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Resistance of primary isolates of human immunodeficiency virus type 1 to neutralization by soluble CD4 is not due to lower affinity with the viral envelope glycoprotein gp120

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ABSTRACT **Recombinant soluble CD4 (rsCD4) has potent antiviral activity against cell line-adapted isolates of the human immunodeficiency virus type 1 (HIV-1) but low activity toward HIV-1 primary isolates from patients. A simple hypothesis proposed to explain this discrepancy, which questions the therapeutic utility of soluble CD4-based approaches, is that the major envelope glycoprotein, gp120, of patient virus has lower affinity for CD4 than does gp120 from laboratory viruses. To test this hypothesis, we have produced pairs of low- and high-passage HIV-1 isolates which, depending on culture passage history, display dramatically different sensitivities to neutralization by rsCD4. Here, we present evidence that the HIV-1 major envelope glycoprotein cDNAs cloned from one such isolate pair show only minor differences in their deduced gp120 primary structures, and these occur outside regions previously shown to be involved in CD4 interactions. In addition, recombinant gp120 from a low-passage rsCD4-resistant patient virus binds rsCD4 with high affinity, equal to that previously measured for recombinant gp120 from high-passage cell line-adapted virus isolates. These data indicate that differences in CD4–gp120 affinity do not account for rsCD4 resistance in HIV-1 recently isolated from patients.**

The antiviral activities *in vitro* of recombinant soluble versions of CD4, the primary cell surface receptor for human immunodeficiency virus type 1 (HIV-1), have been extensively documented (reviewed in refs. 1 and 2). A number of soluble CD4 congeners have been shown to block laboratory isolates of HIV-1, generated after prolonged passage in tumor cell lines, in both replication assays and HIV-induced syncytium assays. The concentration of recombinant soluble CD4 (rsCD4) required to neutralize such laboratory isolates of HIV-1 appears to depend on the dissociation constant (K_d) for the complex of CD4 and the virus major envelope glycoprotein, gp120. For example, replication of cell line-adapted viruses such as HIV-1/IIIB and HIV-1/RF, K_d(gp120/CD4) = 1–5 nM, typically is blocked by rsCD4 at concentrations less than 20 nM (1, 3). However, cell line-adapted isolates of retroviruses such as HIV-2 and simian immunodeficiency virus of macaque monkeys (SIV_{mac}), which also utilize CD4 as a cellular receptor, express envelopes with lower affinity for human CD4 and require commensurately higher concentrations of rsCD4 to block virus replication. For example, HIV-2, K_d(gp120/CD4) = 48 nM, requires more than 100 nM rsCD4 for complete blocking (4, 5), while SIV_{mac}, K_d(envelope/CD4) = 350 nM (6), requires more than 2 μM rsCD4 to achieve total blocking (4, 7). The correlation between intrinsic CD4–gp120 affinity and the concentration of rsCD4 required to block virus replication supports the model that rsCD4 neutralizes HIV-1 by coating the virus envelope, competing with cellular CD4 (1, 8, 9).

A striking difference in relative sensitivity to rsCD4-mediated neutralization has been uncovered by comparing the level of rsCD4 required to inhibit replication of common HIV-1 cell line-adapted isolates and that needed to block HIV-1 in patient plasma, from patient peripheral blood mononuclear cells (PBMCs) grown *in vitro*, or produced after one or a few passages *in vitro*. Such primary, or low-passage, HIV-1 isolates require 100- to 1000-fold higher concentrations of rsCD4 to block virus replication *in vitro* (10). Considering the range of envelope–CD4 affinities described above, an obvious hypothesis for the relative rsCD4 resistance of low-passage HIV-1 is that gp120s from primary isolates have relatively low affinity for CD4, as suggested by Daar *et al.* (10). We have tested this hypothesis by isolating pairs (each pair from independent AIDS patient blood samples) of low-passage rsCD4-resistant and high-passage rsCD4-sensitive HIV-1 and by comparing them with respect to proviral gp120 nucleotide sequence and CD4 binding of the corresponding recombinant gp120s (r-gp120s). Data from these analyses suggest that gp120 affinity for CD4 does not change during HIV-1 passage on cell lines and that other mechanisms must be invoked to explain the relative rsCD4 resistance of primary HIV-1 isolates.

