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New therapeutic agents able to block HIV-1 replication are eagerly sought after to increase the possibilities of treatment of resistant viral strains. In this report, we describe a rational strategy to identify small peptide sequences owning the dual property of penetrating within lymphocytes and of binding to a protein target. Such sequences were identified for two important HIV-1 regulatory proteins, Tat and Rev. Their association to a stabilizing domain consisting of human small ubiquitin-related modifier-1 (SUMO-1) allowed the generation of small proteins named SUMO-1 heptapeptide protein transduction domain for binding Tat (SHPT) and SUMO-1 heptapeptide protein transduction domain for binding Rev (SHPR), which are stable and efficiently penetrate within primary lymphocytes. Analysis of the antiviral activity of these proteins showed that one SHPR is active in both primary lymphocytes and macrophages, whereas one SHPT is active only in the latter cells. These proteins may represent prototypes of new therapeutic agents targeting the crucial functions exerted by both viral regulatory factors.

HIV-1[1] expresses several regulatory proteins that divert key cellular factors to allow rapid and efficient production of viral particles. After two decades of extensive studies, significant progresses have been achieved in the understanding of the molecular mechanisms mediating various actions of these regulatory proteins. Particular attention has been paid to two of them, Tat and Rev. The first potently activates transcription of the integrated provirus by establishing contacts with both transcription factors and the transactivation-responsive element motif located at the 5′ end of the viral RNA (for reviews see Refs. 1 and 2). This activator has been shown to interact with the Sp1 upstream transcription factor (3), general initiation factors including TBP (4, 5), TFIIB (6), and RNA polymerase II (7), and coactivators as p300 and PCAF (8, 9). It has also been shown that Tat is able to interact simultaneously with transactivation-responsive element and the CDK9/cyclin T complex (10, 11), thereby stimulating the phosphorylation of the RNA polymerase II large subunit carboxyl-terminal domain and ensuring a stable elongation (12, 13). These interactions, which are regulated by posttranslational modification of the protein as acetylation (14, 15), allow Tat to potently activate transcription of the provirus by acting both at the initiation and elongation steps (16). Besides its transcriptional activation power, Tat exhibits other properties that are likely to intervene in the onset of the immunodeficiency resulting from HIV-1 infection (17–19).

Rev also has the dual ability to interact with RNA, the Rev response element motif present in intronic position in the 3′ part of the viral RNA in this case, as well as with cellular nuclear factors (for a review see Ref. 20). This viral protein includes both a nuclear localization signal (NLS) and a nuclear export sequence (NES) and, by shuttling between the nucleus and the cytoplasm, allows export and hence translation of unspliced or partially spliced viral RNAs (21–23). Export of Rev from the nucleus depends on association with hCRM1 and RanGTP (24–28). Rev is also known to bind nucleoporins (29–32), and recently, it has been shown that a kinesin-like protein binds to the NES of Rev and stimulates the activity of the protein (33). Although several cofactors are likely to intervene in the RNA export process mediated by Rev, hCRM1 and Ran have been shown to be sufficient to induce translocation to the cytoplasm of incompletely spliced HIV mRNAs (34, 35).

By considering that both Tat and Rev are small proteins that act through protein-protein interactions, we reasoned that it should be feasible to inhibit their function, which is absolutely necessary to viral replication, with peptide ligands competitively interfering with the associations in which they are engaged. Because it has the interest of reporting the interaction

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† This paper is dedicated to the memory of Dr. Dominique Dormont (Deceased November 16, 2003).

[S] The on-line version of this article (available at http://www.jbc.org) contains Supplemental Figs. 1 and 2 and Tables 1–3.

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; NES, nuclear export sequence; NLS, nuclear localization signal; PTD, protein transduction domain; SHP, SUMO-1 heptapeptide PTD; SUMO, small ubiquitin-related modifier; SHPT, SUMO-1 heptapeptide PTD for binding Tat; SHPR, SUMO-1 heptapeptide PTD for binding Rev; RT, reverse transcriptase; PBS, phosphate-buffered saline; PBMC, peripheral blood mononuclear cells; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; MDM, monocyte-derived macrophages; AZT, azidothymidine; TCID50, 50% tissue culture infectious doses; GST, glutathione *S*-transferase; ED, effective doses; PHA-P, phytohemagglutinin-P; rHuIL-2, recombinant human interleukin-2; SEAP, secreted alkaline phosphatase.

in the intracellular environment, especially the nucleus, the two-hybrid approach (36) represented the method of choice for selection of such peptides from a library of random sequences (37). An important problem with peptides is their intracellular penetration. To avoid utilization of complex gene therapy procedures, this property was added at the screening step through adjunction of a protein transduction domain (PTD), the Tat basic domain in this case. In this report, we present data showing that this method led to identification of short peptide sequences that bind to Tat or Rev. Association of these peptide motifs with SUMO-1 for their stabilization led to small proteins named SUMO-1 heptapeptide protein transduction domain (SHP) that efficiently penetrate within lymphocytes and inhibit, for some of them, the function of Rev. These proteins, which were also observed to inhibit viral replication, in both lymphocytes and macrophages might represent new therapeutic agents useful for impairing generation of new viral particles.

