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Binding of Intracellular Anti-Rev Single Chain Variable Fragments to Different Epitopes of Human Immunodeficiency Virus Type 1 Rev: Variations in Viral Inhibition

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Intracellular immunization to target the human immunodeficiency virus type 1 (HIV-1) regulatory protein Rev has been explored as a genetic therapy for AIDS. Efficient intracellular expression of rearranged immunoglobulin heavy and light chain variable regions of anti-Rev monoclonal antibodies, with various vectors, and subsequent inhibition of HIV-1 replication have been previously reported by our laboratories. To further understand the molecular mechanism(s) and effects that intracellular anti-Rev single chain variable fragments (SFvs) have against HIV-1, via blocking of Rev function, two anti-Rev SFvs which specifically bind to differing epitopes of the Rev protein have been cloned. One SFv binds to the Rev activation domain, and the second SFv binds to the distal C terminus of Rev in the nonactivation region. Further studies now demonstrate that both anti-Rev SFvs lead to variable resistance to HIV-1 infection. Although binding affinity assays demonstrated that the SFv which specifically recognizes the Rev activation domain (D8) had an extracellular binding affinity significantly lower than that of the SFv specific to the nonactivation region (D10), the SFv D8 demonstrated more potent activity in inhibiting virus production in human T-cell lines and peripheral blood mononuclear cells than did SFv D10. Thus, extracellular binding affinities of an SFv for a target viral protein cannot be used to directly predict its activity as an intracellular immunization moiety. These data demonstrate potential approaches for intracellular immunization against HIV-1 infection, by efficiently blocking specific motifs of Rev to alter the function of this retroviral regulatory protein. These studies extend the understanding of the effects, on a molecular level, of SFvs binding to critical epitopes of Rev and further suggest that rational design of SFvs, with interactions involving specific viral moieties which mediate HIV-1 expression, may hold promise for the clinical application of genetic therapies to combat AIDS.

Human immunodeficiency virus type 1 (HIV-1) produces a viral transactivator, termed Rev, which is required for the expression of the viral structural proteins and, hence, for HIV-1 replication (22). The primary role of Rev is to allow the nuclear export of sequestered incompletely spliced viral mRNA species, either by antagonizing their interaction with splicing factors (8) or by directly facilitating their interaction with a component of the cellular RNA transport pathway (7, 15, 24). As a posttranscriptional regulator of HIV-1 gene expression, Rev is a 116-amino-acid protein composed of at least two separate functional domains (21). The N-terminal domain is an approximately 40-amino-acid sequence, characterized by an arginine-rich central core which has been shown to function as a Rev nuclear-nucleolar localization signal (17, 21). The C-terminal domain, at residues 78 to 90, is termed an activation domain, and it interacts directly with a component of the nuclear RNA transport or splicing machinery (21, 23). Several recent studies have suggested that the activation domain interacts with a nucleopore-like protein (7, 16). As well, several studies have demonstrated that mutations in the activation or effector region can inhibit Rev function and suppress HIV-1 replication in human T-cell lines (21, 28, 34). Hence, the strategy for inhibiting Rev function, through blocking of Rev domains within infected cells, has been suggested for suppression of HIV-1 production (21, 28, 29).

Genetic therapy may be used for interdiction in a variety of infectious diseases through the expression of specific recombinant constructs within cells. This has been termed "intracellular immunization" (5). The techniques used to inhibit HIV-1 replication via intracellular immunization include RNA- and protein-based modalities (4, 19, 20, 29, 33, 35). One approach to selectively alter viral proteins is through the development of intracellularly expressed single chain variable fragments (SFvs). SFvs represent the variable regions of monoclonal antibodies, which have been conclusively shown to contain the antigen-binding site of the parent antibody (6). Our laboratories have reported initial studies of an anti-Rev SFv moiety which potently inhibits HIV-1 replication in human epithelial lines, T-lymphoid cells, and peripheral blood mononuclear cells (PBMC) (10, 13, 14). In the present report, we demonstrate the inhibitory effects and variations in inactivation of HIV-1 Rev by two separate SFvs which specifically bind to divergent epitopes located in different regions of Rev. Various effects on the inhibition of HIV-1 replication are characterized by utilizing these anti-Rev SFvs.

Two murine-based anti-Rev SFvs (D8 and D10) were uti-

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|--->Light Chain                                                    |<----
M N I M M T Q S P S S L A V S A G E K V T M S C K S
ATGAACATTATGATGACACAGTCGCCATCATCTCTGGCTGTGTCTGCAGGAGAAAAGGTCACTATGAGCTGTAAGTCC

-----------------CDR 1--------------------->|
S Q S V L Y S S N Q K N Y L A W Y Q Q K P G Q S P K
AGCCAAAGTGTTTTATACAGTTCAAATCAGAAGAACTACTTGGCCTGGTACCAGCAGAAACCAGGGCAGTCTCCTAAA

          |<-------CDR 2------>|
L L I Y W T S T R E S G V P D R P T G S G S G T D F
CTGCTGATCTACTGGACATCCACTAGGGAATCTGGTGTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGATTTT

                                                |<-------CDR 3------>|
T L T I S S V Q A E D L A V Y Y C H Q Y L S L F T L
ACTCTTACCATCAGCAGTGTACAAGCTGAAGACCTGGCAGTTTATTACTGTCATCAATACCTCTCCTTATTCACGCTC