MATERIALS AND METHODS

Virus and Cell Culture. Except where noted, culture methods and media have been described (11, 12). HIV-1/IIIB (a gift of M. Popovic and R. Gallo, National Institutes of Health) was propagated as described (11). The primary HIV-1 isolates lymphocytotropic JR-CSF and dual mono- and lymphocytotropic JR-FL (13) were supplied by the National Institutes of Health AIDS Research and Reagent Program. Clinical blood specimens, provided by the Massachusetts General Hospital AIDS clinic, were processed for plasma and PBMC isolations, and primary cultures were grown in R-20 (RPMI-1640 medium with 20% fetal calf serum) supplemented with interleukin 2 essentially as described (12, 14). Primary (i.e., minimally passaged) isolates of HIV-1 were produced by cocultivation of patient plasma or patient PBMCs with pooled PBMCs from seronegative donors (Leukopack; American Red Cross) that had been activated with phytohemagglutinin (PHA) (designated as PBMC.PHA) (12). Primary isolates in these studies are designated with the letter *P*, followed by a patient number. For example, primary

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Abbreviations: HIV, human immunodeficiency virus; HIV-1 and HIV-2, HIV types 1 and 2; rsCD4, recombinant soluble CD4; r-gp120, recombinant gp120; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; TCID, tissue culture infectious dose; HRP, horseradish peroxidase.

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isolates P-17 and P-08, derived from PBMCs of patient 17 and patient 08, respectively, were isolated by cocultivation with PBMC.PHA. After cultures became positive for HIV-1 p24 antigen, as measured by a commercial ELISA (DuPont/NEN), they were expanded to 25 ml, and aliquots of cell-free supernatants were stored at −70°C. Aliquots from each virus stock, thawed on ice for each experiment, were titered in duplicate by using the limiting dilution method on seronegative donor PBMC.PHA. The rsCD4-blocking titer was established by measuring the lowest concentration of rsCD4 required to inhibit 5 tissue culture infectious dose (TCID) units in duplicate infections of PBMC.PHA.

To produce high-passage HIV-1 isolates, 2.5×10^5 C8166 cells (15) (derived from umbilical cord lymphocytes transformed by human T-cell lymphotropic virus type 1) were cocultured with 1-ml aliquots of each of 20 primary HIV-1 isolates (15 from patient PBMCs and 5 from patient plasmas) in 48-well plates (Costar). After 2 days, cultures were washed three times, and, following addition of R-20 medium, further incubated until the cultures were clearly p24 antigen positive (p24 > 100 pg/ml) and displayed obvious cytopathic effect. Only 7/20 primary isolates (6 from patient PBMCs and 1 from patient plasma) produced measurable p24 and cytopathic effect in this assay. The positive infections were maintained for long-term passage, and clonal virus isolates were produced, as outlined in Fig. 1. Virus supernatants collected during passage on C8166 cells are named with the letter *C* followed by the patient identification number and by the number of days in continuous culture with C8166 cells. For example, C-17.44 refers to virus supernatant collected 44 days after initiating C8166 cocultivation with P-17. Primary and C8166-passaged virus isolates were titered on C8166 cells by limiting dilution to determine TCID/ml.

Replication assays to determine the potency of rsCD4 blocking for passaged viruses were carried out across a range of rsCD4 and virus concentrations, as described in Table 2.