EXPERIMENTAL PROCEDURES

Construction of the Library of Peptides of Random Sequence—As a first step, the sequence coding for the Tat PTD was inserted in plasmid pGAD424 (Clontech). The phosphorylated sense, 5′-AATTGGGTGGTGGCGGATCCGGTTTGCCCGGGAGAAAGAAGCGTAGACAAAGAAGACGTGGTTA-3′, and antisense, 5′-GATCTTAACCACGTCTTCTTTGTCTACGCTTCTTTCTCCCGGGCAAACCGGATCCGCCACCACCC-3′, oligonucleotides were hybridized and inserted between the EcoRI and BglII restriction sites of pGAD424, giving plasmid pGAD-CR. DNA fragments coding for heptapeptide motifs of random sequence were generated as follows. The 5′-biotinylated sense, 5′-ACTCGGATCC*NNNNNNNNNNNNNNNNNNN*CCCGGGGTCGCAGTG-3′ (*N* representing any of the four nucleotides A, G, C, or T), and antisense, 5′-CACTGCGACCCCGG-3′, oligonucleotides were hybridized, and the 3′-recessed end was filled-in using the Klenow fragment of the *Escherichia coli* DNA polymerase I. The DNA fragments were digested by BamHI and XmaI restriction enzymes, and the digestion products were incubated with streptavidin paramagnetic beads (Promega) to remove the biotinylated ends. The unbound fragments were inserted between the BamHI and XmaI restriction sites of plasmid pGAD-CR, giving plasmids pGAD-CR-Ps. The ligation products were transformed in the *E. coli* strain XL1-blue and streaked on LB agar plates containing ampicillin. 2×10^6 independent colonies were recovered and grown for 1 h in LB medium containing ampicillin. Plasmids were extracted and purified according standard PEG procedure.

Two-hybrid Screening—Plasmid pLex-Tat was constructed as follows. The pSG-Tat mammalian expression vector (5) was digested by the NcoI restriction enzyme, and the 3′-recessed ends were filled-in using the Klenow fragment of DNA polymerase I. The vector was further digested by BamHI, and the resulting DNA fragment corresponding to the Tat coding sequence was inserted between the SmaI and BamHI restriction sites of plasmid pLex9 (32). For Rev, the pLex-Rev and pLex-NES constructs were used as baits (32). The two-hybrid screens with pLex-Tat, pLex-Rev, or pLexNES as bait and pGAD-CR-P as prey were performed in *Saccharomyces cerevisiae* HF7c strain, and colonies that grew on minimal medium lacking histidine were analyzed for β-galactosidase expression by the filter assay as described previously (38). The pGAD-CR-P plasmid from positive colonies was recovered, and the heptapeptide motifs were characterized by DNA sequencing.

Mammalian Expression Constructs—For two-hybrid assay in mammalian cells, the prey plasmids were constructed by inserting the sequence coding for the heptapeptide motif fused to the Tat PTD in the pSG-FNV mammalian expression vector, which includes sequences coding for the FLAG epitope as well as for a NLS and for the VP16 activation domain (39). Amplification from pGAD-CR-P constructs was performed using the sense, 5′-TGAAGGTCGACCACCAAACCCAAAAAAAGAG-3′, and antisense, 5′-CCTGAGAAAGCAACCTGACC-3′, oligonucleotides, and the amplified fragment was digested with the SalI and BglII restriction enzymes. The digestion product was inserted between the XhoI and BglII restriction sites of pSG-FNV, giving plasmid pSG-FNV-P. Vectors expressing the LexA-Rev and LexA-Tat fusion proteins were pSG5-LexA-Rev (32) and pSG5-LexA-Tat, respectively. This latter construct was generated by inserting the NcoI (filled-in)-BamHI restriction fragment of pSG-Tat between the SmaI and BamHI restriction sites of pSG5-LexA (32). Vectors expressing the SHP proteins were generated in two steps. The human SUMO-1 sequence was amplified from an expressed sequence tag using the following sense and antisense primers, 5′-GGGTCGACGTCCATATGTCTGACCAGGAGG-3′ and 5′-ATCAGATCTGAATCTCGAGCCGTTTGTTCCTGATAAAC-3′, respectively. The amplified DNA fragment was cut by SalI and BglII restriction enzymes and inserted between the XhoI and BglII restriction sites of pTL1, which is a pSG5 derivative, giving plasmid pTL1-SUMO-CP. The heptapeptide-PTD modules were amplified from pGAD-CR-P as described above and after digestion with SalI and BglII restriction enzymes inserted between the XhoI and BglII restriction sites of pTL1-SUMO-CP, giving vectors pTL1-SHP.

Cell Culture and Transfection—All of the cells were incubated at 37 °C in a 5% CO_2-humidified atmosphere. HeLa and COS7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. Transfections were performed by the calcium phosphate coprecipitation method. For mammalian two-hybrid assay, transfections were done in 6-well plate seeded with 80,000 HeLa cells. The DNA mixture included 125 ng of secreted alkaline phosphatase (SEAP) reporter construct, 25 ng of vectors expressing Tat or Rev fused to LexA along with the amount determined as optimum for the prey-expressing plasmids, *i.e.* 250 ng for motifs selected against Tat and 25 ng for motifs selected against Rev with the exception of clones 142 and 190 (50 ng). SEAP activity was measured using the SEAP reporter gene assay chemiluminescent kit (Roche Applied Science). Tat functional assay was performed by transfecting 300,000 HeLa cells in 60-mm Petri dish with the LTR HIV-CAT reporter construct (50 ng) (40), pSG-Tat (2 ng), and SUMO-1 or SHPT expression vectors (0.5 and 2 μg). Rev functional assay was performed similarly by transfecting the pDM128 reporter plasmid (50 ng) (41), pSG-Rev (5 ng), and SUMO-1 or SHPR expression vectors (0.5 and 2 μg). CAT activity was measured using the CAT enzyme-linked immunosorbent assay kit (Roche Applied Science). Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Human peripheral blood mononuclear cells (PBMC) and monocytes were isolated from the blood of healthy seronegative donors by density gradient centrifugation using Ficoll-Hypaque (Eurobio) and adherence. Human PBMC were 3-day-activated with 1 μg/ml phytohemagglutinin-P (PHA-P, Difco) and 5 IU/ml recombinant human interleukin-2 (rHuIL-2, Roche Applied Science). PBMC then were dispensed into 96-well microplates (100,000 cells/well) in 200 μl of medium A (RPMI 1640 cell culture medium (Invitrogen), 10% fetal calf serum (Bio West), and 1% penicillin, streptomycin, and neomycin mixture (Invitrogen) supplemented with 20 IU/ml rHuIL-2). Monocyte-derived macrophages (MDM) were differentiated from monocytes for 7 days by adherence. At day 3, 300,000 cells were dispensed/well into 48-well plates in 1 ml of cell culture medium. Monocyte differentiation and MDM cultures were performed in cell culture medium A′ (Dulbecco's modified Eagle's medium Glutamax™ supplemented with 10% fetal calf serum and 1% penicillin, streptomycin, and neomycin mixture).