G T G T K L E I K R G D A A P T V S I F P P S S K L
GGGACGGGGACAAAGTTGGAAATAAAACGGGGTGATGCTGCTCCTACTGTATCCATCTTCCCACCATCCAGTAAGCTT

      |<----------------------Linker------------------->|--->Heavy Chain
G P G G G G S G G G G S G G G G S E L G R S E V Q L
GGGCCCGGTGGGGGCGGTTCGGGTGGCGGGGGCTCGGGCGGGGGTGGCTCAGACCTCGGCAGATCTGAGGTGCAGCTG

V E S G G D L V K P G G S L K L S C A A S G F T F S
GTGGAGTCTGGGGGAGACTTAGTGAAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAGCCTCTGGATTCACTTTCAGT

|<---CDR 1--->|                                  |<-----------CDR 2----
D Y G M S W V R Q T P D K R L E W V A T I S S G G S
GACTATGGCATGTCTTGGGTTCGCCAGACTCCAGACAAGAGGCTGGAGTGGGTCGCAACCATTAGTAGTGGTGGTAGT

---------------------------->|
Y I Y Y P D S V K G R F T I S R D N A K N T L Y L Q
TACAATTACTATCCAGACAGTGTGAAGGGGCGATTCACCATCTCCAGAGACAATGCCAAGAACACCCTGTACCTGCAA

                                                |<----------------------------
M S S L K S E D T A M Y Y C A R H G G K D A L G L L
ATGAGCAGTCTGAAGTCTGAGGACACAGCCATGTATTACTGTGCAAGACATGGGGGTAAGGATGCTTTGGGACTACTG

--------------CDR 3---------------------------------------->|
G S R N L S H R L L S Q N N T T T R L S P G P W K L
GGGTCAAGGAACCTCAGTCACCGTCTCCTCAGCCAAAACAACACCACCCGTCTATCCCCTGGCCCCTGGAAGCTT

G N P Y *
GGGAATCCATATTAG
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FIG. 1. Nucleotide and amino acid sequences of the variable light (V_l) and variable heavy (V_h) chains of the anti-Rev SFv D10. CDR, complementarity defining region.

lized in these studies. The anti-Rev SFv D8 was cloned and constructed from a murine hybridoma expressing a parent monoclonal antibody which bound to recombinant HIV-1 Rev. This construct has been previously described in detail (13). The newly constructed anti-Rev SFv D10 was constructed and cloned from a separate murine hybridoma cell line (provided by Intracel, Inc., Cambridge, Mass.). In brief, 5×10^6 murine hybridoma cells were first transduced with a murine leukemia virus (MLV)-based shuttle vector encoding a ribozyme which cleaves mutant kappa-chain RNA, expressed from Sp2/0-derived hybridoma cells, as previously described (12, 32). As such, the mRNA isolated from these murine hybridoma cells, after treatment with the ribozyme-encoding MLV vector, is enriched for the correct kappa light-chain mRNA. Total cellular RNA was prepared by an acid-guanidinium method (2). Reverse transcriptase-initiated PCR (RT-PCR) with degenerate primers was utilized to amplify cDNA transcripts of the murine immunoglobulin (Ig) gene variable regions by using the Ig Prime System (Novagen, Inc.). The murine Ig variable heavy-chain (V_h) and variable light-chain (V_l) fragments were then cloned into the pT7 blue (R) vector. After DNA sequencing to confirm the proper V_h and V_l sequences, these genes were reamplified by PCR using oligonucleotides D10H1 (5′-AGATC TGAGTGCAGCTGGAGTC-3′), D10H2 (5′-ATGGATCCT AATATGGATTCCCAAGC-3′), D10κ1 (5′-CTCGAGCATA TGAACATTATGATGACACAGTC-3′), and D10κ2 (5′-CT AGGGCCCAAGCTTACTGGATGGTG-3′) and constructed into an SFv with the insertion of $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ as a flexible linker (13). The final full-length anti-Rev SFv D10 cDNA sequence was confirmed by sequencing in the sense and antisense directions (Fig. 1).

Both the anti-Rev SFv D8 and D10 constructs were cloned into MLV-based amphotropic shuttle vectors, as previously described (11, 14). These constructs were entitled pSLXCMV-SFv-D8 and pSLXCMV-SFv-D10. As well, the chloramphenicol acetyltransferase (CAT) gene was cloned into the pSLX CMV vector and the resulting construct was entitled pSLX CMV-CAT. In these vectors, the genes of interest were expressed from the internal cytomegalovirus (CMV) promoter and a neomycin resistance gene (*neo*) was expressed from the 5′ MLV long terminal repeat. The anti-Rev SFvs (869 bp) were each cloned into the pSLXCMV vector by using blunt ligation, downstream of the CMV promoter, at the *Hpa*I and *Bam*HI sites. pSLXCMV-CAT was constructed by inserting a 726-bp fragment containing the CAT gene via *Hpa*I-*Bgl*II sites (Fig. 2).

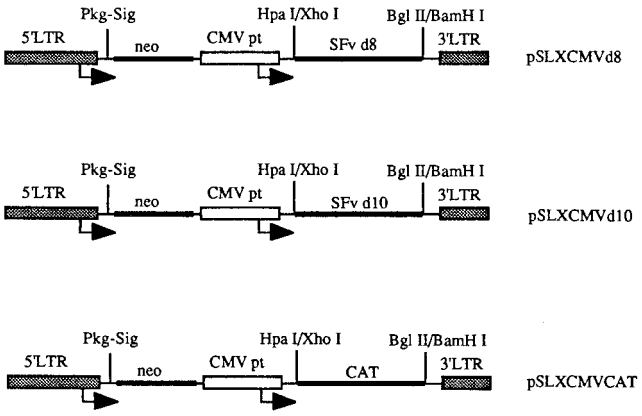

FIG. 2. Schematic diagram of anti-Rev SFv- and CAT-expressing retroviral constructs. The SFv D8-, SFv D10-, and CAT-encoding genes were placed downstream of the CMV promoter (pt) in the amphotropic murine retroviral shuttle vector pSLXCMV (31). The neomycin resistance gene (neo) was expressed from the 5′ long terminal repeat of the MLV vector. Pkg-Sig, packaging signal.

