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The CD4-Independent Tropism of Human Immunodeficiency Virus Type 2 Involves Several Regions of the Envelope Protein and Correlates with a Reduced Activation Threshold for Envelope-Mediated Fusion

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Several human immunodeficiency virus type 2 (HIV-2) strains have been shown to infect some CD4-negative cell lines (P. R. Clapham, A. McKnight, and R. A. Weiss, J. Virol. 66:3531–3537, 1992). Using molecular clones of HIV-2 with a CD4-independent tropism, we have identified critical amino acid residues in the envelope protein which are required for CD4-independent infection. Mutations located immediately upstream of a proposed coiled coil domain in the transmembrane protein (A526T or I528M) and flanking the base of the V4 loop (L378F and K403R) are crucial for the CD4-independent phenotype. Of several mutations conferring a positive charge in V1, V2, and V3, only the change in V3 (Q310K) helped to enhance the CD4-independent phenotype but could not mediate it on its own. These mutations reduce the amount of soluble CD4 required to trigger CD4-independent cell-cell fusion, suggesting that they lower the activation threshold for the fusion process. After binding to cell surface-anchored CD4, a CD4-independent recombinant envelope protein showed an increased binding of anti-envelope protein antibodies, suggesting either an enhanced binding to cell surfaces or more extensive conformational changes in CD4-independent compared to CD4-dependent envelope proteins. The reduced activation threshold of CD4-independent envelope proteins may thus enable them to utilize a membrane molecule for entry which is not as efficient as CD4 in triggering the conformational changes required for the membrane fusion process. CD4-independent HIV-2 variants may be conceptually similar to influenza virus variants capable of fusing at a higher than normal pH (R. S. Daniels, J. C. Downie, J. A. Hay, M. Knossow, J. J. Skehel, M. L. Wang, and D. C. Wiley, Cell 40:431–439, 1985).

All primate lentiviruses use the CD4 molecule as their main receptor (61, 75). Extensive work on human immunodeficiency virus type 1 (HIV-1) has shown that in addition to serving as a docking site for the outer envelope protein of these viruses, CD4 is involved in postbinding events and induces conformational changes in the tetrameric envelope protein complex which lead to the exposure of domains in both the outer envelope protein (SU) and the transmembrane protein (TM) (50, 60). These conformational changes precede and are required for the triggering of the fusion event.

HIV-1 entry into human cells requires an additional factor which is absent from most nonhuman cells (2, 13, 44). Several chemokine receptors, in particular CXCR4, CCR-3, and CCR-5, have recently been shown to act as coreceptors for HIV-1 (1, 10, 20, 22, 23, 26). HIV-2 and the related SIV_{mac} also require additional components, which may, however, differ from those required for HIV-1 entry (13, 46).

HIV-1 and HIV-2 isolates can differ in their relative tropism for CD4-positive human cells. Isolates with a preferential tropism for primary human CD4-positive T cells, monocytes/macrophages, or permanent T-cell lines can be established from patients (27, 62, 63, 65, 74, 76). Viruses with an increased fusogenic and replicative potential are more readily isolated from patients with advanced disease stages (15, 27, 63, 74). The outer envelope protein of HIV-1, in particular amino acid changes in the V3 loop, determines the tropism for macrophages versus T cells (62, 65, 71, 74), but other envelope protein regions also contribute (4, 16, 68). Continuous passage in vitro, particularly adaptation to established T-cell lines, will select for virus variants with an increased fusogenicity (4). These T-cell-line-adapted isolates are often easier to inhibit by neutralizing antibodies (45) or recombinant soluble CD4 (sCD4) (18) than are primary isolates, and this may be related to a change in the conformation of their envelope protein complex, which becomes more accessible to these reagents (3, 67, 68). This more accessible conformation may facilitate the interaction between viral envelope protein and CD4, thus increasing infectivity (48). Adaptation of HIV-2 and SIV_{agm} isolates to human T-cell lines can cause a premature truncation of the cytoplasmic domain of the transmembrane protein (36, 42). At least some of these truncations result in increased fusogenicity (55), increased affinity for CD4 (37), and an increased density of the viral glycoprotein on the cellular and virion membrane (43, 79). T-cell-line-adapted HIV-1 isolates use CXCR4 as a coreceptor (26), whereas primary isolates use mainly CCR-5 and on occasion also CCR-3 and CCKR-2b (1, 10, 20, 22, 23). Other cell membrane molecules may further modulate the efficiency of HIV-1 entry (25, 39, 57).

Both HIV-1 and HIV-2 are capable of infecting CD4-negative cell lines. For HIV-1, this has been demonstrated for cell lines of nervous system, fibroblast, and liver origin (9, 11, 21, 31, 32, 73). In the case of some neural cell lines, this may be mediated by a glycolipid, galactosyl ceramide (32), and sequences in either V3 or V4/V5 determine this tropism (33).

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Efficient CD4-independent infection has been documented for several HIV-2 isolates (14). The cellular receptor mediating this route of entry (72) is mainly CXCR4 (36a, 58a). Most, if not all, strains of HIV-2 can bind to the surface of some human cells, possibly via the same structure that mediates CD4-independent infection, and, following binding, can be induced to fuse by treatment of bound virions with sCD4 (14). This suggests that CD4-independent strains of HIV-2 could depend less on the conformational changes induced by sCD4 and may thus be able to utilize a membrane component for entry which, unlike CD4, is not capable of triggering the conformational changes required for the activation of the fusion process.

To define the envelope determinants responsible for the CD4-independent phenotype, we used a previously described, laboratory-adapted variant of the prototypic HIV-2 strain ROD/A, ROD-B, previously referred to as LAV-2/B (14). We generated infectious molecular clones from ROD/B and determined sequence differences in the envelope protein between these clones and a CD4-dependent molecular clone derived from HIV2/ROD. We then studied the contribution of individual sequence changes to the CD4-independent phenotype by inserting them, alone and in combination, into a CD4-dependent molecular clone. Our results suggest that a combination of mutations, involving a region in the transmembrane protein in close proximity to a predicted coiled-coil domain (28, 77), regions flanking the V4 loop, and V3, are required for efficient CD4-independent infection. We further provide evidence that CD4-independent envelope proteins are more easily induced to mediate fusion by pretreatment with sCD4 and postulate that this reduced "activation threshold" allows CD4-independent HIV-2 strains to use a cell surface molecule for entry which is not as effective as CD4 in triggering the conformational changes required for the fusion process.

MATERIALS AND METHODS

Cells. The CD4-negative human B-cell line Daudi (58) and the CD4-positive human T-cell line SupT1 (66) were grown in suspension in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (FCS), 60 μg of penicillin per ml, and 100 μg of streptomycin per ml. The CD4-negative human rhabdomyosarcoma cell line RD/TE671 (69) was grown in monolayers in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 5% FCS, 60 μg of penicillin per ml, and 100 μg of streptomycin per ml. The insect cell line SF9, from *Spodoptera frugiperda*, was grown at room temperature in spinner flasks containing supplemented Grace's insect medium (GIBCO-BRL), 10% FCS, 60 μg of penicillin per ml, and 100 μg of streptomycin per ml.