Production in Bacteria and Purification of SHP—SHP sequences were cloned between the HindIII and BglII restriction sites in the bacterial expression vector pFLAG.MAC (IBI). Vectors were used to transform *E. coli* BL21-CodonPlus™-RP (Stratagene), which were cultured in 2 liters of LB medium up to an optical density of 0.9. After 30 min at 18 °C, 0.1 mM isopropyl-1-thio-β-D-galactopyranoside was added to the cultures that were pursued overnight at 18 °C. Bacteria were sonicated in lysis buffer (200 mM NaCl, 0.1 M Tris-HCl, pH 7.4, 10 mM $MgCl_2$), which was completed with Complete antiprotease (Roche Applied Science), 0.5 mg/ml lysozyme, and 20 units/ml Benzonase (Sigma). After centrifugation at 4,000 rpm for 30 min, the supernatant was loaded on a 5-ml column of Heparin HyperD (Biosepra). Elution was done with DE 600 buffer (600 mM KCl, 20 mM Hepes, pH 7.9, 10 μM ZnCl2, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol). The eluate was fractionated through a Hiload 16/60 Superdex 200 gel filtration column (Amersham Biosciences). Fractions containing the SHP were pooled, and after dialysis against DE 50 buffer (same composition as DE 600 with the exception that KCl concentration was 50 mM), fractions were loaded on a 5-ml Mono Q HyperD (Biosepra) column. The flow-through was dialyzed against 0.1× phosphate-buffered saline (PBS), concentrated 10 times by lyophilization, and sterile-filtered.

Immunoblot and Immunofluorescence—Proteins were separated on polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Immunoblots were carried out using the M2 monoclonal antibody directed against FLAG (Sigma) diluted 1:1000 and revealed using ECL (Amersham Biosciences). After incubation with the SHPs, Jurkat cells were added to slides coated with polylysine (1 mg/ml solution added for 5 min). Cells were fixed for 10 min in 4% paraformaldehyde in PBS, incubated for 10 min in 0.1 M glycine in PBS, and then permeabilized for 5 min in 1% Triton X-100 in PBS and blocked with 1%

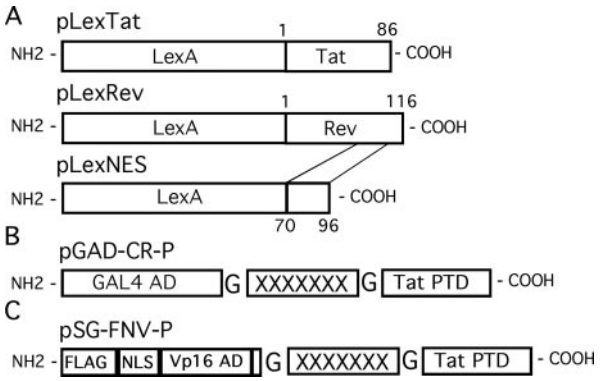

FIG. 1. **Schematic representation of the proteins used as baits (A) and preys (*B* and *C*) for two-hybrid assays in yeast (*A*) or in mammalian cells (*C*).** *A*, the baits consisted of the LexA protein fused to Tat (amino acids 1–86), Rev (amino acids 1–116), or the nuclear export domain of Rev (amino acids 70–96). *B*, yeast preys comprised the GAL4 activation domain, a stretch of seven amino acids of random sequence, and the Tat PTD. *C*, mammalian prey included the FLAG epitope, the SV40 large T NLS, the herpes simplex virus Vp16 activation domain, and the heptapeptide-Tat PTD module.

bovine serum albumin in PBS. The antibody to FLAG was used at 1:500 dilution and incubated for 2 h at room temperature. Secondary antibody coupled to Alexa Fluor 488 (Molecular Probes) diluted at 1:1000 was further added to the cells for 1 h. Slides were mounted in medium containing Mowiol (Calbiochem) and observed with a LSM 510 confocal microscope (Zeiss).

Virus and Antiviral Assays—PBMC were infected with the reference lymphotropic HIV-1-LAI strain (42), and MDM was with the reference macrophage-tropic HIV-1/Ba-L strain (43). These viruses were amplified *in vitro* with PHA-P-activated umbilical blood mononuclear cells. Cell-free supernatants were ultracentrifugated at 360,000 × *g* for 10 min to eliminate soluble factors such as cytokines, and pellets were resuspended in cell culture medium. Viral stocks were titrated using PHA-P-activated PBMC, and 50% tissue culture infectious doses (TCID50) were calculated using Kärber's formula (44).