For bacterial expression of the anti-Rev SFv D10, an 873-bp *Nde*I-*Bam*HI cDNA fragment of D10 SFv was inserted downstream of the T7 RNA polymerase promoter, with a polyhistidine-encoding tag region, in pET19b vector (Novagen) to create pET19B-D10. Plasmid pET19B-D8 has been previously described (13). To express recombinant anti-Rev SFvs in *Escherichia coli*, a methodology similar to that previously described

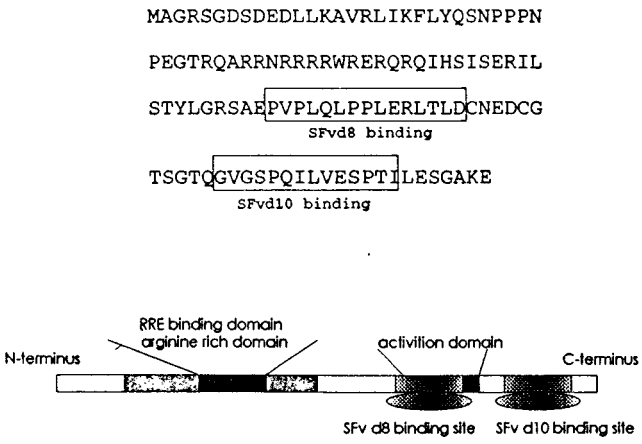

FIG. 3. HIV-1 Rev functional domains and binding sites for two anti-Rev SFvs (D8 and D10). The upper panel illustrates the binding sites for SFv D8 and D10, based on amino acid residues of the HIV-1 Rev protein. These binding sites were demonstrated by epitope mapping using overlapping HIV-1 Rev peptides via an ELISA technique. The lower panel shows the locations of various functional domains and the D8 and D10 SFvs' binding sites on the HIV-1 Rev molecule.