Generation of molecular clones with a CD4-independent phenotype. Stocks of the CD4-independent HIV-2 variant ROD/B, obtained as previously described (14) by passaging HIV-2/ROD/A in CD4-positive C8166 cells, contain a mixture of CD4-dependent and -independent viruses. To select for viruses with a CD4-independent phenotype, stocks of ROD/B from C8166 cells were passaged twice through CD4-negative RD/TE671 cells. After extraction of genomic DNA from infected RD/TE671 cells, a 2,972-bp amplicon, containing both *tat* and *rev* exons as well as the entire *env* reading frame, was amplified by PCR with primers TAGACATGGAGACACCCTTGAAGG (bp 5840 to 5863; numbering as in reference 30) and TGAATCTACATCATCCATATTTTG (bp 8832 to 8809). A *Sac*I-*Bam*HI restriction fragment (nucleotides 5871 to 8574) was obtained from this amplicon and substituted for the corresponding fragment of the molecular clone pACR23, a CD4-dependent infectious proviral clone of LAV2/ROD (40). As neither *Sac*I nor *Bam*HI are unique in pACR23, the *Sac*I-*Bam*HI restriction fragments obtained from HIV-2/ROD/B-infected cells were first cloned into plasmid pACRΔgag-pol, obtained by deleting a *Nar*I fragment (nucleotides 305 to 5863) containing *gag*, *pol*, *vif*, and *vpx*. After substituting the *Sac*I-*Bam*HI fragment containing the *env* gene, this *Nar*I fragment was reinserted. All DNA manipulations were carried out by following standard protocols, with *Escherichia coli* TOP 10F (Invitrogen) and growing bacterial cultures at 32°C to minimize cloning artifacts when assembling full-length proviral clones.

The ability of proviral clones to produce infectious HIV-2 exhibiting a tropism for CD4-negative cells was tested initially by transfection (calcium phosphate method) into RD/TE671 cells followed by cocultivation with Daudi cells and monitoring of virus production by a previously described reverse transcriptase (RT) assay (59). To quantitate their infectivity, viral stocks were generated by transfection of proviral clones into RD/TE671 cells and cell-free virus stocks were harvested after 3 days and calibrated by the RT assay.

After subcloning of individual *Sac*I-*Bam*HI fragments into M13, their sequence was obtained by dideoxy sequencing with internal primers. In this communication the numbering of amino acid residues in the envelope protein refers to the mature envelope protein after removal of the leader sequence; i.e., numbering starts at codon 20 of the *env* gene (threonine).

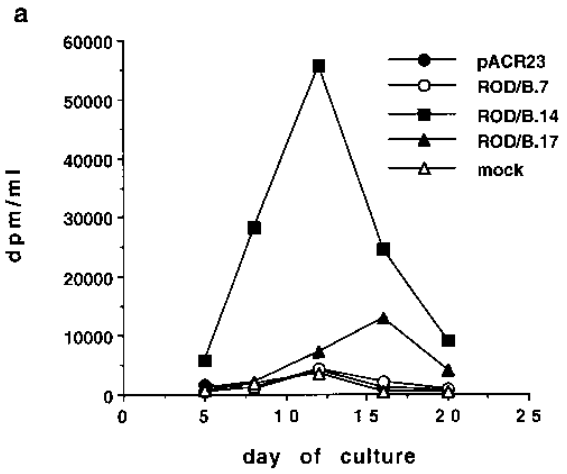
Construction of intraenvelope chimeras and site-directed mutants. To map sequence changes responsible for the CD4-independent phenotype, molecular clones containing chimeric envelope genes were constructed by exchanging restriction fragments between CD4-independent clones and the CD4-dependent proviral clone pACR23. We used the *Pst*I sites at positions 7323 and 8466 and the *Mae*I site at position 7692 to exchange restriction fragments after subcloning of the 2.7-kb *env*-containing *Sac*I-*Bam*HI fragments into a pUC19-derived plasmid from which the *Pst*I site had previously been deleted. Site-directed mutagenesis was carried out with the Sculptor in vitro mutagenesis kit (Amersham, Little Chalfont, United Kingdom). Mutations were introduced individually into subcloned restriction fragments, whose sequences were verified by automated sequencing. To construct proviral clones containing combinations of mutations, either additional mutations were introduced into previously mutated restriction fragments or suitable restriction fragments were joined. After envelope clones containing combinations of mutations were reconstructed, their sequence was always verified.

Assaying the biological phenotype of proviral clones. Cell-free viral stocks derived from HIV-2/ROD/B molecular clones, intraenvelope chimeras, and site-directed mutants were produced by transfection of full-length molecular clones into RD/TE671 cells by the calcium phosphate method. Supernatants were harvested after 3 days, and the amount of virus was quantified by measuring RT activity as previously described (59). To assay cell-free infection of Daudi cells in suspension, 5×10^5 cells/well of a six-well plate were infected with 20,000 dpm of viral stocks for 16 h, washed, and cultured for 2 to 3 weeks. Supernatants were harvested every 3 to 4 days and assayed for RT activity.

To assay cell-free infection of adherent RD/TE671 cells, 10^4 cells/well of a 96-well plate were infected with 4,000 dpm of viral stock for 16 h, washed, and incubated for 3 days. At 3 days postinfection, infected cells were immunostained with pooled rat monoclonal antibodies to the HIV-2 envelope protein (47), a β-galactosidase-conjugated anti-rat antibody (Seralab; dilution 1:600), and 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal; 0.5 mg/ml in phosphate-buffered saline (PBS) containing 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 1 mM magnesium chloride) as previously described (14).

Fusion assay. Molecular clones containing a range of mutations were transfected into RD/TE671 cells, plated at 2×10^5 per well in a six-well tray. After removal of the transfection mixture, cultures were incubated in the presence of different concentrations of sCD4 (a gift from R. Sweet, SmithKline Beecham) for 3 days and then stained for envelope expression as described above. The percentage of envelope-expressing cells forming syncytia with more than three nuclei relative to the total number of envelope-expressing cells was determined by counting at least 300 envelope-expressing cells.

Expression and quantitation of recombinant envelope proteins. To express soluble recombinant envelope precursor proteins of the CD4-dependent envelope of pACR23 and the CD4-independent envelope of ROD/B.14 in insect cells, a stop codon was inserted into the *env* gene by PCR, replacing the codon for tryptophan at position 654 (nucleotide 8164), using primers AGA CAA GGA TCC ATG ATG AAT CAG CTG (nucleotides 6147 to 6161) and AAT ATG AAT TCA CTA GGA GGT TAA GTC AAA (nucleotides 8163 to 8148). The resulting truncated *env* gene was inserted into the *Xho*I/*Sma*I sites of the baculovirus transfer vector pVL941, and the resulting constructs were transfected into SF9 cells, derived from *S. frugiperda*, with the BaculoGold Transfection kit (Pharmingen). Recombinant baculoviruses were obtained, plaque purified once, and used to infect 500-ml cultures of SF9 cells for large-scale production of recombinant envelope proteins. Supernatants were harvested after 3 to 4 days, and the concentration of recombinant envelope proteins was quantitated by capture enzyme-linked immunosorbent assay (ELISA). ELISA plates (Immulon 2; Dynatech Laboratories) were coated overnight at 4°C with 100 μl of *Galanthus nivalis* agglutinin (GNA; Boehringer Mannheim) per well at 10 μg of GNA per ml in 0.1 M $NaHCO_3$–0.15 M NaCl (pH 8.5). The plate was blocked twice for 10 min with 2% dried skimmed milk (Marvel) in Tris-buffered saline (TBS) before addition of serial dilutions of tissue culture supernatants containing recombinant envelope proteins, to which 0.05% Empigen had been added. As a standard, we used a serial dilution of recombinant baculovirus-derived HIV-2 gp105 (outer envelope glycoprotein), produced with a previously described recombinant baculovirus (51) and purified to 90% purity by affinity chromatography on GNA-coupled glass beads as reported previously (29). The plates were washed three times with TBS before addition to each well of 100 μl of pooled rat monoclonal antibodies to the outer envelope glycoprotein of LAV2/ROD diluted in TBS–2% dried skimmed milk–0.5% Tween 20–10% sheep serum. Antibody binding was quantitated with an alkaline phosphatase-conjugated anti-rat immunoglobulin G (IgG; Seralab) and the AMPAK substrate kit (Dako) as previously described (49). Recombinant envelope glycoproteins produced in this study were also purified by lectin affinity chromatography on GNA coupled to glass beads (29).