Provirus Sequence Analyses. DNA for polymerase chain reaction (PCR) amplifications was prepared from cells acutely infected with the appropriate virus. Cells, harvested ≈48 hr after infection, were lysed in Sarkosyl buffer and DNA was extracted as described (16). Except where noted, PCR was performed according to the supplier's specifications. PCRs were set up with 250 ng of DNA, combined with

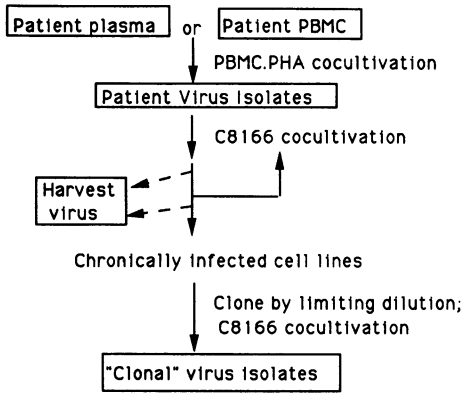

FIG. 1. Production of clonal virus isolates. Patient virus isolates were produced as described in *Materials and Methods*. The patient virus isolates were used to infect C8166 cells, and at 3- to 4-day intervals culture supernatants were harvested and frozen as aliquots. Uninfected cells (5×10^5) were added to maintain the culture, as many of the infected C8166 cells were killed. Within 2 weeks the infected cultures no longer displayed obvious cell death, and a chronic infection was established. By diluting chronically infected cells into 96-well microtiter wells at 0.5 cell per well and cocultivating with C8166 cells, chronically infected cultures were used to make clonal isolates of passaged virus.

25 pmol of each oligonucleotide primer and 1 unit of DNA polymerase (Amplitaq; Perkin–Elmer/Cetus), in a final reaction volume of 100 μl. All oligonucleotide primers were chosen from the HIVHXB2CG GenBank sequence file, and gp120 gene amplifications were carried out in two steps by using a model 480 thermal cycler (Perkin–Elmer/Cetus). For gp120 PCR cloning, the first amplification step consisted of 30 cycles using oligonucleotide primers REC-07 (5′-ATGGCAGGAAGAAGCGGAGA-3′, coordinates 5969–5988) and REC-09 (5′-AATTGTCTGGCCTGTACCGT-3′, coordinates 7835–7854). The second amplification step of 30 cycles used 5% of the product from the first amplification together with primers REC-27 (5′-TAACGGATCCGAGCAGAAGACAGTGGCAAT-3′, including coordinates 6206–6225 and a 5′ sequence that specifies a *Bam*HI site) and REC-12 (5′-GAAATTGAATTCTTATCTTTTTTCTCTCTGCACCA-3′, including coordinates 7737–7756 and a 5′ sequence that specifies an *Eco*RI site). The 1550-base-pair (bp) gp120 PCR products were purified by agarose gel electrophoresis prior to digestion with *Bam*HI and *Eco*RI and cloning. Bulk PCR nucleotide sequence analyses were performed as described (17), using PCR amplification products produced in two consecutive amplification steps of 30 cycles each (see above). Oligonucleotides used for bulk sequence PCR were REC-02 (5′-TTGTGGGTCACAGTCTATTA-3′, coordinates 6323–6342) and REC-06 (5′-CTAATTCCATGTGTACATTG-3′, coordinates 6959–6978) for the first step and REC-05 (5′-CAATGTACACATGGAATTAG-3′, coordinates 6959–6978) and REC-04 (5′-TTATCTTTTTTCTCTCTGCACCA-3′, including coordinates 7737–7756 and a 5′ sequence that specifies a stop codon) for the second step.

Expression of r-gp120. Amplified gp120 genes were subcloned in the expression vector pBG311 (16) between *Bam*HI and *Eco*RI endonuclease restriction sites. Large-scale transient expression of gp120 was accomplished by electroporation of 200 μg of circular plasmid DNA into 4×10^8 COS 7 cells with a Gene Pulser (Bio-Rad) set at 0.28 kV. Transfected cells were maintained for 48 hr in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (Hazelton Biologics, Lenexa, KS), in 2-liter cell factories (Nunc).