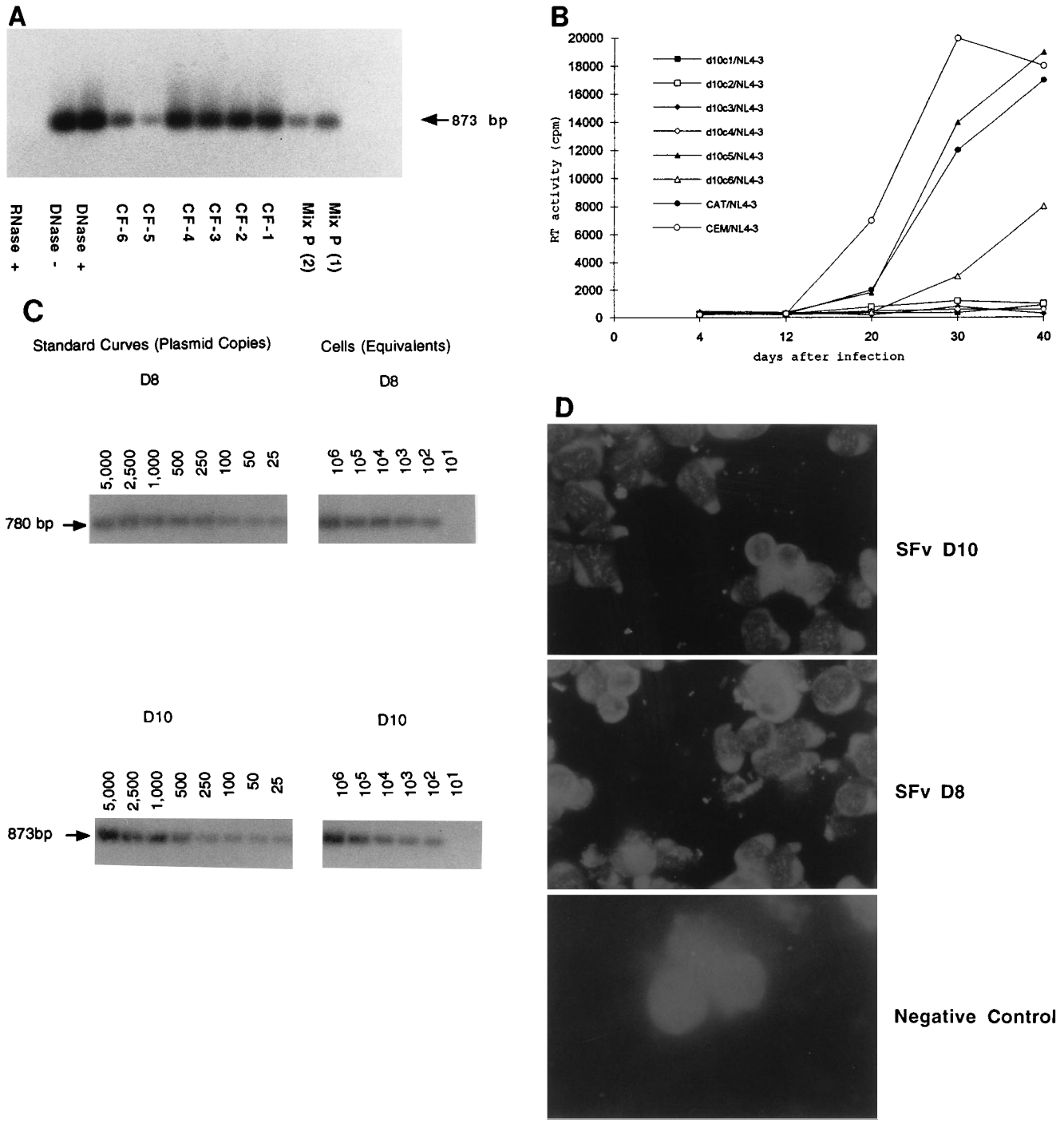

FIG. 4. Anti-Rev SFv D10 expression in T-lymphocytic cells. (A) RT-PCR of D10 SFv mRNA in a transduced T-lymphoid cell line (CEM). Lanes 1 and 2 from the right, mixed populations of transduced CEM cells (MIX-P); lanes 3 to 8, single cell clones from D10 SFv-transduced CEM cells (CF-1 to -6); lane 9, DNase$^+$ control; lane 10, DNase$^-$ control; lane 11, RNase$^+$ control. (B) HIV-1 replication in CEM cell clones. After infection of CEM single cell clones, transduced with SFv D10 (CF 1 to CF 6) or CAT or nontransduced, with cell-free HIV-1_{NL4-3} (multiplicity of infection, 0.15), the supernatants were periodically sampled for virion-associated RT activity. (C) Comparison of D8 and D10 SFv mRNA expression in T-cell lines by RT-PCR. A representative mixed population of CEM cells transduced with the D10 and D8 retroviral vectors was analyzed by RT-PCR, with comparison with plasmid standard control curves for each construct. Specific bands were 873 bp for the D10 amplicon and 780 bp for D8. (D) Indirect immunofluorescent staining for anti-Rev SFv protein expression in T-lymphoid cells (D8 and D10). Upper panel, SFv D10-transduced CEM cells; middle panel, SFv D8-transduced CEM cells; lower panel, nontransduced CEM cells. Magnification, ×296. (E) Western blotting analysis for anti-Rev SFv protein levels in T-lymphoid cell lines (D8 and D10). Mixed populations of CEM cells, transduced with SFv D10 (30.9 kDa) or SFv D8 (29.7 kDa), were analyzed (2, 14a). The upper panel illustrates Western blotting for SFvs, while the lower panel is a loading control for β-actin (rabbit anti-actin IgG; Sigma). The primary antibody used in these Western blotting studies for intracellular SFvs was a goat anti-mouse kappa chain IgG (1:200), and the secondary antibody was a peroxidase-conjugated rabbit anti-goat IgG (1:4,000; Sigma). Separation of proteins was performed via sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis, and after transfer and blotting, signals were detected with a chemiluminescence detection system (NEN-Dupont).

FIG. 4—*Continued.*

for SFv D8 was utilized (13). In brief, a bacterial strain, BL21 $(DE)_3$, was transformed with pET19B-D10 and induced with IPTG (isopropyl-β-D-thiogalactopyranoside). The soluble and insoluble protein fractions were harvested, and the SFv molecules were found to be primarily localized in the insoluble fraction of the protein isolates. Purification of the SFv D10 moiety was carried out on a histidine-binding metal chelation resin column. The extracellular binding affinities of the anti-Rev SFvs were determined by an enzyme-linked immunosorbant assay (ELISA) using recombinant biotinylated Rev protein (Intracel, Inc.). Different dilutions of renatured SFvs were resuspended in phosphate-buffered saline (PBS), and the wells were coated overnight at 4°C. The plates were then blocked with 10% bovine serum albumin for 2 h at 37°C. The plates were washed three times with 1× PBS containing 0.5% Tween 20. To each plate's wells dilutions of biotin-labelled Rev were added, and the plates were incubated at 37°C for 1 h. After vigorous washing with PBS, avidin-conjugated horseradish peroxidase was added to each well. Color was developed at room temperature for 30 min and quantitated with a spectrophotometer (optical density at 590 nm) in triplicate. In addition, for peptide mapping, overlapping purified 15-amino-acid peptides of HIV-1 Rev (Intracel, Inc.) were used in the ELISA format. The anti-Rev SFv D10 was found to have an in vitro binding affinity to recombinant Rev of 1.2×10^{-8} M, while the affinity of extracellular binding to Rev by SFv D8 was 1×10^{-7} M. As such, SFv D10 was shown to have a binding affinity to recombinant HIV-1 Rev approximately 10-fold higher than that of D8. In addition, peptide mapping confirmed that anti-Rev SFv D8 binds, in vitro and extracellularly, to a peptide containing amino acids 70 to 84 of Rev (PVPLQLPPLERLTLD), overlapping the 5′ portion of the Rev activation domain. The SFv D10 bound specifically to a peptide containing amino acids 96 to 110 of Rev (GVGSPQILVESPTI), near the carboxyl terminus of Rev distal to the activation domain (Fig. 3).

To transduce human T-lymphocytic cell lines and PBMC with the anti-Rev SFv, helper-free, recombinant MLV stocks were produced by transient transfection of the retroviral vector plasmids into PA317 packaging cells (27). Briefly, 5-μg portions of purified plasmids were transfected into 10^6 PA317 cells per 100-mm-diameter dish by using a standard lipofectamine reagent (Gibco-BRL). At 48 h posttransfection, the supernatants were harvested and used to transduce CEM and Sup-T1 T-lymphocytic cells, which were then subjected to selection in G418 (1 mg/ml)-containing RPMI 1640 medium supplemented with 10% fetal calf serum for 2 weeks. Mixed cellular populations and single cell clones were isolated and maintained in G418-containing media for 2 further weeks. For transduction of PBMC, 2×10^6 phytohemagglutinin (5 μg/ml)- and interleukin-2 (50 U/ml)-stimulated primary cells were infected with 10^7 CFU of recombinant retroviral vectors. This procedure was repeated daily for 3 days with fresh packaging cell line supernatant prior to culturing in fresh media.