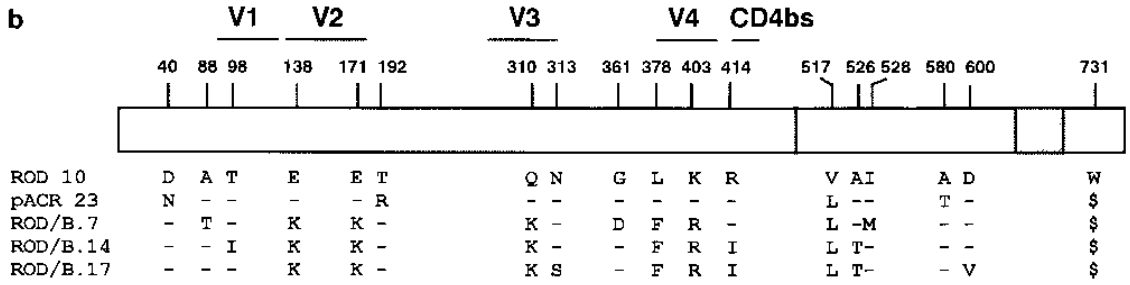


FIG. 1. Replication in Daudi cells and sequence of molecular clones derived from CD4-independent HIV-2. (a) RD/TE671 cells transfected with 5 μg of proviral DNA were cocultivated overnight with 5×10^5 Daudi cells. The Daudi cells were then passaged twice a week, and supernatants were collected for determination of RT activity. (b) The envelope protein sequences deduced from three CD4-independent clones were compared to those of HIV2/ROD (30) and ACR23 (40). The amino acid numbering is from the start of the mature envelope protein, i.e., at codon 20 of the *env* gene (threonine).

Affinity of recombinant envelope proteins for sCD4 and sCD4IgG. Recombinant envelope proteins were captured on GNA-coated ELISA plates as above, except that no Empigen was added to the tissue culture supernatants. After blocking as above, increasing concentrations of sCD4 produced in baculovirus (MRC ADP Reagents Repository) or sCD4IgG (6) in TBS–2% dried skim milk–0.1% Tween 20–10% sheep serum were added for 2 h. Binding was quantified by using, in the case of sCD4, a monoclonal antibody to CD4, L120 (34), followed by an alkaline phosphatase-conjugated antibody to mouse IgG (Seralab) or, in the case of sCD4IgG, an alkaline phosphatase-conjugated antibody to human IgG (Seralab). The AMPAK (Dako) substrate was used in both cases.

Binding of recombinant envelope proteins to the surface of CD4-positive and -negative cells. The binding of recombinant envelope proteins to CD4-positive SUPT1 and CD4-negative Daudi cells was measured by immunofluorescence. A 50-μl sample of tissue culture supernatant, diluted in PBS–1% FCS–0.1% sodium azide to contain 0.5 μg of recombinant protein per ml, was added to 50 μl of cells (at 2×10^7 cells/ml; preincubated in PBS–1% FCS–0.1% sodium azide for 30 min at room temperature) and incubated at room temperature for 2 h. The cells were washed twice in PBS-FCS-sodium azide before resuspension in 100 μl of rat monoclonal antibody to the envelope protein of HIV-2/ROD (47) or a pooled HIV-2-positive patient serum sample and incubation for 1 h at room temperature. After two washes in PBS-FCS-sodium azide, the cells were resuspended in 100 μl of anti-rat IgG fluorescein isothiocyanate FITC or anti-human IgG FITC (Seralab) diluted 1:80 in PBS-FCS-sodium azide and incubated for 1 h at room temperature. The cells were then washed once in PBS-FCS-sodium azide and twice in PBS-sodium azide, resuspended in 100 μl of PBS-sodium azide, and added to 300 μl of formol saline (4% of formaldehyde in 0.5% NaCl and 1.5% Na_2SO_4) before analysis by fluorescence-activated cell sorting (Becton-Dickinson).

RESULTS

Generation and envelope gene sequence of molecular clones derived from ROD/B. Stocks of the CD4-independent HIV-2 variant ROD/B, as described previously from this laboratory (14), had been grown in the CD4-positive T-cell line C8166. To enrich for CD4-independent viruses, ROD/B was passaged twice through the CD4-negative rhabdomyosarcoma cell line RD/TE671. DNA extracted from infected cells after the second passage was used for PCR to generate a 2.9-kb amplicon containing both *tat* and *rev* exons as well as the entire *env* gene. A 2.7-kb *Sac*I-*Bam*HI fragment was obtained from this amplicon and substituted for the corresponding fragment in molecular clone pACR23 of the CD4-dependent strain HIV-2/ROD. After transfection of three resulting molecular clones into RD/TE671 cells and cocultivation with Daudi cells, two, ROD/B.14 and ROD/B.17, produced infectious virus capable of replicating in Daudi cells (Fig. 1a). All three clones showed various degrees of syncytium formation in RD/TE671 cells (not shown), with the two replication-competent clones, ROD/B.14 and ROD/B.17, being the most potent. We compared the *env* gene sequences of all ROD/B-derived clones to that of the CD4-dependent molecular clone pACR23 and the prototypical HIV2/ROD sequence (ROD 10) (Fig. 1b). Compared to ACR 23 and ROD 10, all clones shared mutations in the variable loops V1 (E138K), V2 (E171K), and V3 (Q310K) which would confer an increase in positive charge to these regions of the envelope protein. All ROD/B-derived molecular clones also contained two changes flanking the V4 loop (L378F and K403R). In the transmembrane protein, both ROD/B.14 and ROD/B.17 had an A526T change. One of the other clones, ROD/B.7, had an I528M change in the same region, i.e., a short distance upstream of a proposed coiled-coil domain previously shown to be important for HIV-1 infectivity (24, 77). Like pACR23, all ROD/B-derived molecular clones had a premature stop codon in the cytoplasmic tail of the transmembrane protein at amino acid (aa) 731 (W731$). Changes observed in only one or two clones comprised A88T, T98I, N313S, G361D, R414I, and D600V, but, as shown below, none of these are required for the CD4-independent phenotype.