Expression of r-gp120 was measured by ELISA. Immulon-2 96-well microtiter plates (Dynatech) were coated overnight at 4°C with rsCD4 (Biogen) at 50 μg/ml in 50 mM Na_2CO_3, pH 9.6. Plates were blocked with phosphate-buffered saline (PBS)/2% dry milk (Carnation) for 30 min, then washed three times with PBS/0.03% Tween-20. Samples (clarified conditioned medium or column fractions) in PBS/2% milk were added to the wells, and after 2 hr at 23°C plates were washed three times with PBS/0.03% Tween-20. Bound gp120s were detected with pooled HIV-1 seropositive donor serum (heat inactivated) diluted 1:500 and incubated at 23°C for 4 hr. After three washes, as above, bound antibody was detected with a conjugate of goat anti-human IgG and horseradish peroxidase (HRP) diluted 1:100 and incubated for 2 hr at 23°C. After washes, bound HRP conjugate was detected with urea/hydrogen peroxide/*o*-phenylenediamine, and, after quenching with 0.5 M H_2SO_4, absorbance at 490 nm was measured with a Dynatech plate reader. The gp120 ELISA was linear in the range of 5–65 ng/ml.

Purification of r-gp120. Forty-eight-hour conditioned media from transiently transfected COS cells were filtered through 0.22-μm-pore filters and stored at −20°C. Four hundred milliliters of each gp120-containing medium was diluted with 400 ml of PBS and 16 ml of 1 M Tris·HCl, pH 7.7. Proteins were batch-loaded onto 6 ml of lentil lectin coupled to Sepharose 4B (Pharmacia) in an overnight incubation at 4°C with continuous stirring. The gel was collected in a 1-cm-diameter column and washed with PBS, and the total glycoprotein pool was released by treatment with 10% *N*-ace-

tylglucosamine/300 mM NaCl/100 mM Tris·HCl, pH 7.7. Fractions were monitored by absorbance at 280 nm and by ELISA (see above). Following concentration of peak fractions, the gp120s were further purified on a CD4 affinity column that had been prepared by coupling rsCD4 on Affi-Gel-10 (Bio-Rad) at 10 mg of protein per ml of gel. Then 200 μl of washed CD4 beads was added to the 1.5 ml of concentrate and incubated overnight at 4°C. The product was eluted from the gel with 50 mM glycine, pH 3.0/250 mM NaCl and immediately neutralized by adding Hepes, pH 7.5, to 100 mM. Fractions were analyzed by SDS/PAGE and by ELISA. Peak fractions were pooled and stored in aliquots at −70°C. The overall yield and fold purification for the two r-gp120s were 28% and >59,000 for HIV-1/IIIB and 54% and 38,000 for P-17 gp120, respectively.

rsCD4/gp120 Affinity Measurements. The affinity of gp120 for rsCD4 was assessed in an ELISA format (18). All manipulations were performed at room temperature. Immulon-2 96-well microtiter plates were coated with the 6C6 anti-CD4 monoclonal antibody [which blocks gp120 binding (P. Chisholm, personal communication)] at 20 μg/ml in 50 mM sodium carbonate, pH 9.6 (50 μl per well, incubation overnight), and then washed five times with water. The plates were blocked with PBS/2% milk (150 μl per well for 90 min) and washed three times with PBS/0.025% Tween-20. Test samples in PBS/2% milk, containing 25 ng of rsCD4 and various concentrations of gp120, were incubated for 60 min and then added to the coated plates (50 μl per well). Each sample was assayed in triplicate. After 120 min, plates were washed five times with PBS/0.025% Tween-20. Bound rsCD4 was detected with a monoclonal antibody OKT4–HRP conjugate by first incubating samples for 75 min with the conjugate in PBS/2% milk (50 μl per well) and then visualizing the tagged products with urea/hydrogen peroxide/*o*-phenylenediamine, as described above. Under the conditions used, rsCD4 gave a linear response over a range of 6–50 ng (A_{490} range of 0.2–1.2).