To analyze anti-Rev SFv intracellular expression, total cellular RNA was extracted, as previously described (10, 27), from SFv-transduced cells. Both cell clones and mixed cellular populations were analyzed by utilizing RT-PCR, as previously described (36). For anti-Rev SFv D10 RT-PCR, the primers used were SFv-1-D10 (5′-GAATTCCCGGGGAACATTATGATG ACACAGTC-3′) and SFv-2-D10 (5′-CGAACCCTTAGGTA TAATCCTAGGTA-3′) (3′ end of the heavy chain and 5′ end of the light chain). The ^{32}P-labelled probe was SFv-Dκ2 (5′-GTGGTAGGTCATTCGAACCCGGG-3′). Amplification was conducted for 30 cycles at 94°C for 1 min, 52°C for 1 min and 30 s, and 72°C for 1 min and 30 s. The PCR products were then analyzed by standard Southern blotting. As shown in Fig. 4A, most mixed populations and cell clones maintained stable SFv D10 mRNA expression. As illustrated in Fig. 4A, four of six representative CEM cellular clones transduced with the anti-Rev SFv D10 vector expressed high levels of D10-SFv mRNA (CF 1 to 4) while lower levels were observed in two clones (CF 5 and 6), as assayed by RT-PCR. Of note, the degrees of protection of individual CEM clones, transduced with SFv-D10, against HIV-1 replication directly correlated with the levels of SFv D10 mRNA expression (compare SFv D10 mRNA expression shown in Fig. 4A with levels of HIV-1 expression shown in Fig. 4B).

To compare SFv-D8 and SFv-D10 mRNA expression, mixed cellular populations of CEM and Sup-T1 cells transduced with these constructs were analyzed by RT-PCR (Fig. 4C and data not shown). The primers for D10 mRNA were as noted above, and the primers for D8 mRNA were SFv-1-D8 (5′-GAATTC CCGGGGTTGGTGCTGACGTTCTGG-3′) and EAR-6 (5′-TTGGATCCTCAGGATAGACGGGTGGGGGTG-3′). A common probe was then used for detection of both D8 and D10 (EAR-3 [5′-CCGGGCCCGGTGGGGGCGGTTCGG GTGGCGGGGGCTCG-3′], which was located in the middle of both SFvs' linker regions). As illustrated in Fig. 4C, the levels of D10 and D8 mRNA expression were not significantly different in these cells, although mRNAs expressed from the MLV long terminal repeat and the CMV promoter could not be distinguished. As we could not tell from the RT-PCR whether the amplicon was from the MLV long terminal repeat

or the CMV promoter, further protein-based studies were performed.

In addition, indirect immunofluorescence was performed, as previously described (3), to evaluate anti-Rev SFv expression in the cytoplasm of transduced cells. Briefly, transduced cells (5×10^4) were cultured in six chamber slides. The cells were fixed with 95% ethanol–5% acetic acid at 4°C overnight. Cells were incubated for 1 h at 37°C with a goat anti-murine (κ) Ig. These cells were washed and incubated with fluorescein isothiocyanate-conjugated polyclonal rabbit anti-goat IgG antibody (1:400 dilution; Sigma Chemical Co., St. Louis, Mo.) for 30 min at 37°C. The samples were then examined by epifluorescence microscopy. As demonstrated in Fig. 4D, both anti-Rev SFv D8 and D10 were shown to be efficiently expressed in the cytoplasm of most transduced cells (both mixed cell populations [not illustrated] and cellular clones). The D8- and D10-transduced cells showed diffuse yellow-green cytoplasmic staining, while nontransduced cells demonstrated no specific staining (Fig. 4D). In an experiment in which T-cell lines were selected in G418 (1 mg/ml)-containing media for 2 weeks, the various mixed populations of cells showed very similar numbers of D8- and D10-expressing cells by immunofluorescence. Of the CEM cells, 94% were positive for the D10 SFv and 95% were positive for the D8 SFv per low-power field; of the SupT-1 cells, 96% were positive for the D10 SFv and 95% were positive for the D8 SFv per low-power field (the data represent the averages of those for five low-power fields, evaluated by epifluorescence microscopy; standard errors were 1 to 2%). Thus, the newly constructed anti-Rev SFv D10 was shown to have significant intracellular cytoplasmic expression in transduced cells, as had been previously demonstrated for the prototype anti-Rev SFv D8. Of note, very sensitive assays are necessary to quantitate intracellular SFv expression localized to the cytoplasmic compartment, as levels of SFvs demonstrated in the cytoplasm are lower than those demonstrated for nucleus-targeted SFvs (6a).

Finally, to further confirm similar expression levels of D8 and D10 anti-Rev SFvs, Western blotting (immunoblotting) techniques were also utilized (2, 14a). As illustrated in Fig. 4E, protein levels of D8 and D10 in stably transduced cell lines were quite similar. As such, three independent lines of evidence suggest that no significant differences between D8 and D10 expression existed in transduced T-lymphocytic cells.

