (central panel), and with GST beads (right panel). (C) DNA templates used were as described for panel B. PCR reamplification of templates incubated with GST-Vpr beads in the presence of HeLa cell extracts (left panel), with GST-Vpr beads in the absence of HeLa cell extracts (central panel), and with GST beads in the presence of HeLa cell extracts. (D) PCR reamplification of the (dU)+ template (left panel) and (dT)+ template (right panel) preincubated with GST-Vpr beads (lanes 1 and 5), with UNG-containing HeLa cell extracts bound to GST-Vpr beads (lanes 2 and 6), with UNG-containing HeLa cell extracts bound to GST-Vpr beads in the presence of anti-UNG antibody PU101 (lanes 3 and 7), and with UNG-containing HeLa cell extracts bound to GST-Vpr beads in the presence of nonimmune normal rabbit serum (NRS; lanes 4 and 8).

UNG, as expected, the (dT)+ but not the (dU)+ template could be reamplified (Fig. 5B, left panel). The GST-UNG fusion protein immobilized on GSH-agarose beads also exhibited the UNG enzymatic activity easily detectable by the PCR assay (central panel). Indeed, GST used as a negative control displayed no UNG activity (right panel). These results indicate that the enzymatic activity of UNG encoded from cDNA clone 16 can be revealed when UNG is in a chimeric protein form immobilized on GSH-agarose beads. Also, these results indicate that proteins immobilized on GSH-agarose beads can be used in the PCR.

To detect the enzymatic activity of cellular UNG bound to Vpr, GST- or GST-Vpr beads were first incubated with HeLa cell extracts to allow binding of cellular UNG, extensively washed, and then analyzed by PCR as depicted in Fig. 5A. As shown in Fig. 5C, GST-Vpr beads incubated with HeLa cell extracts exhibited UNG activity, since the (dU)+ DNA template could not be amplified anymore, in contrast to the amplification of the (dT)+ DNA template (left panel). GST-Vpr immobilized on GSH beads, by itself and in the absence of preincubation with cell extracts, did not impair the PCR with either of the DNA templates used (central panel), indicating that the UNG activity detected was indeed due to the cellular UNG from HeLa cells specifically bound to Vpr. No cellular UNG activity was retained on GST beads preincubated with HeLa cell extracts (right panel).

To confirm that the identification of the UNG enzymatic activity retained on GST-Vpr corresponded to the true UNG, PCRs were performed in the presence of an anti-UNG specific antibody. We used anti-UNG antibody PU101, which specifically inhibited the human UNG enzymatic activity (48), and, as a control, nonimmune rabbit serum. As expected, the UNG enzymatic activity was detected in the presence but not in the absence of HeLa cell extracts (Fig. 5D, lanes 1 and 2). The UNG enzymatic activity was abolished when PCR amplification of the (dU)+ DNA template was performed in the presence of anti-UNG antibody PU101 (lane 3), while nonimmune rabbit serum had no effect (lane 4). As a control, no inhibition was observed when the (dT)+ DNA template was used for the PCR (right panel).

Taken together, these results clearly indicate that (i) authentic cellular UNG from human HeLa cells can interact with GST-Vpr and (ii) UNG retains its enzymatic activity upon association with Vpr.

Coimmunoprecipitation of Vpr and cellular UNG in human cells. To demonstrate that Vpr can associate with cellular UNG in human cells, coimmunoprecipitation experiments were performed with HeLa cells expressing Vpr following transient transfection. Cells were transiently transfected with the pTEJ-Vpr expression vector, and cell extracts were prepared from either transfected or mock-transfected HeLa cells and subjected to immunoprecipitation with flag M2 antibodies. Proteins present in the immunocomplexes were resolved by SDS-PAGE and then subjected to Western analysis with anti-Vpr and anti-UNG antibodies (Fig. 6). As expected, anti-Vpr antibodies revealed the Vpr protein in the immunoprecipitate from transfected cells (lane 2) and not from untransfected cells (lane 1). Anti-UNG antibody PU101 revealed a protein with the expected size for the mature form of UNG in the immunoprecipitate from transfected cells (lane 4) but not from untransfected cells (lane 3). These results indicate that cellular UNG coprecipitates with Vpr, confirming that a specific interaction between the two proteins takes place in human cells.