RESULTS

The ability of rsCD4 to block replication by patient primary virus isolates has been evaluated in tissue culture, using seronegative donor PBMCs as host cells. Table 1 shows that all patient primary HIV-1 isolates tested are relatively rsCD4 resistant, compared with the extensively passaged laboratory isolate HIV-1/IIIB. The lack of correlation between TCID and p24 values among primary isolates has been previously documented (19), and it did not appear to be a factor for the degree of rsCD4 resistance (Table 1). Because primary virus isolates are refractory to rsCD4, even at limiting dilution (data not shown), it is likely that the resistant population represents the major fraction of virus. Fig. 1 illustrates how patient plasmas or HIV-1 isolates generated from patient PBMCs were utilized to produce high-passage, cell line-adapted HIV-1 isolates. Primary isolates, in each case, were cultured with $CD4^+$ C8166 cells for extended periods, as described in Fig. 1. When this approach is used, the potency of rsCD4 for blocking virus replication apparently increases dramatically within 6–10 weeks of passage (Table 2). For example, a primary isolate from the plasma of patient 17 (P-17), which requires rsCD4 at >100 μg/ml to block replication of 5 TCID *in vitro*, becomes approximately 100-fold more sensitive to rsCD4 blocking by 75 days of passage (Fig. 2).

Table 1. Relative rsCD4 resistance of primary HIV-1 isolates

Isolate	Source	TCID/ml	p24, ng/ml	rsCD4 blocking conc., μg/ml
P-08	PBMCs	20	13	300
P-15	PBMCs	100	29	100
P-38	Plasma	>250	62	100
P-39	PBMCs	>250	70	100
P-40	Plasma	>250	196	100
P-17	PBMCs	50	8	100
P-0104B	Plasma	200	43	100
JR.CSF	CSF	10^5	58	300
JR.FL	FL	10^5	39	300
HIV-1/IIIB	PBMCs	3×10^6	300	<1

HIV-1 isolates produced during this study are preceded by the letter *P*. The isolation of JR.CSF and JR.FL (13) and the rsCD4 refractoriness of these isolates (10) have been previously described. CSF, cerebrospinal fluid; FL, frontal lobe. The extensively passaged HIV-1/IIIB is listed for comparison. HIV-1 core p24 antigen concentrations were determined from supernatants of patient PBMCs or plasmas after 10–14 days of cocultivation with HIV-1 seronegative donor PHA-treated PBMCs. rsCD4 blocking concentrations are those required to prevent infection of 5 TCID on HIV-1 seronegative donor PBMCs.

Table 2. Prolonged passage of patient HIV-1 isolates in a $CD4^+$ cell line yields rsCD4-sensitive virus

Virus	Passage day	rsCD4 blocking conc., μg/ml			
		1 TCID	5 TCID	25 TCID	125 TCID
HIV-1/IIIB	>100	<1	<1	<1	<1
C-08	14	ND	10–100	10–100	100
	44	1	1	3	10
	67	0.3	0.3	1	ND
C-17	14	100	100	100	300
	44	3	3	10	30
	67	0.3	1	3	ND

Cell line-adapted isolates C-08 and C-17 (both produced in this study from patient PBMCs) were kept in continuous culture with HIV-1 seronegative PHA-treated PBMCs. Virus (1, 5, 25, or 125 TCID) and rsCD4 (300, 100, 30, 10, 3, 1, or 0.3 μg/ml, final concentration) were mixed in triplicate and incubated in 48-well plates for 60 min at 37°C prior to the addition of 2.5×10^5 C8166 in R-20. Infection mixtures (total volume 1 ml) were allowed to incubate for 10–14 days, and virus growth was assessed by both the appearance of a cytopathic effect and the production of HIV-1 p24 antigen. ND, not done.

Two possible explanations for this phenomenon are (*i*) a trans-acting inhibitory factor (e.g., defective interfering particles) abrogates rsCD4 activity and (*ii*) resistance is due to a host cell-specific factor. The presence of a trans-acting factor only in low-passage patient HIV-1 isolates was tested by mixing an rsCD4-resistant virus isolate (P-0104B) and an rsCD4-sensitive virus isolate (HIV-1/IIIB) prior to titrating against rsCD4 in the C8166 cell replication assay described above. Because P-0104B cannot replicate in the C8166 cell line in our assay, it was possible to measure directly the sensitivity of HIV-1/IIIB in the presence the rsCD4-resistant isolate P-0104B. Replication of the mixed virus inoculum was iden-