PBMC were pretreated for 30 min by five concentrations of each molecule and infected with 75 TCID50 of the HIV-1-LAI strain. AZT (1.6, 8, 40, 200, and 1,000 nM) and indinavir (1.6, 8, 40, 200, and 1,000 nM) were used as control. SHPT-8, SHPR-15, SHPR-190, and SHPR-142 were tested at 31, 62.5, 125, 250, 500, 1000, and 2000 nM. Molecules were maintained throughout the culture, and cell supernatants were collected at day 7 postinfection and stored at −20 °C to measure viral replication by reverse transcriptase (RT) activity dosage.

MDM were pretreated for 30 min by five concentrations of various molecules and infected with 30,000 TCID50 of the HIV-1/Ba-L strain. AZT (0.8, 4, 20, 100, and 500 nM) and indinavir (0.8, 4, 20, 100, and 500 nM) were used as control. SHPT-8, SHPR-15, SHPR-190, and SHPR-142 were tested at 62.5, 125, 250, 500, 1000, and 2000 nM. Molecules were maintained until 7 days after infection, and cell supernatants were collected at days 7, 14, and 21 and stored at −20 °C to measure viral replication by RT activity dosage.

Both PBMC and MDM were microscopically observed before supernatant harvest to assess possible drug-induced cytotoxicity. RT activity was measured in supernatants using the RetroSys RT kit (Innovagen). Experiments were performed in triplicate, and results were expressed as the mean of RT activity ± S.D. 50, 70, and 90% effective doses (ED_{50}, ED_{70}, and ED_{90}) were calculated using percents of untreated controls and microcomputer software (Biosoft).

RESULTS

Identification of Peptide Sequences Binding to Tat and Rev—A search for short peptide sequences, which bind the regulatory proteins Tat and Rev, was performed by two-hybrid screening in yeast. Baits consisted of the LexA sequence fused to the entire sequence of Tat, Rev, or the NES of Rev (Fig. 1*A*). The prey included three different parts: 1) the GAL4 activation domain; 2) a random sequence of seven amino acids; and the PTD of Tat (Fig. 1*B*). The random sequence was selected of restricted length to limit the occurrence of stop codons and the presence of unnecessary sequences. The various domains were separated by proline and glycine residues to allow some flexibility between the different functional parts. The library of prey expression vectors was constructed by the cloning of DNA fragments prepared from synthetic oligonucleotides including a series of 21 degenerate positions (see "Experimental Procedures"). This library was used in three different screens with Lex-Tat, Lex-Rev, or Lex-NES as baits (see Supplementary Table 1*a*). Prey vectors present in clones, which grew on histidine minus medium and which expressed β-galactosidase, were isolated and tested again. None of these vectors triggered β-galactosidase expression when LexA alone was used as bait (see Supplementary Table 1, *b–d*). Four vectors selected against Tat were found positive again (see Supplementary Table 1*b*). This was also the case for 3 and 19 vectors obtained after screening against Rev and NES, respectively (see Supplementary Table 1, *c* and *d*). The peptide motifs of these Tat- and Rev-positive clones along with those of three of the NES clones were analyzed further by the two-hybrid assay in mammalian cells. The heptapeptide-PTD modules were inserted in a mammalian expression vector downstream of a NLS linked to the Vp16 activation domain (Fig. 1*C*). When LexA, either alone or together with these prey proteins, was transiently expressed, no expression of SEAP under the control of LexA binding sites was observed (data not shown). Both Lex-Tat and Lex-Rev were by themselves able to trigger some expression of SEAP, but cotransfection of the prey vectors increased this production. This was the case for the peptides selected against Tat (Fig. 2*A*) with the Lex-Tat bait and for those issued from either the Rev or NES screens when Lex-Rev was used as bait (Fig. 2*B*). These results showed that the sequences selected in yeast also bind Tat and Rev proteins in the nucleus of human cells.

Inhibition of the Tat and Rev Function—Considering the binding properties of the isolated peptide sequences, it was anticipated that they might antagonize the activity of Tat and Rev by impairing association with cellular effectors. To examine this point, functional assays were performed. As small peptides are often instable, they were fused to a stabilizing protein. Expected properties for this latter one were to be small, abundant, ubiquitously expressed, and of human origin. For these reasons, we selected members of the ubiquitin family. Constructs were made with both ubiquitin itself and SUMO-1 (45, 46). Because better results were obtained with it, SUMO-1 was chosen (data not shown). The entire coding sequence including a mutation of the carboxyl-terminal diglycine motif was inserted upstream of that coding for the heptapeptide-PTD module (Fig. 3*A*). As stated earlier, the resulting artificial proteins were named SUMO-1 heptapeptide protein transduction domain, abbreviated SHPT or SHPR for those binding Tat or Rev, respectively. The effect of the four different SHPTs on the transactivation of the HIV-1 promoter by Tat was evaluated by transient expression experiments in HeLa cells. As control, the effect of overexpression of wild-type SUMO-1 was also tested. At low concentration, the various SHPT did not reduce the Tat transactivation and even slightly increased it as compared with wild-type SUMO-1 (Fig. 3*B*, *light gray bars*). At the highest concentration, a moderate reduction of the Tat transactivation was observed, especially for SHPT-8 (Fig. 3*B*, *dark gray bars*). These data showed that the selected SHPT are poor inhibitors of the Tat function, possibly because of a limited affinity for the transactivator that hampers efficient competition with the different transcription factors known to associate with Tat. It is also possible that Tat does not exhibit a particular structure in the absence of its natural partners as cyclin T, thereby impairing selection of efficient inhibitory peptides. The activity of SHPR was evaluated using the pDM128 reporter construct that includes the CAT-coding sequence along with