The human T-cell lines CEM and Sup-T1, which were transduced with various retroviral vectors, were next utilized in further HIV-1 infectivity studies. Both mixed populations and representative single cell clones were evaluated after removal of the lines from G418-containing media for 2 weeks prior to HIV-1 infection. Cell-free HIV-1 isolates, NL4-3 (containing all open reading frames intact) and R7-HXB2, were utilized in these infectivity studies (1). In addition, PBMC, stimulated with phytohemagglutinin and interleukin-2 and transduced with the retroviral vectors, were also used in HIV-1 infectivity analyses. After infection with these HIV-1 strains, the cells were analyzed at various time points over the next 2 weeks for HIV-1 expression by HIV-1 p24 antigen ELISAs (Dupont) and by measurement of virion-associated reverse transcriptase (RT) activity in the cellular supernatants (2). The virion-associated RT activity was measured by incubating 10 μl of culture supernatants with 50 μl of an RT reaction mixture containing a poly(A)-poly(dT) template primer (0.3 U/ml; Sigma Chemical Co.) in a solution composed of 50 mM Tris-HCl (pH 7.8), 75 mM KCl, 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.005% Nonidet P-40, 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], and 20 μCi of [^{32}P]TTP (3,000 Ci/mmol) per ml. Following a 2-h incubation at 37°C, 15 μl of the reaction mixture was spotted onto DE81 paper and washed to remove unincorporated nucleotides with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) five times for 5 min each, with two subsequent 3-min washes in 95% ethanol. The dried filters were analyzed by autoradiography and also in a scintillation counter.

As demonstrated in Fig. 5A to D, mixed populations of both CEM and Sup-T1 cells were efficiently protected from HIV-1 replication after transduction with the anti-Rev SFv D8, as previously reported (13). This was observed by analysis of HIV-1 p24 antigen expression in the cell supernatants. Of importance, although suppression of HIV-1 replication was noted in cells transduced with the anti-Rev SFv D10, significantly less inhibition of HIV-1 replication was consistently demonstrated in cells expressing the anti-Rev D10 than in cells expressing the anti-Rev D8. This finding was remarkably consistent in all experiments in which the anti-Rev SFv D8 and SFv D10 were compared in the same infectivity studies, both those utilizing cellular clones and those utilizing mixed populations (Fig. 5A to D and data not shown). HIV-1 virion-associated RT analyses also confirmed a higher level of HIV-1 replication in CEM cells transduced with SFv D10, compared with those transduced with SFv D8 (Fig. 5E and F). Nevertheless, the RT assays did demonstrate a suppression of HIV-1 replication induced by SFv D10 (Fig. 5E and F; also see below). Importantly, studies of transduced PBMC also consistently demonstrated that the anti-Rev SFv D8 moiety was significantly more potent in inhibiting HIV-1 replication than was SFv D10 (Fig. 6).

As initial transduction efficiencies of PBMC were routinely between 20 and 30%, as measured by immunofluorescence (14), these data represent only SFv expression at relatively high levels. As well, the PBMC are highly mixed cellular populations. Thus, we do not know the transduction and expression levels of the SFvs solely in CD4-positive lymphocytes. In addition, transduction and expression of SFvs in quiescent versus proliferating lymphocytes are not yet understood. These and other factors may explain the relatively impressive decreases in HIV-1 replication in anti-Rev SFv-transduced PBMC (14). Thus, the PBMC data support, but do not conclusively prove, the findings with the T-lymphocytic cell lines.

These studies suggest that an anti-Rev SFv moiety which has a higher extracellular binding affinity to HIV-1 Rev is, nevertheless, a weaker moiety for inhibiting HIV-1 infection, when expressed intracellularly, than the less strongly binding anti-Rev SFv (D8). Of note, the anti-Rev D8 binds to a critical motif in Rev, the activation domain, while the anti-Rev D10 binds to an area of Rev which has not been shown to have significance in Rev function. These data suggest that select epitope binding may be more important than extracellular binding affinity in intracellular immunization utilizing SFvs against HIV-1 regulatory proteins. There also remains a formal possibility that the intracellular binding affinities of these anti-Rev SFvs differ from their extracellular binding affinities (27a).

The major objectives of this study were to gain insights into the anti-HIV-1 activities of two SFvs which bind to different sites on the HIV-1 Rev molecule by utilizing stable expression in transduced T-cell lines and human PBMC. Of note, Ig molecules are responsible for two major biological functions: (i) a receptor function which entails the recognition of foreign antigens and (ii) an effector function which results in the elimination or inactivation of a foreign antigen. The variable domains of antibodies demonstrate the molecular heterogeneity necessary to allow differentiation between antigens. Thus, it is the variable regions which encode the receptor function of the