DISCUSSION

To identify cellular partners interacting with Vpr, we used the GAL4 two-hybrid system. Although Vpr has been reported

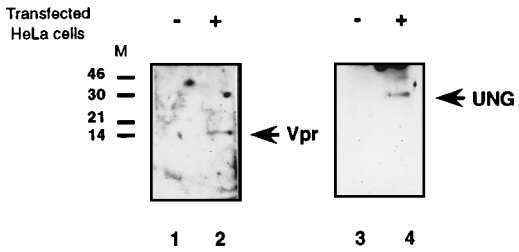

FIG. 6. Coimmunoprecipitation of Vpr and UNG in human cells. HeLa cells were transfected with the expression plasmid pTEJ-Vpr, encoding a chimeric tagged Vpr protein. Cell extracts were prepared 48 h posttransfection and immunoprecipitated with flag M2 antibodies. Immunoprecipitates were resolved on an SDS–10% polyacrylamide gel. In the Western blot analysis of coimmunoprecipitated proteins with flag M2 antibody, Vpr was immunodetected with an antiserum raised against recombinant Vpr fused to GST protein. Lane 1, untransfected cells; lane 2, transfected cells. UNG was immunodetected with anti-UNG antibody PU101. Lane 3, untransfected cells; lane 4, transfected cells. M, molecular mass markers (in kilodaltons).

to act as a transcriptional activator of several viral promoters (11), we did not find any ability of the GAL4BD-Vpr hybrid by itself to activate the transcription in yeast of the two GAL4-inducible reporter genes (HIS3 and *lacZ*) used in the two-hybrid system as previously reported (7). This observation, which allowed us to use the Vpr hybrid as a bait in the screen without any background, confirmed that Vpr did not possess, on its own, a transcriptional activator domain, as demonstrated by transient transfection experiments using HeLa cells and chimeric proteins containing GAL4BD fused to different portions of Vpr (1).

In the two-hybrid screen for cDNAs encoding cellular proteins that interact with HIV-1 Vpr, we isolated a cDNA clone (clone 16) which encodes human UNG. The sequence of this clone was completely homologous to that of a cDNA clone coding for human UNG previously isolated from a human placenta cDNA library (38). Interaction between Vpr and UNG was confirmed both in vitro by protein-protein binding studies and ex vivo by coimmunoprecipitation experiments with HeLa cells expressing transiently Vpr. We demonstrated also that the enzymatic activity of UNG was retained upon association of UNG with Vpr.

Using a panel of N- and C-terminal Vpr deletion mutants, we mapped the UNG-Vpr interaction region in the core of Vpr between residues 15 and 77. In this region, two domains, from amino acids 15 to 27 at the N terminus and from amino acids 63 to 77 at the C terminus, seem to be critical for interaction. Interestingly, essential determinants for Vpr functions have been mapped within both regions. Mutations of the highly conserved acidic residues between amino acid positions 17 and 34 eliminated Vpr virion incorporation (32), as did deletion in the C-terminal part of Vpr (39). In addition, the domain between positions 63 and 77, which contains the motif HS/FRIG, has been implicated in the effect of Vpr on cell growth arrest and gross cell enlargement of the yeast *S. cerevisiae* (31). Additional mutants including point mutations within Vpr are needed to identify the residues involved in the interaction with UNG.

Clone 16 is not full length and corresponds to a UNG polypeptide of 253 amino acid residues devoid of 52 residues at the N terminus. Two isoforms of UNG have been described; a precursor form, 304 amino acids long, is essentially located in the cytoplasmic compartment, while the mature predominant form of UNG (227 amino acids long) lacks the first 77 residues and is localized in the nucleus and mitochondria (47). In vitro protein-protein binding experiments indicated that Vpr can interact with UNG (UNGΔ52) encoded by clone 16 as well as with purified UNG (UNGΔ84), suggesting that the N-terminal part of UNG is not required for the interaction. This means that the mature form of UNG is probably the main target for interaction with Vpr.