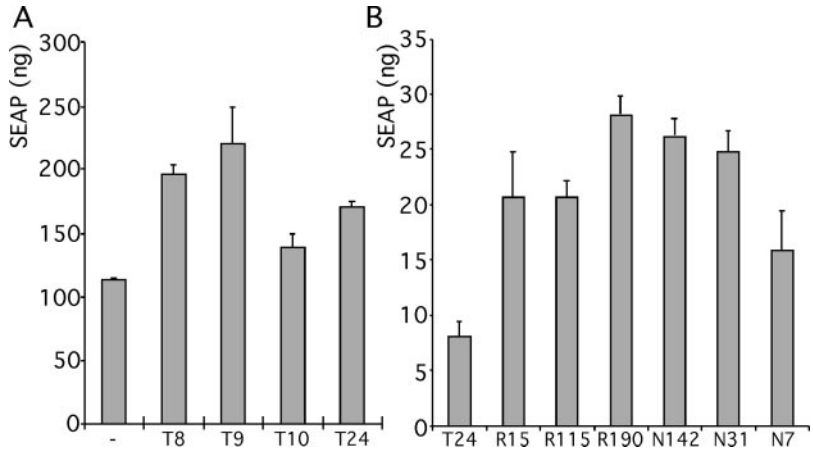

FIG. 2. **Analysis of heptapeptide-Tat PTD modules selected against Tat and Rev by mammalian two-hybrid assay.** *A*, HeLa cells were transfected with the pSEAP LEX5X reporter construct and pSG5-LexA-Tat, alone or with pSG-FNV-P-T8, pSG-T9, pSG-T10, or pSG-T24. The amount of SEAP was measured, and the mean of the values obtained for two independent point of transfection is represented. The *error bar* corresponds to half of the difference between the two values. *B*, the same assay was performed with pSEAP LEX5X and pSG5-LexA-Rev together with pSG-FNV-P-T24, pSG-FNV-P-R15, pSG-FNV-P-R115, pSG-FNV-P-R190, pSG-FNV-P-N142, pSG-FNV-P-N31, and pSG-FNV-P-N7. The results are represented as in *A*. *WT*, wild type.

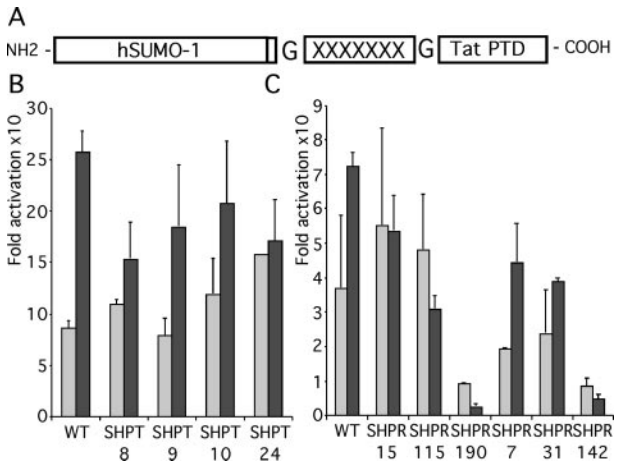

FIG. 3. **Inhibition of the Tat and Rev activities by the SHPT and SHPR constructs.** *A*, schematic representation of the SHP construct, which included the entire human SUMO-1 sequence, of which the diglycine (GG) motif was mutated in Ala-Arg, associated at its COOH terminus with the heptapeptide-Tat PTD module. *B*, HeLa cells were transfected by the LTR HIV-CAT construct together with pSG-Tat and pTL1-SUMO-1, pTL1-SHPT-8, pTL1-SHPT-9, pTL1-SHPT-10, and pTL1-SHPT-24. The amount of these latter plasmids was either 0.5 (*light gray bars*) or 2 μg (*dark gray bars*). The CAT activity was measured, and the mean of the values obtained for two independent points of transfection is represented. The *error bar* corresponds to half of the difference between the two values. *C*, for evaluating the Rev activity, HeLa cells were transfected with plasmid pDM128 and pSG-Rev together with either pTL1-SUMO-1 or pTL1-SHPR-15, pTL1-SHPR-115, pTL1-SHPR-190, pTL1-SHPR-7, pTL1-SHPR-31, and pTL1-SHPR-142. The CAT activities are represented as in *A*.

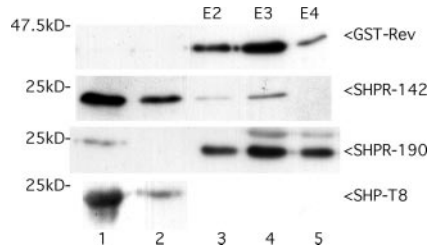

FIG. 4. **Direct protein-protein interaction between SHPR and Rev.** GST-Rev was produced in bacteria and loaded on a glutathione-agarose column. SHPR-142, SHPR-190, and SHPT-8 were loaded onto the column. After a wash, proteins were eluted with glutathione. An aliquot of the flow-through (*lane 1*), wash (*lane 2*), and elution fractions (*lanes 2–4*) was analyzed by immunoblot for GST-Rev (*upper panel*) and for SHP using antibodies to GST or to FLAG, respectively.