Mapping of mutations determining the CD4-independent phenotype. To map the amino acid changes determining the CD4-independent tropism of ROD/B.14, we initially used the

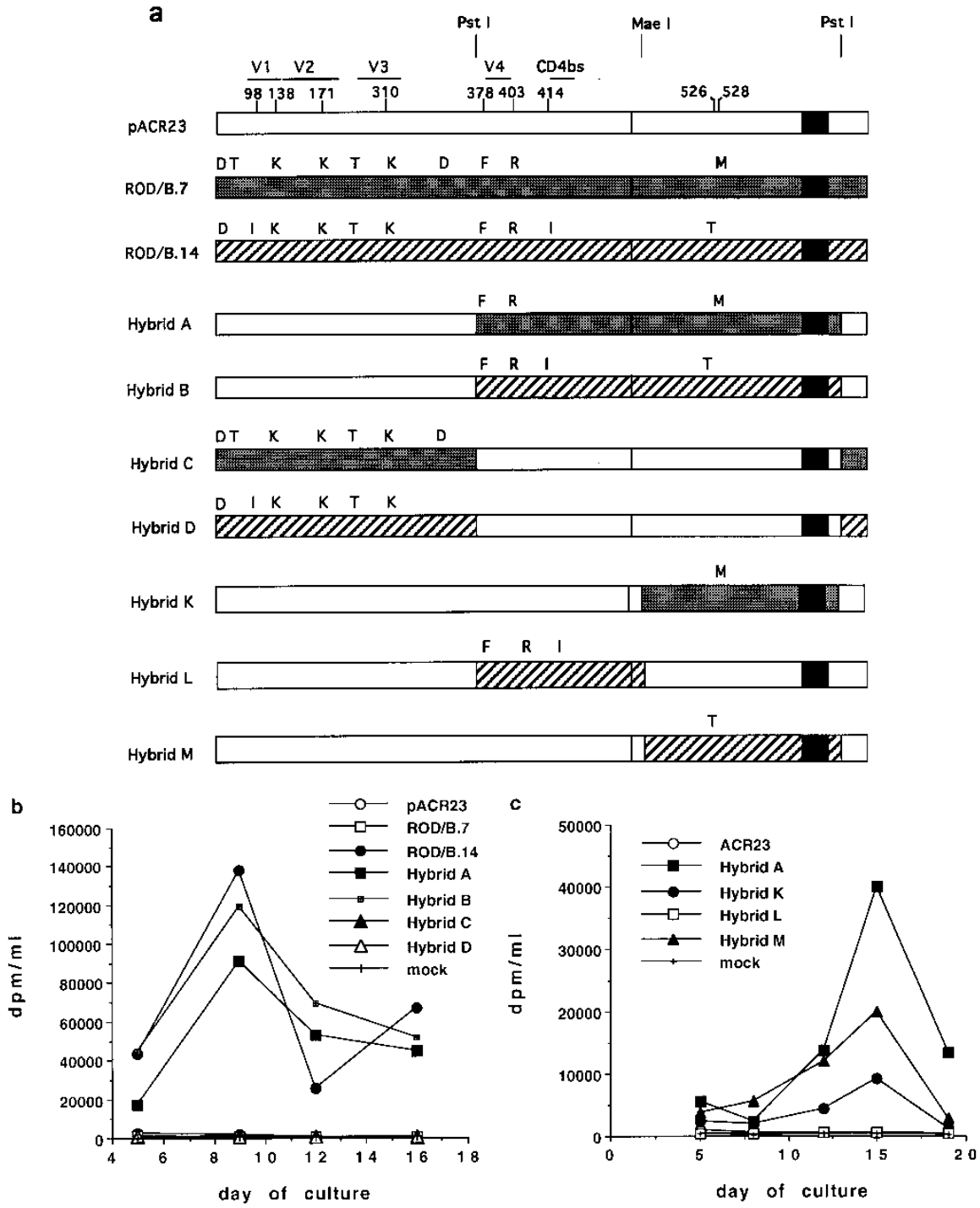


FIG. 2. Construction of intraenvelope chimeric clones and their replication in Daudi cells. (a) Molecular clones containing only the indicated subsets of mutations were constructed by exchanging restriction fragments as described in Materials and Methods. (b and c) The replication potential in Daudi cells of clones containing different chimeric envelope proteins was assayed by transfection and cocultivation as in Fig. 1. For cell-free infection of Daudi cells with viruses containing a similar set of mutations, see Fig. 3.

*Pst*I sites at positions 7323 and 8466 to construct a series of proviral clones containing chimeric envelope genes (Fig. 2a). After cocultivation of transfected RD/TE671 cells with Daudi, it emerged that only hybrids containing aa 374 to 731 from a CD4-independent envelope protein (i.e., nucleotides 7323 to 8466; hybrids A and B in Fig. 2b) were capable of replicating in Daudi cells whereas those that contained aa 1 to 373 from a CD4-independent envelope protein joined to aa 374 to 731 of the CD4-dependent ACR23 envelope protein (hybrids C and D in Fig. 2b) were incapable of replication, even in CD4-positive MOLT4 cells (Fig. 2b and data not shown). However, if supernatant was harvested from transfected RD/TE671 cells

and used for cell-free infection of Daudi cells, hybrids A and B replicated with delayed kinetics compared to the molecular clone ROD/B.14, from which hybrid B was derived (data not shown). We show below that while changes within aa 374 to 731 are required for the CD4-independent tropism, additional mutations enhance this phenotype. To further define the crucial changes within aa 374 to 731, we used a *Mae*I site at position 7692 to separate the two changes flanking the V4 loop (L378F and K403R) and in C4 (R414I) from the change in the transmembrane protein of ROD/B.14 (A526T) or ROD/B.7 (I528M). After transfection and cocultivation, hybrids M and K, containing, respectively, only the transmembrane protein mutation A526T or I528M, showed weak, sometimes inconsistent, replication in Daudi cells, whereas hybrid L, containing the changes flanking the V4 loop and in C4 (L378F, K403R, and R414I) did not produce infectious virus (Fig. 2c). However, none of these constructs, when transfected into RD/TE671 cells, gave rise to virus capable of cell-free infection (data not shown). This indicates that either of the two transmembrane protein mutations (A526T or I528M), observed initially in different molecular clones (ROD/B.14, ROD/B.17, and ROD/B.7), is a minimal requirement for CD4-independent tropism but that further changes are required to enable efficient CD4-independent infection.

Relative importance of individual mutations for the CD4-independent phenotype. To confirm these findings and to investigate the relative contributions of individual mutations to the CD4-independent phenotype in a more quantitative manner, we introduced the mutations observed in clone ROD/B.14 into the CD4-dependent clone pACR23 by site-directed mutagenesis. We constructed molecular clones containing these changes alone and in various combinations. As shown in Fig. 3a, we obtained a CD4-independent virus capable of efficient cell-free infection by combining the change in the transmembrane protein (A526T) with two changes flanking the V4 loop (L378F and K403R), positive-charge-conferring mutations in V1, V2, and V3 (E138K, E171K, and Q310K), and an additional change in V1 (T98I) (Fig. 3a). The R-I change in the linear part of the CD4 binding site (R414I), observed in ROD/B.14 and ROD/B.17, does not increase the replication potential in Daudi cells (Fig. 3a). However, if the mutation in the transmembrane protein (A526T) is not present, the virus is not capable of replicating in Daudi cells (Fig. 3a), supporting the conclusion that this change is necessary for CD4-independent tropism. Similarly, if both mutations flanking the V4 loop (L378F and K403R) are removed (Fig. 3a), the resulting virus is not capable of replicating in Daudi cells, and removal of only L378F results in a virus replicating in Daudi cells with very delayed kinetics (Fig. 3b). This suggests that the K403R mutation also contributes to the CD4-independent phenotype.