UNGs belong to the specific class of DNA repair enzymes and have been identified in a wide variety of prokaryotic and eukaryotic organisms. Their biological function is to specifically remove from DNA the RNA base uracil, the N-glycosidic bond linking the base to the deoxyribose sugar by hydrolysis, followed by initiation of the base excision repair process. By monitoring the UNG enzymatic activity with an indirect PCR approach, we demonstrated that UNG associated with Vpr was still able to degrade dU-containing DNA templates. These data indicate that the catalytic site of UNG and the DNA binding region of UNG (34) are not affected upon association between Vpr and UNG, although a minor positive or negative modulation of the enzymatic activity could not be excluded. Nevertheless, the lack of competition between Vpr and UGI for binding to UNG allows us to exclude the hypothesis of a UGI-like function for Vpr as a strong inhibitor of UNG.

Uracil may arise in DNA by two independent pathways (for a review, see reference 19). It may become misincorporated during DNA synthesis when the dUTP pool is high or may arise by deamination of cytosine (for a review, see reference 46). If not repaired, uracil resulting from cytosine deamination would give a G:C-to-A:T transition mutation in the next round of replication. Although not shown to be mutagenic, misincorporated uracil could be cytotoxic as a result of alteration of sequences specific for binding of transcription factors, leading to alterations in gene expression (17, 50). Another distinct class of proteins, the dUTPases, is also implicated to prevent dUMP incorporation into DNA during DNA synthesis. They act directly on the pool of intracellular nucleotides, maintaining a low ratio of dUTP to dTTP in the cell.

Both UNG and dUTPase are encoded by some DNA viruses, like poxviruses and herpesviruses (33, 43). Genomes of retroviruses encode only dUTPase (15). Lentiviruses from nonprimate species, such as feline immunodeficiency virus, caprine arthritis-encephalitis virus, visna-maedi virus, or equine infectious anemia virus, contain in their genomes, as an integral part of the *pol* gene, a dUTPase-encoding sequence, located between the RNase H and integrase sequences (15). In contrast, the genomes of lentiviruses from primate species do not exhibit gene sequences coding for either dUTPase or UNG. This genetic difference between primate and nonprimate lentiviruses is not understood, and our data concerning interaction of HIV-1 Vpr with cellular UNG could help to assess this question. One hypothesis could be that the association between Vpr and UNG plays a role similar to that played by the dUTPases of nonprimate lentiviruses, i.e., the reduction of uracil misincorporation into proviral DNA. Studies to determine the uracil content within the proviral DNA, as well as studies to monitor the enzymatic activity of UNG, in cells infected with either wild-type or Vpr-defective virus should help to assess this question.

We have to mention that attempts to identify UNG incorporated within virions through its specific association with Vpr were unsuccessful. In four independent experiments, HIV-1 virions were concentrated by centrifugation from cell-free supernatant of acutely infected H9 T cells and highly purified on a sucrose density gradient. In all virus preparations, although Vpr always has been immunodetected, the UNG protein was never identified either by Western blotting analysis or by its enzymatic activity in our PCR assays (data not shown). The most probable interaction of Vpr with UNG occurs mainly

intracellularly, during the replication cycle of HIV. Colocalization experiments by confocal microscopy should help to confirm this assumption.

Although some cellular proteins have been reported to interact with Vpr by affinity chromatography (54), and in particular a protein of 41 kDa, RIP1 (41), which is linked to the glucocorticoid receptor type II complex, these proteins have not been molecularly identified and their coding cDNAs have not been cloned. It should be noted that they do not correspond to UNG, since they possess distinct biochemical characteristics. Thus, UNG is the first protein characterized at the molecular level which associates with Vpr. However, the full functional relevance of such interaction remains to be elucidated.

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