the Rev response element motif in an intron (41). Rev expression stimulates CAT expression by allowing export of the unspliced messenger in the cytoplasm. Using this assay, the various SHPR exhibited different activities. SHPR-15 and SHPR-115 were inefficient at the lowest amount of tranfected plasmid as compared with wild-type SUMO-1 but caused a moderate inhibition at a higher concentration (Fig. 3*C*). For SHPR-7 and SHPR-31, a limited inhibition was observed at both amounts of transfected plasmid (Fig. 3*C*). For SHPR-142 and SHPR-190, a strong reduction in the level of CAT expression was observed and this inhibition was augmented when the amount of protein was increased (Fig. 3*C*). These observations clearly showed that two SHPRs, SHPR-142 and SHPR-190, were potent inhibitors of the Rev function. It was verified whether this inhibition correlates with a direct protein-protein interaction as expected. To this end, both Rev and SHPR were produced in bacteria. A protocol was developed to purify the SHPs (see Supplementary Fig. 1). Rev was expressed as a GST fusion and coupled to glutathione-agarose beads. SHPR-142 and SHPR-190 were loaded onto this column as well as SHPT-8 as control. When GST-Rev was unloaded by glutathione, coelution of this protein with both SHPR-142 and SHPR-190 was observed, whereas no SHPT-8 was present in the fractions containing GST-Rev (Fig. 4, *lanes 3–5*). This finding showed that the interaction of SHPR-142 and SHPR-190 with Rev is direct and does not involve any bridge factor.

Penetration of the SHPs within the Cell from the Extracellular Milieu—Given these positive results, it was evaluated whether SHPs can be active from the extracellular environment. It was first examined whether they cause cytotoxicity. Up to a concentration of 1 μM, these molecules were not observed to modify the percentage of dying cells in a population of *in vitro* cultured PBMC activated by PHA-P and rHuIL-2 (see Supplementary Fig. 2). To evaluate the ability of SHP to penetrate into cells, PBMCs were cultured in a medium supplemented with 2 μM SHPR-190. At various times, an aliquot of the supernatant was taken and the cells were collected. After several washes, cells were lysed in radioimmune precipitation assay buffer and the supernatant along with this extract was analyzed by immunoblot using an antibody directed against the FLAG epitope present at the amino terminus of the bacterially produced SHPs. In both resting (Fig. 5*A*) and activated (Fig. 5*B*) lymphocytes, it was observed that SHPR-190 is stable

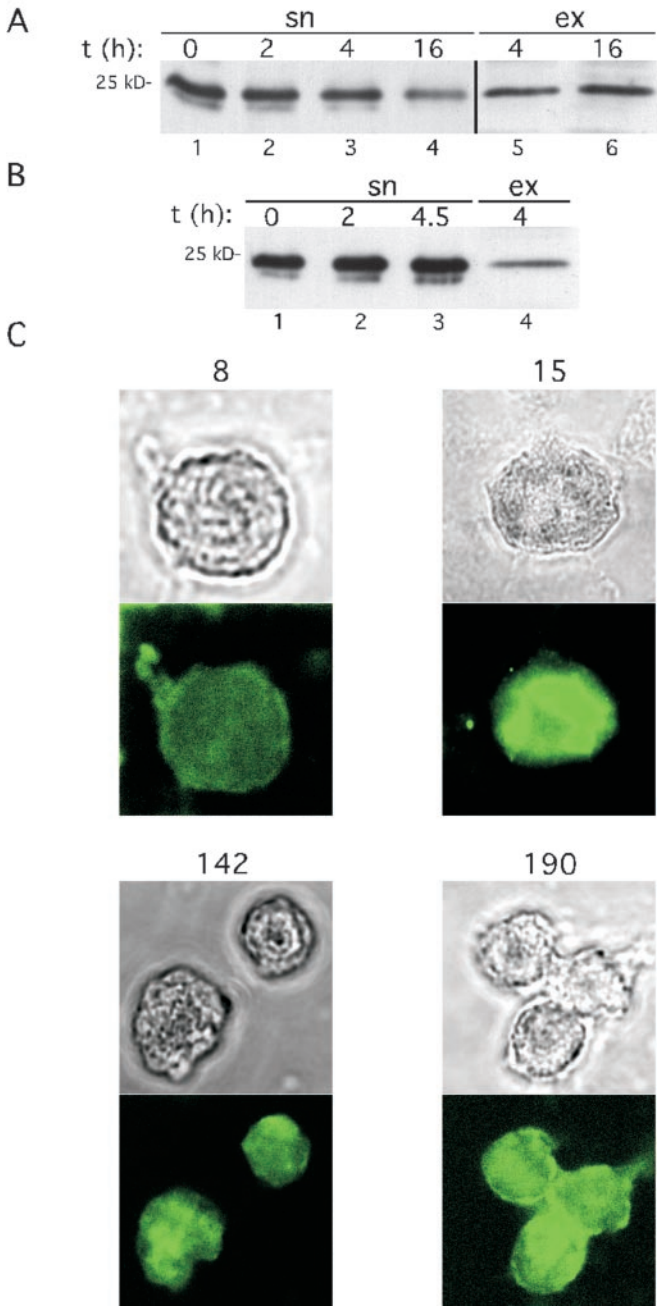

FIG. 5. **Intracellular entry of SHP.** Mononuclear cells were prepared from peripheral blood and either directly (*A*) or after activation by PHA-P and rHuIL-2 (*B*) were incubated with 2 μM SHPR-190. At the indicated times after the addition of the protein, an aliquot of the supernatant (*sn*) was analyzed by immunoblot using the antibody to FLAG (*A*, *lanes 1–4*; *B*, *lanes 1–3*). Cells were also collected and lysed in radioimmune precipitation assay buffer. The resulting extract (*ex*) was also analyzed by immunoblot (*A*, *lanes 5* and 6; *B*, *lane 4*). In *panel A*, exposure times is different between *lanes 5* and *6* (30 s) and *lanes 1–4* (5 s). *C*, Jurkat cells were incubated with 2 μM SHPT-8, SHPR-15, SHPR-142, or SHPR-190 for 1 h, and 24 h later, cells were collected and analyzed by immunofluorescence using the antibody to FLAG. Light transmission and fluorescence images of representative cells are shown.