Amino acid changes increasing the positive charge of V3 or V1/2 determine the ability of laboratory-adapted HIV-1 isolates to replicate in CD4-positive T-cell lines (38). However, removal of all three positive-charge-conferring mutations in V1, V2, and V3 (E138K, E171K, and Q310K) and the change in V1 (T98I) is still compatible with replication in Daudi cells (Fig. 3b), albeit with delayed kinetics. This molecular clone contains the same mutations and has the same phenotype as hybrid B (Fig. 2). When we introduced these positive-charge-conferring mutations individually into a proviral clone already containing the changes identified as essential for the CD4-independent tropism (i.e., L378F, K403R, and A526T), as well as the nonessential mutation R414I, only the V3 loop mutation (Q310K) caused a marked acceleration in viral replication in Daudi cells (Fig. 3b) whereas the changes in V1 (E138K) and V2 (E171K) (Fig. 3c) had no significant accelerating effect.

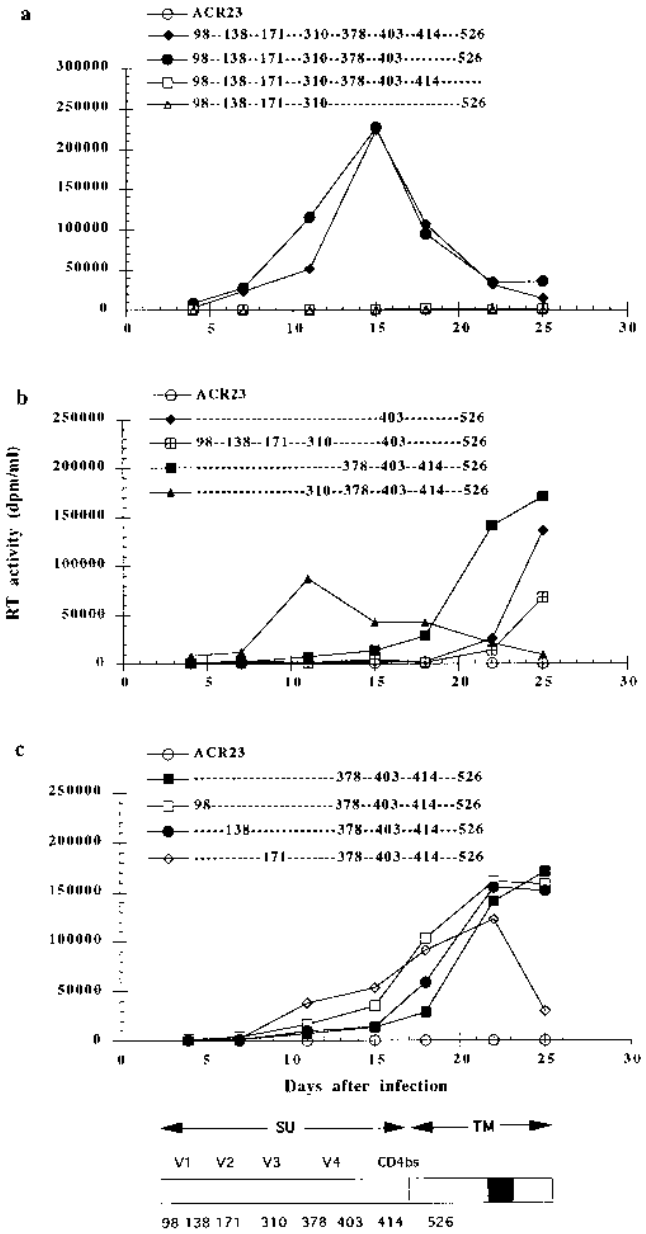


FIG. 3. Contribution of individual mutations to the CD4-independent replication in Daudi cells. Individual mutations were introduced, alone and in combination, into the CD4-dependent molecular clone pACR23, and their effect on CD4-independent replication was assayed by cell-free infection of Daudi cells, using viral stocks generated by transfection of RD/TE671 cells, as outlined in Materials and Methods. (a) Removal of either the transmembrane mutation (A526T) or the two mutations flanking the V4 loop (L378F and K403R) completely eliminates the CD4-independent tropism, whereas R414I is not required. (b) A combination of L378F, K403R, R414I, and A526T allows replication with delayed kinetics, and addition of a positive charge conferring mutation in V3 (Q310K) accelerates this phenotype. Removal of L378F markedly delays the replication kinetics, whether or not the charge-conferring mutations in the variable loops are present. (c) In contrast to the V3 loop mutation (Q310K) in panel b, similar positive-charge-conferring changes in V1 (E138K) and V2 (E171K) have no accelerating effect on a virus containing the minimal requirements for CD4-independent tropism (L378F, K403R, and A526T) and R414I, nor has a change at the base of V1 (T98I).

The simultaneous presence of several such charge-conferring mutations may, however, enhance the ability of HIV-2 variants to replicate in Daudi cells, but these mutations are not required for and cannot on their own cause CD4-independent

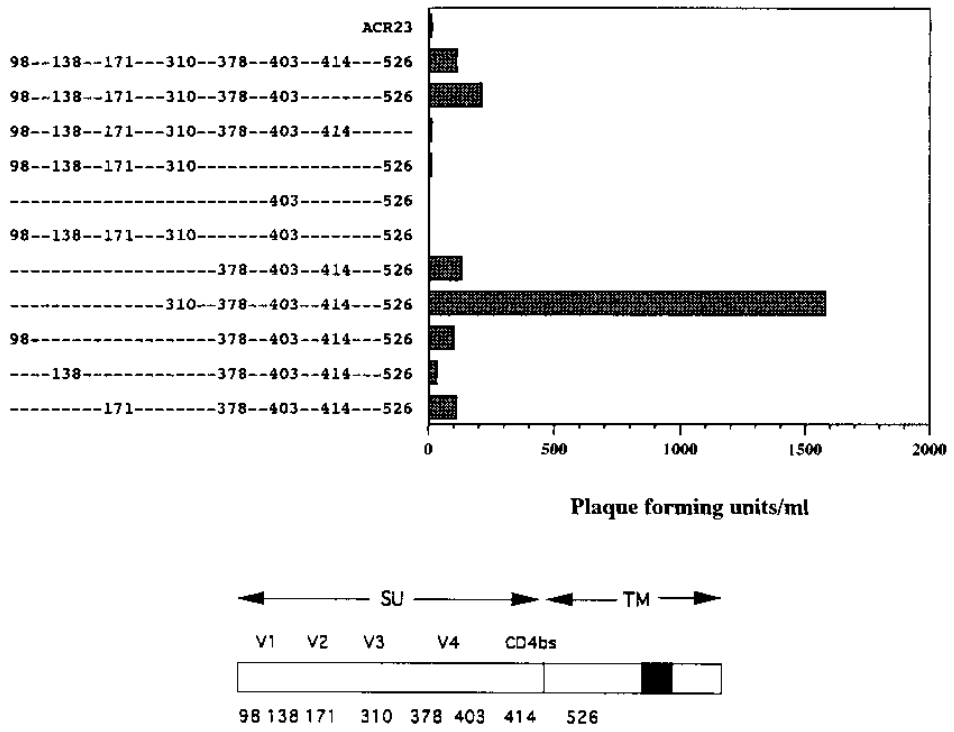


FIG. 4. Effect of individual mutations on CD4-independent infection of RD/TE671 cells. The ability of envelope proteins containing different combinations of mutations to mediate cell-free infection of RD/TE671 cells was assayed in a focus-forming assay with β-galactosidase staining to visualize infected cells (see Materials and Methods).

tropism. Likewise, the T98I mutation, found only in ROD/B.14, did not enhance the replication of the L378F, K403R, A526T, R414I virus (Fig. 3c) and is thus unlikely to play a major role in the CD4-independent phenotype.