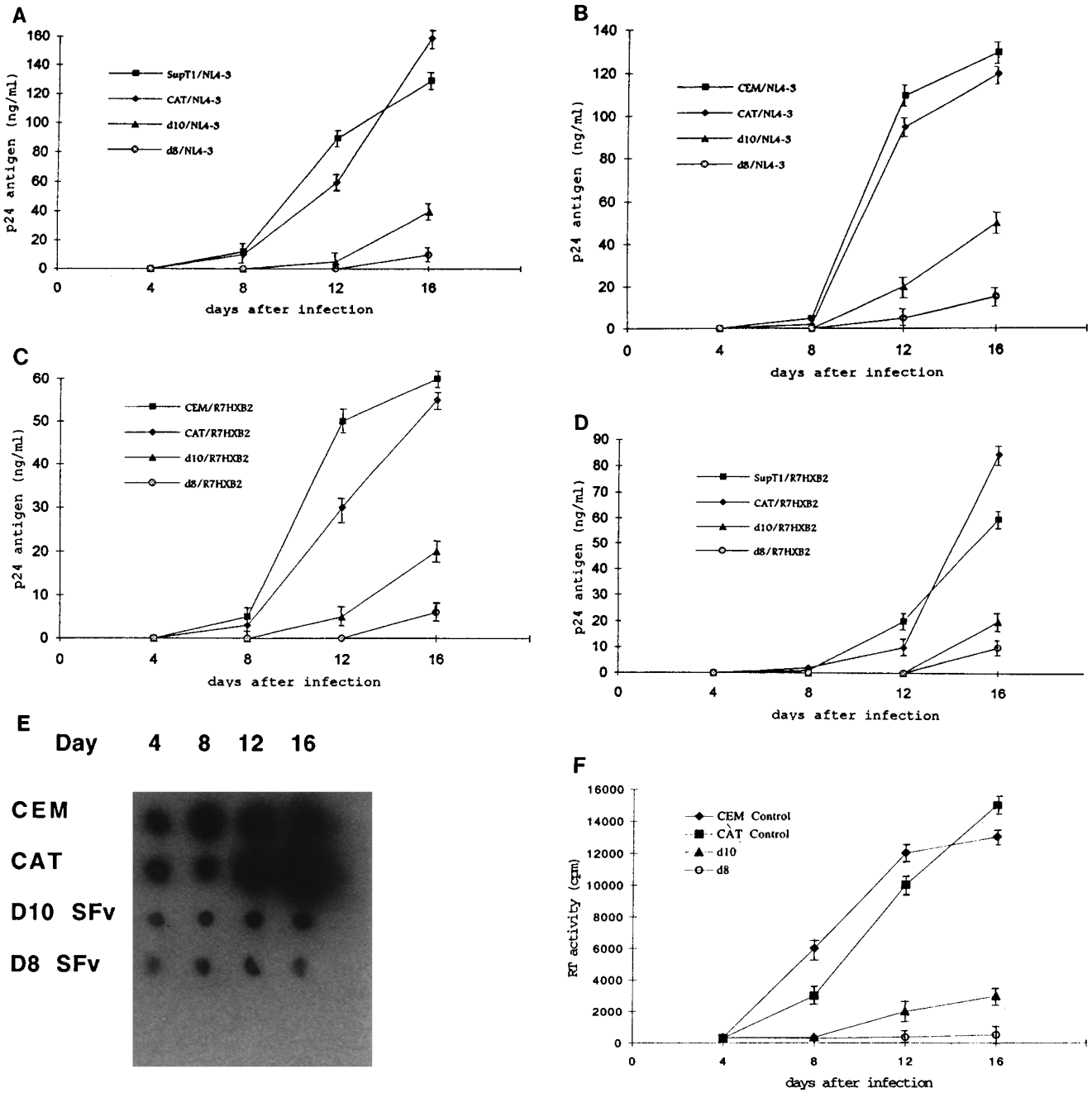

FIG. 5. Infections of CEM and Sup-T1 cell lines, transduced with anti-Rev SFvs (D8 and D10), with HIV-1. (A and B) Representative mixed populations of Sup-T1 (A) and CEM (B) T-cell lines transduced with murine retroviral vectors expressing SFv D8, SFv D10, or CAT or nontransduced and infected with cell-free HIV-1_{NL4-3} virions (multiplicity of infection [MOI], 0.2). HIV-1 p24 antigen expression in the supernatants was then monitored via ELISAs (Dupont). (C and D) Representative mixed populations of CEM and Sup-T1 cells challenged with HIV-$1_{R7-HXB2}$ (MOI, 0.2). Panels A to D are representative of at least two independent experiments performed in duplicate. Standard deviations are represented as bars above and below the datum points. (E and F) HIV-1 virion-associated RT assay of infections of anti-Rev SFv (D8 and D10)-transduced mixed CEM cellular populations. These panels represent a time course, analyzed by autoradiography (E) and scintillation counting (F), of exogenous HIV-1 RT activities in supernatants of CEM cells, either transduced with CAT-, SFv D8-, or SFv D10-expressing vectors or nontransduced.

antibody and allow the relatively specific binding of the antibody to a portion of a particular antigen, termed the epitope (6).

Various techniques have been utilized to inhibit HIV-1 replication via intracellular immunization (29, 30). One especially attractive protein-based modality to inhibit viruses in general, and HIV-1 in particular, is through the development of intracellularly expressed SFvs. SFvs represent the variable regions of antibodies, with the light- and heavy-chain variable portions bound together with a flexible linker, maintaining the ability to bind to specific antigenic epitopes. Our laboratories, and others, have demonstrated the potential utility of anti-Rev, anti-Env, and anti-Tat SFv molecules, expressed in various intracellular compartments, to efficiently inhibit HIV-1 replication (9, 10, 13, 14, 25, 26).