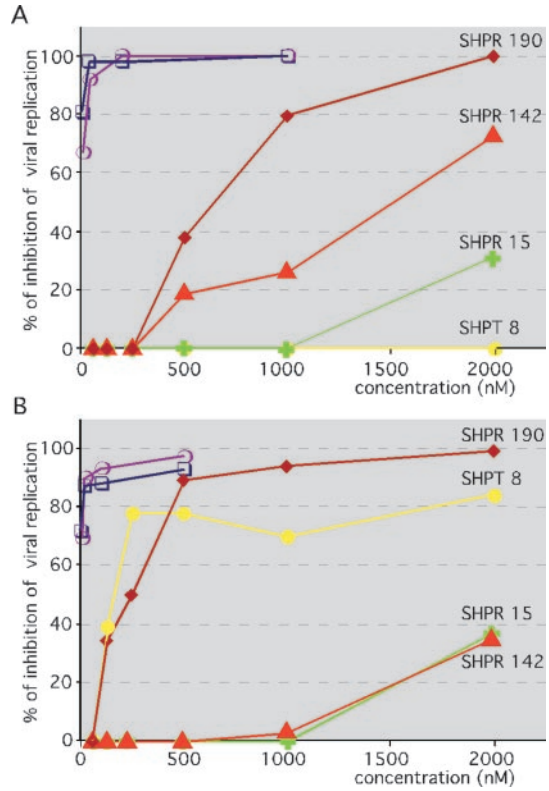

FIG. 6. **Inhibition of HIV-1 replication by SHP in PHA-P-activated PBMC and monocyte-derived macrophages.** *A*, mononuclear cells were prepared from peripheral blood and activated. Cells were treated by various amounts of AZT (*blue line*), Idinavir (*purple*), SHPR-15 (*green*), SHPR-142 (*orange*), SHPR-190 (*red*), or SHPT-8 (*yellow*) and infected with HIV-1-LAI. Viral replication was measured by dosage of the RT activity in supernatants, and percentages of inhibition are represented as a function of concentration. *B*, macrophages were prepared from monocytes, treated with drugs and SHPs, and infected by HIV-1/Ba-L. Viral replication was measured, and percentages of inhibition at day 7 postinfection are represented as shown in *A*.

in the culture medium and that a fraction of the protein is present within the cell (Fig. 5, *A*, *lanes 5* and *6*, *B*, *lane 4*). Considering the lymphocyte as a 12 μ-diameter sphere, quantification experiments performed with fluorescently labeled antibodies showed that a 2 μM external concentration led to a 15 μM intracellular concentration (data not shown). This indicates that the SHPs are likely to be concentrated within cells. To confirm that SHPs enter within cells, immunofluorescence analyses were also done. Jurkat cells were incubated with 1 μM SHP for 4 h, and after two washes, cells were cultured for 24 h. Immunofluorescence analysis using the antibody to FLAG showed a clear fluorescence present diffusely within the entire cell for all four SHPs tested (Fig. 5*C*). This was not observed in control cells (data not shown). Taken together, these observations show that lymphocytes can be cultured with purified SHPs and that these molecules effectively enter cells.

Inhibition of HIV-1 Replication by SHPs—In an ultimate step, it was examined whether the SHPs can inhibit HIV-1 replication. This was done in both PHA-P-activated PBMCs and MDM. In our model of PHA-P-activated PBMC, HIV-1-LAI (42) replication was optimal at day 7. As a consequence, the effects of different SHPs were tested at this date. AZT and indinavir were used as controls. As expected, HIV-1-LAI replication was effectively inhibited by AZT and indinavir (Fig. 6*A* and Supplementary Table 2*a*). SHPT-8 did not display any anti-HIV activity, and SHPR-15 showed a poor activity, only at the highest dose (Fig. 6*A*). In contrast, SHPR-142 and SHPR-190 decreased viral replication in PHA-P-activated PBMCs (Fig. 6*A*). HIV-1 replication was inhibited by SHPR-142 at 2 μM (73 $\pm$ 6%; see Supplementary Table 2*a*) and by SHPR-190 at 1 and 2 μM (80 $\pm$ 7 and 100% respectively; see Supplementary Table 2*a*). 90% effective doses were, respectively, equal to 4,600 and 927 nM (see Supplementary Table 2*a*), confirming that SHPR-190 is more potent than SHPR-142.