Viruses containing similar combinations of mutations were also tested for their cell-free infectivity on RD/TE671 cells by a plaque-forming assay (Fig. 4). Although the titers, measured in focus-forming units, of these viruses were low on this cell line, the mutations identified as crucial for infection of Daudi cells were also found to be important for infection of RD/TE671 cells. Interestingly, a virus combining one single-charge-conferring change in V3 (Q310K) with the three essential changes (i.e., L378F, K403R, and A526T) and R414I had a higher cell-free titer on RD/TE671 than did a virus carrying all the mutations observed in ROD/B.14. In this system, too, the other positive-charge-conferring mutations in V1/2 (E138K and E171K) or the T98I mutation again did not enhance infectivity beyond that conferred by the three essential changes (L378F, K403R, and A526T).

Mutations determining CD4-independent tropism lower the amount of sCD4 required to trigger fusion. The envelope proteins of many laboratory-adapted HIV-2 isolates, including those restricted to infecting CD4-positive cells, can be induced, by pretreatment with sCD4, to fuse with several CD4-negative cell lines (14). We therefore investigated the effect of the mutations identified above on the ability of the corresponding envelope proteins to induce cell-cell fusion. As shown in Fig. 5 and 6, an envelope protein containing all the mutations identified in the CD4-independent molecular clone ROD/B.14 (Fig. 5, panels b; Fig. 6a) was capable of fusing with RD/TE671 cells in the absence of sCD4, whereas the CD4-dependent envelope protein from pACR23 (Fig. 5, panels a; Fig. 6a) did not show any fusion, even in the presence of 5 μg of sCD4 per ml. (The pACR23 envelope protein required sCD4 concentrations of ≥10 μg/ml to induce noticeable fusion in RD/TE671 cells.) As for cell-free infectivity for Daudi cells, removing the R414I mutation in the linear part of the CD4 binding site (Fig. 6a) did not alter this phenotype. In contrast, if only the mutation A526T in the transmembrane protein was absent from a virus carrying all the other mutations (Fig. 5, panels d; Fig. 6a), fusion was not seen in the absence of sCD4 but could be induced by 5 μg of sCD4 per ml. An envelope protein lacking only the mutations flanking the V4 loop (L378F and K403R), (as well as the nonessential R414I mutation) (Fig. 5, panels c; Fig. 6a) behaved in a similar manner. A combination of the four changes in V1 to V3, including the three positive-charge-conferring mutations E138K, E171K, and Q310K, did not induce fusion in the absence of the other changes (Fig. 6a), even at the highest sCD4 concentration tested. However, their removal from ROD/B.14, resulting in a construct containing only the changes flanking the V4 loop (L378F and K403R), the "nonessential" mutation in the linear part of the CD4bs (R414I), and the change in the transmembrane protein (A526T), showed little fusion in the absence of sCD4 but could be induced to do so by very low sCD4 concentrations (Fig. 5, panels f; Fig. 6b). Furthermore, when we introduced the changes in V1 to V3 individually into an envelope construct containing L378F, K403R, R414I, and A526T, only the V3 change (Q310K) resulted in an increased level of fusion in the absence of sCD4 (Fig. 5 panels g; Fig. 6b), whereas T98I and E171K did not (Fig. 6b). E138K (Fig. 5, panels h; Fig. 6b) reduced sCD4-induced syncytium formation. Qualitatively, at least, these results are very similar to those obtained for cell-free infectivity of RD/TE671 and Daudi cells and confirm the conclusion reached above, i.e., that the two mutations flanking the V4 loop, L378F and K403R, together with the transmem-