In the present experiments, the activities of two anti-Rev SFvs, D8 and D10, which recognize differing epitopes of the

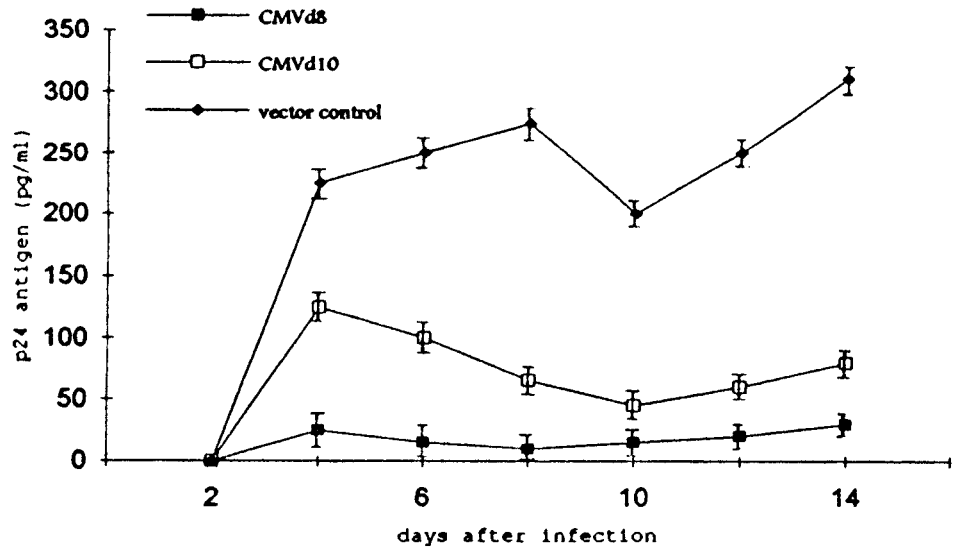

FIG. 6. Inhibition of HIV-1 expression in anti-Rev SFv-transduced human PBMC. Human PBMC, stimulated with phytohemagglutinin and interleukin-2, were transduced with pSLXCMV-SFv-D8, pSLXCMV-SFv-D10, or pSLXCMV (lacking an SFv insert), as previously described (11, 14). The transduction efficiencies were routinely between 20 and 30% as determined by immunofluorescence, and cell viability was approximately 90 to 95%, as described previously (14). Mixed populations of these transduced human PBMC were then infected with HIV-1_{NL4-3} (multiplicity of infection, 0.024). HIV-1 production was monitored by quantitation of HIV-1 p24 antigen expression in the culture supernatants via ELISA. The data are representative of at least two independent series of experiments, performed in duplicate, using PBMC from different HIV-1-seronegative blood donors.

Rev molecule, were evaluated for their abilities to inhibit HIV-1 replication. The ability to transduce various human T-lymphocytic cells with a murine retroviral shuttle vector, which was utilized to express both anti-Rev SFvs, rendered these cells relatively resistant, to variable degrees, to high-level HIV-1 expression. We previously demonstrated that anti-Rev D8, which binds to the Rev activation domain in the C terminus of the protein, potently inhibited HIV-1 replication in HeLa-T4 cells, human T-cell lines, and PBMC (10, 14). The anti-Rev SFv D10, which binds further downstream from the Rev activation domain in the nonactivation region of the C terminus, repeatedly showed weaker suppression of HIV-1 replication in all assay systems. However, by extracellular binding affinity assays, it was demonstrated that anti-Rev SFv D10 had a binding affinity for Rev approximately 10-fold higher than that of anti-Rev SFv D8. Lower-level intracellular expression of D10, compared with D8, did not account for this difference in activity against HIV-1 growth. If anything, the less-efficient D10 may be even slightly better expressed than D8 (Fig. 4C and E). As mentioned above, by utilizing epitope mapping, we determined that the SFv D8 bound to Rev peptides including amino acids 70 to 84 of HIV-1 Rev, a region which overlaps the activation domain, and SFv D10 bound to Rev peptides containing amino acids 96 to 109 of the far C terminus of Rev. Thus, these data demonstrate that extracellular binding affinity does not directly correlate with intracellular inhibition of HIV-1 expression by an anti-Rev SFv. It appears that the precise molecular epitope on Rev targeted by the SFv was more directly correlated with anti-HIV-1 effects. This relative variation in resistance to HIV-1 infection in transduced T-cell lines and PBMC, with SFvs which act on different Rev epitopes, further demonstrates that the Rev activation domain is a molecular moiety which may represent an excellent target for intracellular genetic therapy against HIV-1 (21, 34).

Recent studies have demonstrated that binding of an anti-Rev SFv to the activation domain of Rev both changes the subcellular compartmentalization of Rev and decreases the intracellular half-life of this viral regulatory protein (10, 18a). Of note, other laboratories have demonstrated potent inhibition of HIV-1 replication through the use of a transdominant-negative mutant Rev protein (M10), with mutations in the activation domain (34). In addition, viruses with mutations in Rev have been recently detected in the bloodstreams of certain HIV-1-infected-individuals (18). These Rev-deficient viruses appeared to have a mutation at codon 78 in the Rev activation domain, which could be restored by site-direct mutagenesis, yielding a wild-type phenotype (18).

Although anti-Rev SFv D10 was consistently weaker than anti-Rev SFv D8 in inhibiting HIV-1 replication, D10 did demonstrate significant activity in inhibiting HIV-1 production, especially when the less-sensitive RT analyses were utilized. This suggests, therefore, that although interactions of SFvs with particular Rev epitopes are important towards inhibiting viral replication, these interactions do not fully explain the inhibitory effects of SFvs on Rev function. Further mechanistic studies will be necessary to fully understand how SFv-Rev intracellular binding alters HIV-1 production (29).

In summary, this series of experiments demonstrates that rational molecular design of intracellular immunization technologies will be necessary to efficiently generate gene therapeutic approaches to inhibit HIV-1 in vivo. The extracellular binding affinities of SFvs may not be easily correlated with intracellular effects on retroviral replication. Thus, design of anti-HIV-1 SFvs should be based on binding to specific functional epitopes within selected target viral proteins.

Y.W. and L.D. contributed equally to this work.

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