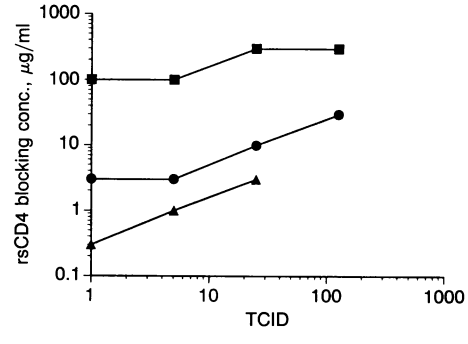

FIG. 2. Changes in rsCD4 resistance of patient 17 HIV-1 after passage in C8166 cells. Data represent the concentration of rsCD4 required to block 1, 5, 25, or 125 TCID of C-17 isolates after 14 (■), 44 (●), or 75 (▲) days in continuous culture.

tical to HIV-1/IIIB alone, being blocked completely by rsCD4 at <1 μg/ml (data not shown). To determine if resistance to rsCD4 blocking is host cell specific, blocking assays with equal amounts of primary and passaged viruses were carried out in PBMC.PHA and C8166, in parallel. In these assays, relative resistance to rsCD4 is roughly equivalent (within a factor of 3), regardless of the host cell (data not shown).

The results described above suggest that during passage in $CD4^+$ cell lines primary virus isolates undergo selection leading to altered interaction with CD4. If affinity between the virus envelope and CD4 increases during passage, nucleotide sequence changes in the gp120-encoding portion of the virus envelope gene could be responsible. To determine the changes in envelope sequence that occurred during passage of P-17, provirus *env* genes from pairs of HIV-1 isolates were subjected to DNA sequence analyses using PCR. To address the problem of virus heterogeneity (20), both "bulk" sequence from the unfractionated PCR product and sequences