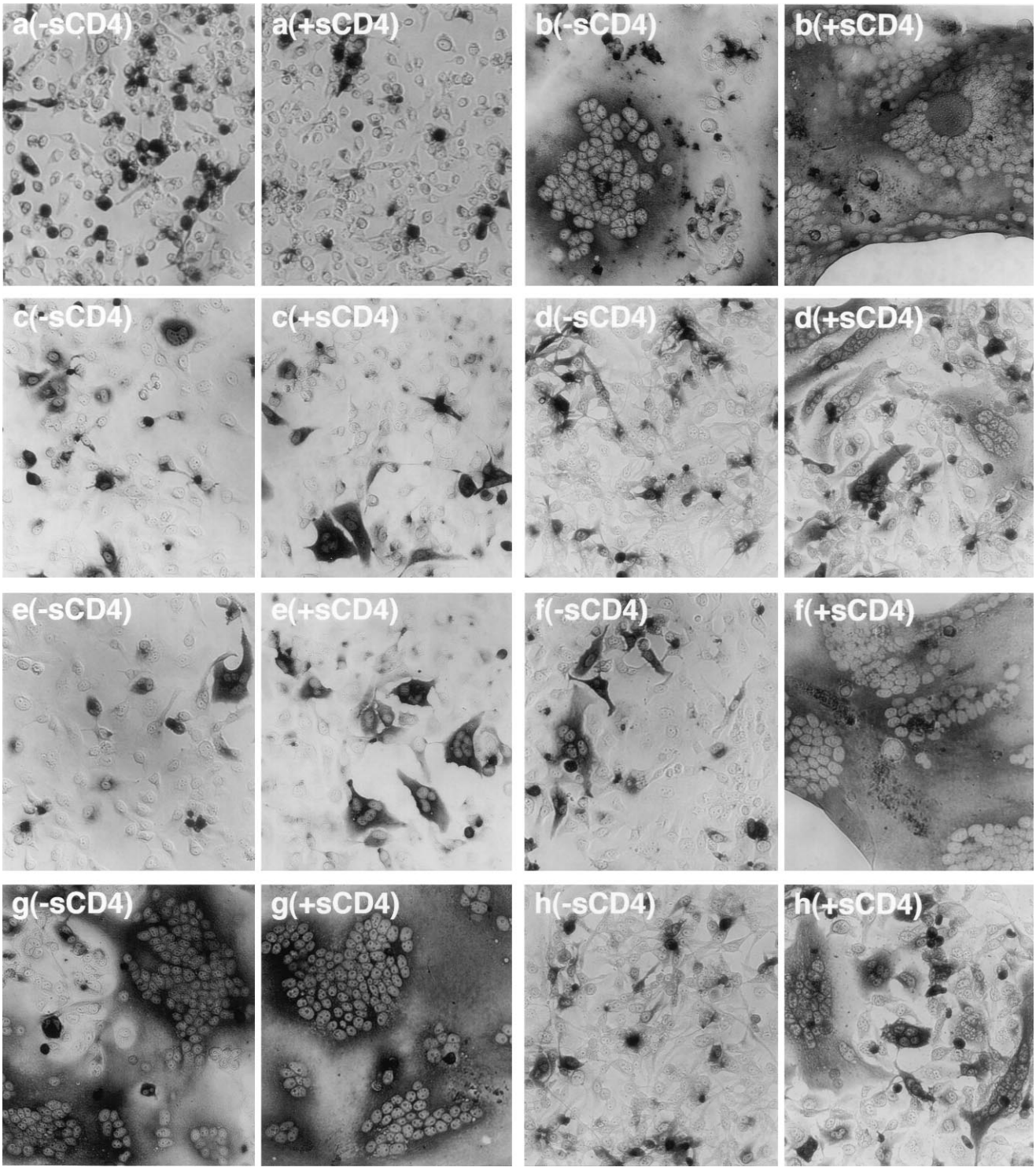


FIG. 5. Effect of individual mutations on cell-cell fusion in the presence and absence of sCD4. Molecular clones containing combinations of mutations were transfected into RD/TE671. Transfected cultures were incubated with (+sCD4) and without (−sCD4) 5 μg of sCD4 per ml for 2 days and then stained with monoclonal antibodies to the envelope protein and β-galactosidase-conjugated second antibody as described in Materials and Methods. (a) pACR; (b) molecular clone containing T98I, E138K, E171K, Q310K, L378F, K403R, R414I, and A526T; (c) molecular clone containing T98I, E138K, E171K, Q310K, and A526T; (d) molecular clone containing T98I, E138K, E171K, Q310K, L378F, and K403R; (e) molecular clone containing T98I, E138K, E171K, Q310K, K403R, and A526T; (f) molecular clone containing L378F, K403R, R414I, and A526T; (g) molecular containing Q310K, L378F, K403R, R414I, and A526T; (h) molecular clone containing E138K, L378F, K403R, R414I, and A526T.

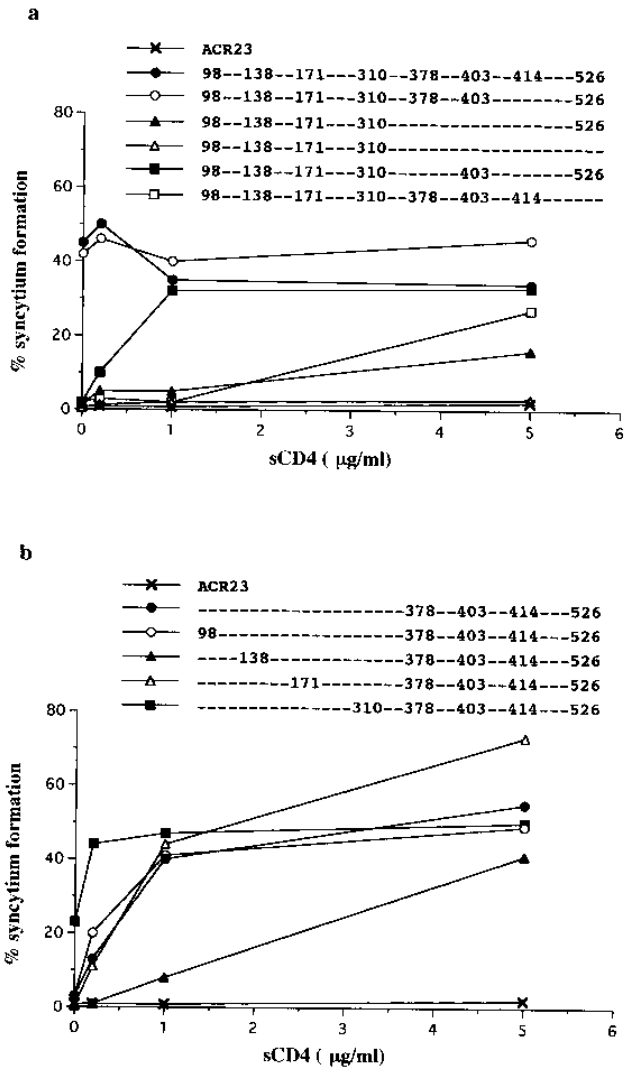


FIG. 6. Mutations mediating CD4-independent tropism reduce the amount of sCD4 required to trigger envelope-mediated fusion. Molecular clones containing different mutations were transfected into RD/TE671 cells as described in the legend to Fig. 5, and transfected cells were grown in the presence of different sCD4 concentrations for 2 days. Envelope protein-expressing cells were stained as described in the legend to Fig. 5, and syncytium formation was quantitated by calculating the percentage of envelope protein-expressing cells forming syncytia with more than three nuclei as outlined in Materials and Methods.

brane protein mutation A526T, are essential for fusion with certain CD4-negative cells, whereas the positive charge-conferring change in V3 has an enhancing effect.

In the presence of these three critical mutations (L378F, K403R, and A526T), the envelope protein induces only minimal syncytium formation without pretreatment with sCD4, but fusion can be induced by very low sCD4 concentrations (Fig. 5, panels f; Fig. 6b). This observation could suggest that these three mutations subtly alter the conformation of the envelope protein such that its threshold for subsequent conformational changes required for the fusion event is lowered.

Recombinant envelope protein from CD4-dependent and -independent viruses. Previously published results from this laboratory (14) suggested that a recombinant outer envelope protein from the CD4-dependent ROD/A showed only very little binding to RD/TE671. To investigate whether, in addition to having a reduced activation threshold, CD4-independent envelope proteins would bind better to susceptible CD4-negative cell lines, we produced recombinant envelope proteins of ROD/B.14 and pACR23. As the transmembrane protein mutation A526T is crucial for the CD4-independent phenotype and thus likely to be required for any conformational change, we expressed the extracellular part of the envelope precursor protein, gp140, in insect cells. To this end, tryptophan 654 of the envelope protein was changed to a stop codon by PCR. Recombinant soluble envelope proteins were purified from tissue culture supernatants by lectin affinity chromatography. Figure 7a shows Western blots of recombinant envelope protein preparations used in this study. We analyzed the binding of a series of monoclonal antibodies to these envelope protein preparations after they had been captured on ELISA plates coated with GNA. These studies revealed that antibodies to several different envelope regions, including C1 (monoclonal antibody 44.5j [47]), V3 (monoclonal antibody 28.8e [47]), and aa 570 to 588 in the extracellular portion of the transmembrane protein (KK41 [41]), bound nearly equivalently to the CD4-dependent and -independent recombinant envelope protein (Fig. 7b). Similarly, the CD4-independent envelope protein of ROD/B.14 had only a slightly (two- to threefold) higher affinity than did the CD4-dependent envelope protein of ACR23 to both sCD4 (half-maximal saturation at 0.8 μg/ml [16 nM] and 2.5 μg/ml [50 nM], respectively) and CD4-IgG (half-maximal saturation at 0.4 and 0.8 μg/ml, respectively) (Fig. 7c).

We then examined the binding of these recombinant envelope proteins to cell surfaces (Fig. 8). We did not observe any significant binding to Daudi (Fig. 8a) or RD/TE671 (not shown) cells with either ROD/A gp140 or ROD/B gp140. We thus conclude that any conformational differences that may exist between a CD4-dependent and a CD4-independent envelope protein do not induce an increased binding to CD4-negative cell surfaces.