The reference HIV-1/Ba-L strain (43) replicated efficiently in MDMs. Reverse transcriptase activity was detected in culture

supernatants from 7 days postinfection and was maximal between days 14 and 21 postinfection. As expected, HIV-1/Ba-L replication was dose-dependently inhibited by AZT and indinavir (Fig. 6*B* and Supplementary Table 3*a*). In these cells as in PBMCs, SHPR-15 showed a poor activity, only at the highest dose at day 7 (Fig. 6*B* and Supplementary Table 3*a*). Contrary to the results obtained in activated PBMC, SHPR-142 also inhibited poorly HIV replication (Fig. 6*B*). From these results, the specificity of these effects of SHPR-15 and SHPR-142 was not established. In contrast, viral replication was inhibited by both SHPT-8 and SHPR-190 at 2 μM (84 $\pm$ 1 and 99% of inhibition at day 7, respectively, and 69 $\pm$ 6 and 92 $\pm$ 2% at day 14, see Supplementary Table 3a). Unlike SHPT-8, the effects of SHPR-190 on HIV replication were dose-dependent (Fig. 6*B*). Moreover, as illustrated by ED_{90} values (see Supplementary Table 3*b*; ED_{90} = 5,500 nM for SHPT-8 *versus* ED_{90} = 1,450 nM for SHPR-190 at day 14), the SHPR-190 compound exhibited a higher anti-HIV activity. However, these anti-HIV effects of SHPR-190 decreased after 14 days, probably as a consequence of the degradation of the protein (ED_{90} >2,000 nM for day 21 of culture *versus* ED_{90} = 1,450 nM for day 14 of culture, see Supplementary Table 3*b*).

These results show that SHPR-190 displays clear anti-HIV effects in both major cell targets of HIV, *i.e.* T CD4+ lymphocytes and macrophages. SHPR-142 was ~2-fold less active than SHPR-190 in lymphocytes and poorly efficient in macrophages. These experiments also revealed that SHPT-8 has some antiviral activity in macrophages despite its poor ability to impair Tat activation as evaluated by functional assay in HeLa cells.

DISCUSSION

Because of viral strains resistant to available therapeutic agents and to the spreading epidemic occurring in developing countries, the search for new drugs that are able to treat or prevent HIV-1 infection remains a major scientific challenge. The Tat and Rev regulatory proteins are clearly important targets in this regard. Indeed, the functions they exert are necessary to viral replication and their expression happens very early in the expression of viral RNA from the integrated provirus. Moreover, it is known that these proteins intervene at the onset of the pathology resulting from the infection. Various strategies have been proposed previously to inhibit these proteins including RNA decoys (47–51) and more recently RNA interference (52–54). A major problem that precludes so far medical use of these RNA-based approaches is intracellular delivery. Because both Tat and Rev act by contacting cellular factors through protein domains of limited size, we undertook the development of a strategy aiming at identifying peptide sequences exhibiting the dual property of binding to these regulatory proteins and also of entering lymphocytes. Because this method has proven its usefulness in screening peptides binding to target proteins (37), the first step of our approach involved a two-hybrid screen of a library of vectors expressing peptides fused to a protein transduction domain. Inclusion of the PTD at this initial step avoids modification of the binding properties of the peptide by its adjunction at later steps. In the case of molecules destined to treat HIV-1 infection, the choice of the Tat PTD of which properties are well documented (55–58) was natural. Conceptually, the same method can be used with another PTD or with another position of this domain (*i.e.* amino-terminally fused to the peptide). The length of random sequence was selected of limited length to avoid generation of long artificial protein epitopes, but clearly it is also a parameter that can be modified depending on the protein domain targeted. Our constructs in which the different domains are separated by flexible arms allowed the identification of molecules binding to both Tat and Rev. The binding properties selected in yeast were retained in human cells. To further use these peptides, they were fused to a stabilizing protein. Several possibilities certainly exist for this domain, but SUMO-1 worked efficiently in our hands. The resulting SHPT or SHPR appeared to enter cells efficiently and to lack cytotoxic effects at concentrations <2 μM. Because leptomycin B is highly toxic, it is believed that CRM1-mediated nuclear export is vital (59, 60). Hence, it is likely that the action of the SHPRs is specific of Rev and does not affect export of endogenous proteins. Two of these SHPRs were able to efficiently inhibit the function of Rev. Because one of the most efficient of them, SHPR-142, was selected against the NES of Rev and also given that the other, SHPR-190, also binds the NES as evaluated by the two-hybrid assay (data not shown), it is likely that these two molecules interfere with the export properties of Rev, probably by competing with cellular proteins as CRM1 and RanGTP.

In agreement with their ability to efficiently inhibit Rev, SHPR-142 and SHPR-190 were effective in inhibiting HIV-1 replication in lymphocytes but at concentrations higher than those of standard drugs as AZT and indinavir. Interestingly, SHPR-190 was also clearly effective in human macrophages. These observations indicate that these SHPR have potential therapeutic applications. In this goal, it should be possible to further optimize these agents by acting on all three parts. Indeed, the PTD appeared to be partially cleaved during the production process, this being likely to alter the cell penetration ability of the molecule. Adequate mutations in this domain or inhibition of involved proteases might bring a solution. The heptapeptide sequence might also be improved by introducing mutations or chemical modifications augmenting the affinity for Rev. Finally, SUMO-1 might be replaced by another more effective stabilizing protein or even by a chemical modification of the peptide ensuring its stability, for instance, cyclization. Such modifications might reduce doses required for full inhibition of HIV-1 replication. It is interesting to note that a functional part of Rev can be targeted by binding peptides, which interfere with its activity. Determination of the structure of the SHPR in association with Rev might help to understand which are the key motifs of the NES domain, thereby allowing the design of small chemical molecules impairing Rev function. The ability of the SHPRs to interfere with the functioning of a particular protein domain of Rev also indicates that the approach developed in this work is likely to represent an interesting tool for analyzing the functional domains of a given protein within the cell.

In conclusion, the data presented in this report show the feasibility of a rational approach for the development of new therapeutic agents targeted against HIV-1 proteins. In this principle, this method is also applicable to other viral or nonviral diseases requiring intracellular inhibition of specific proteins.

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