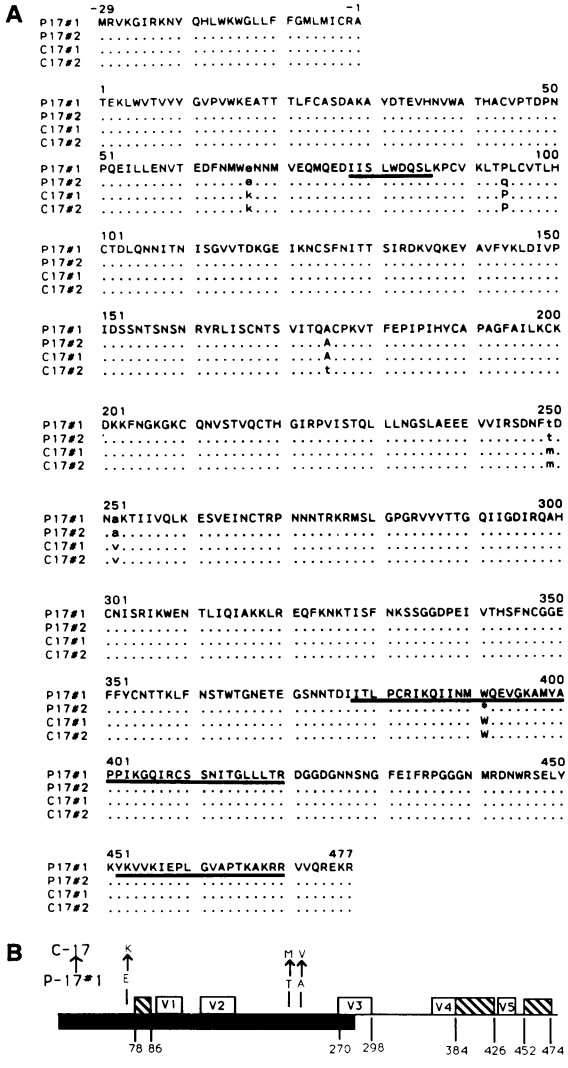

FIG. 3. Primary structure comparison of gp120 from primary and cell line-adapted HIV-1 isolates from patient 17. (*A*) Amino acid sequences deduced from cloned gp120 gene sequences. Lowercase letters indicate differences. Underlined sequences correspond to regions involved in CD4 binding (2). The asterisk shows the position of a stop codon found in P-17#2, an apparently defective provirus. (*B*) Location of amino acid heterogeneities on a functional map of gp120. V-region boxes correspond to variable regions; hatched boxes correspond to regions required for CD4 binding; solid box corresponds to the region involved in gp120–gp41 binding (21). Coordinates for P-17 to C-17 heterogeneities are presented in Table 3.

Table 3. Sequence heterogeneities for bulk PCR products and PCR subclones of passaged virus pairs from patient 17

PCR product	gp120 changes		
	67	249	252
P-17 bulk	E	T	A
C-17 bulk	K	T	A
P-17#1 and #2	E	T	A
C-17#1 and #2	K	M	V
HIV-IIIB	K	T	A

Summary of the sequencing results shown in Fig. 3. Amino acid coordinates correspond to the gp120 map in Fig. 3*A*.

from PCR subclones of gp120 genes (two from each isolate) were compared. Bulk sequence of the *env* PCR product should be representative of the predominant (≈90%) sequence at any nucleotide position from the total provirus population (R.T., unpublished results). Fig. 3*A* shows amino acid sequence comparisons deduced from DNA sequence analyses for P-17.14 and C-17.75. As summarized in Table 3, bulk gp120 sequence comparisons show that P-17.14 and C-17.75 differ in gp120 by only one amino acid, while comparison of PCR subclones shows three amino acid heterogeneities, one of which is identical to that found in the bulk sequence analysis. Fig. 3*B* shows that the three heterogeneities map outside regions previously shown to be involved in CD4 binding (2).

Sequence analysis of the patient 17 virus shows that acquisition of rsCD4 sensitivity to blocking does not correlate with major changes in the primary structure of gp120. To determine whether or not the three gp120 sequence heterogeneities affect CD4 binding, a direct measurement of P-17 gp120/CD4 affinity was performed using P-17 r-gp120 that was produced in COS cells. As a high-affinity control, r-gp120 corresponding to that from HIV-1/IIIB was produced in parallel. Affinity purification yielded relatively pure glycoproteins with similar apparent molecular weights, as shown in Fig. 4*A*. In the gp120 competition ELISA described in *Materials and Methods*, P-17-derived gp120 has an apparent rsCD4 binding affinity not significantly different from that of HIV-1/IIIB gp120. The competition curves presented in Fig. 4*B* show that the apparent K_d for both gp120s is ≈2 nM, in agreement with previously published measurements (1, 6, 22, 23).

DISCUSSION

The HIV-1 rsCD4 resistance documented here and elsewhere (10) raises questions about the potential therapeutic utility of rsCD4, a drug candidate that has yielded rather equivocal

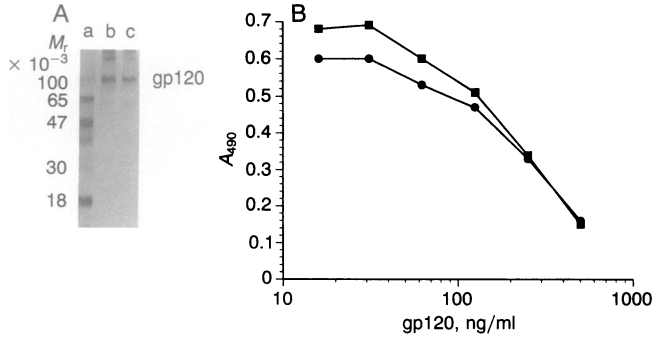

FIG. 4. Expression and affinity measurement of patient 17 HIV-1 gp120. (*A*) Coomassie blue stain of SDS/PAGE of r-gp120s. Lane a, prestained molecular weight markers (Bethesda Research Laboratories); lane b, purified HIV-1/IIIB r-gp120; lane c, purified P-17 r-gp120. (*B*) Competition ELISA comparison of HIV-1/IIIB r-gp120 (■) and P-17.75 r-gp120 (●). K_d = 2 nM corresponds to a gp120 concentration of ≈200 ng/ml.

results in early clinical trials (14, 24). A key assumption behind CD4-based therapeutic strategies is high-affinity binding between CD4 and virus envelope (1). Therefore, testing the validity of the hypothesis that CD4/patient HIV-1 affinity is much lower than that measured with laboratory HIV-1 could be crucial for defining the appropriate therapeutic setting for CD4 congeners.

By generating matched pairs of virus isolates that differ with regard to *in vitro* passage number and, correlatively, differ in refractoriness to rsCD4, it has been possible to search for *env* gene heterogeneities that might account for altered interaction with CD4. The results presented above reveal that the deduced amino acid changes found in gp120 from HIV-1 P-17, subjected to prolonged passage *in vitro*, do not occur within regions of gp120 previously mapped for CD4 interaction (2). We have subjected a second patient virus isolate pair (P-08/C-08) to the sequence analysis described above, and we have confirmed the results found with P-17/C-17—i.e., sequence heterogeneity occurs outside regions previously mapped to interact with CD4 (data not presented). Consistent with the nucleotide sequence comparison, r-gp120 from the P-17 isolate binds rsCD4 with affinity equivalent to r-gp120 derived from extensively passaged, rsCD4-sensitive HIV-1/IIIB. This strongly implies that gp120/rsCD4 affinity does not change during passage of HIV-1 *in vitro*, although it does not rule out the possibility that virus-associated envelope from primary patient viruses might have lower affinity for CD4. Indeed, CD4 affinity for HIV-1 could be determined by the unique conformation of the gp120/gp41 heterooligomer that forms the virus envelope spike (23, 25–27). No such discrepancy in rsCD4 binding affinity has been found when HIV-1/IIIB and the corresponding r-gp120 were compared (18), but primary virus envelope–CD4 interactions may not follow this pattern (25, 26). What is clear from our analysis is that gp120/CD4 affinity alone does not predict HIV-1 susceptibility to neutralization by rsCD4.

Several recent studies have implicated an unexpected mechanism for rsCD4-mediated HIV-1 neutralization. Experiments with cell line-adapted HIV-1 show that such isolates have differential rates of envelope gp120 shedding (5) that can be greatly enhanced by rsCD4 binding (9, 28), a phenomenon that could be likened to CD4-induced envelope "stripping." The physiological correlate to this process could be initiating virus entry (25, 26). If rsCD4 neutralizes laboratory HIV-1 by inducing gp120 shedding, or "stripping," it is possible that patient HIV-1 is somehow "strip-proof" and therefore not subject to irreversible blocking by CD4-based antiviral agents. If this mechanism accounts, at least in part, for the dramatic shift in rsCD4 sensitivity of primary isolates upon passage, it is likely that gp120–gp41 interactions are altered during passage *in vitro*. Interestingly, we find that the gp120 sequence heterogeneities found by comparing P-17 and C-17 occur within in a region involved in gp120–gp41 interactions (21) (Fig. 3*B*).

Such variable sensitivity to rsCD4 might be explained if there are unique receptor complexes utilized by HIV-1 on tumor cells and PBMCs. One model (29, 30) for CD4–envelope interactions suggests that initial virus binding to CD4 initiates a series of conformational changes that expose the gp120 V3 loop and the putative gp41 fusogenic domain, and exposure of these two domains is required for critical virus entry events that might be mediated by separate receptors (25, 29–31). It will be of interest to define the genetic determinants of HIV-1 rsCD4 resistance.

While this manuscript was being reviewed, two studies (32, 33) independently demonstrated equivalent and high-affinity interactions between CD4 and r-gp120 from either cell line-adapted or primary HIV-1 isolates. We have extended these observations by comparing r-gp120s derived from viruses within a defined lineage.

We thank Gary Jaworski for oligonucleotides and Theresa Liu and Beatrice Burrus for certain HRP conjugates.

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