However, when we compared the binding of these recombinant gp140 proteins to the surface of CD4-positive SupT1 cells, we noticed (Fig. 8c) that with increasing concentrations of ROD/A gp140, the fluorescence signal (after staining with pooled HIV-2-positive patient sera and FITC-conjugated anti-human IgG) reached a maximum level, whereas with ROD/B gp140, the signal continued to increase to much higher values. When using individual monoclonal antibodies to measure the binding of gp140 to SupT1 cells, the difference between ROD/A and ROD/B gp140 was particularly strong for monoclonal antibodies 44.5j and KK41 to, respectively, a region in C1 and aa 570 to 588 in the transmembrane protein (Fig. 8b). In contrast, antibody 28.8e to V3 gave an approximately equivalent immunofluorescence signal with ROD/A and ROD/B gp140, the small difference most probably being due to the slightly increased affinity for CD4 of the latter (see above). These observations suggest either that binding of CD4-independent gp140 to CD4 results in conformational changes which promote its secondary interaction with another cell surface component(s) or the aggregation of several gp140 molecules. An alternative explanation is that at least two regions of the envelope protein, which are equally accessible to monoclonal antibodies on both envelope proteins (by ELISA), become more accessible on the CD4-independent envelope protein after its interaction with CD4. Both interpretations would be in agreement with the conclusion reached above, i.e., that CD4-independent envelope proteins are more easily triggered by contact with sCD4 to undergo conformational changes.

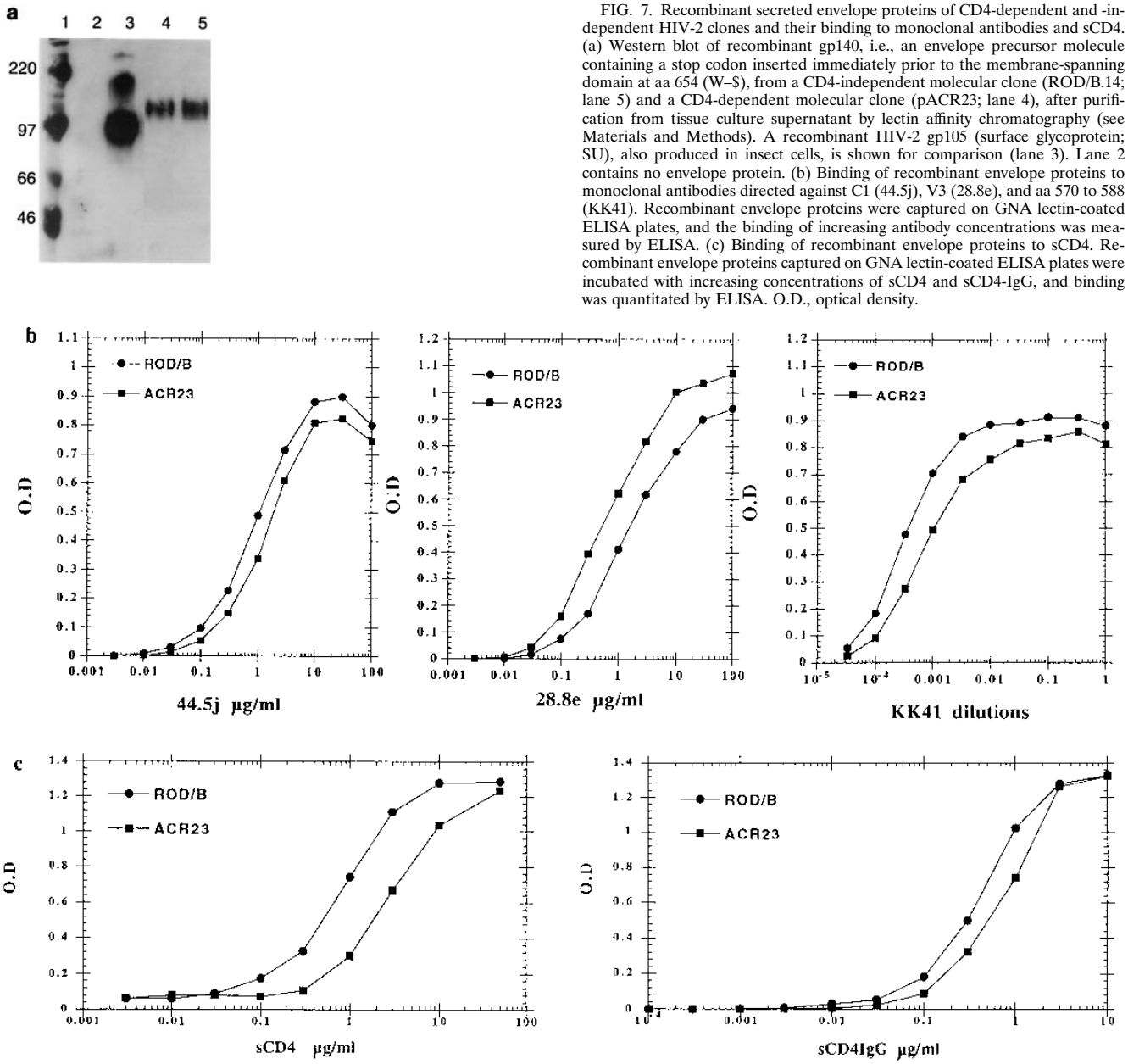


FIG. 7. Recombinant secreted envelope proteins of CD4-dependent and -independent HIV-2 clones and their binding to monoclonal antibodies and sCD4. (a) Western blot of recombinant gp140, i.e., an envelope precursor molecule containing a stop codon inserted immediately prior to the membrane-spanning domain at aa 654 (W–$), from a CD4-independent molecular clone (ROD/B.14; lane 5) and a CD4-dependent molecular clone (pACR23; lane 4), after purification from tissue culture supernatant by lectin affinity chromatography (see Materials and Methods). A recombinant HIV-2 gp105 (surface glycoprotein; SU), also produced in insect cells, is shown for comparison (lane 3). Lane 2 contains no envelope protein. (b) Binding of recombinant envelope proteins to monoclonal antibodies directed against C1 (44.5j), V3 (28.8e), and aa 570 to 588 (KK41). Recombinant envelope proteins were captured on GNA lectin-coated ELISA plates, and the binding of increasing antibody concentrations was measured by ELISA. (c) Binding of recombinant envelope proteins to sCD4. Recombinant envelope proteins captured on GNA lectin-coated ELISA plates were incubated with increasing concentrations of sCD4 and sCD4-IgG, and binding was quantitated by ELISA. O.D., optical density.

DISCUSSION

We have generated infectious molecular clones of HIV-2 exhibiting the CD4-independent tropism of the HIV-2/LAV2B phenotype (14). By comparing their sequence to that of a closely related CD4-dependent clone, pACR23, we have identified several amino acid changes which act in combination to generate the CD4-independent phenotype. We have further obtained evidence suggesting that the same mutations render the envelope protein more susceptible to conformational changes induced by sCD4. After binding of the envelope protein to cell surface CD4, these conformational changes result in an increased secondary binding to other membrane components, in an increased aggregation of individual gp140 molecules, or in an increased exposure of certain epitopes in the outer as well as the transmembrane glycoprotein.

We observed two mutations, A526T and I528M, in the same region of the transmembrane protein of two different molecular clones obtained from LAV2B-infected cells. Our results show that mutations in this region are crucial for the CD4-independent phenotype, since removal of the A526T change abrogates infectivity for Daudi or RD/TE671 cells, and either of these two mutations alone could, in some experiments, induce low-level virus replication in Daudi cells. Two changes flanking the V4 loop, L378F and K403R, are also important for the CD4-independent phenotype and, in combination with the transmembrane protein change A526T, allow replication with delayed kinetics. The positive-charge-conferring mutation in V3, Q310K, enhances this phenotype. Efficient infection of susceptible CD4-negative cells therefore requires a set of four mutations, located in different regions of the envelope protein.

Interestingly, the two mutations found in the transmembrane protein, A526T and I528M, are only 2 aa apart and are

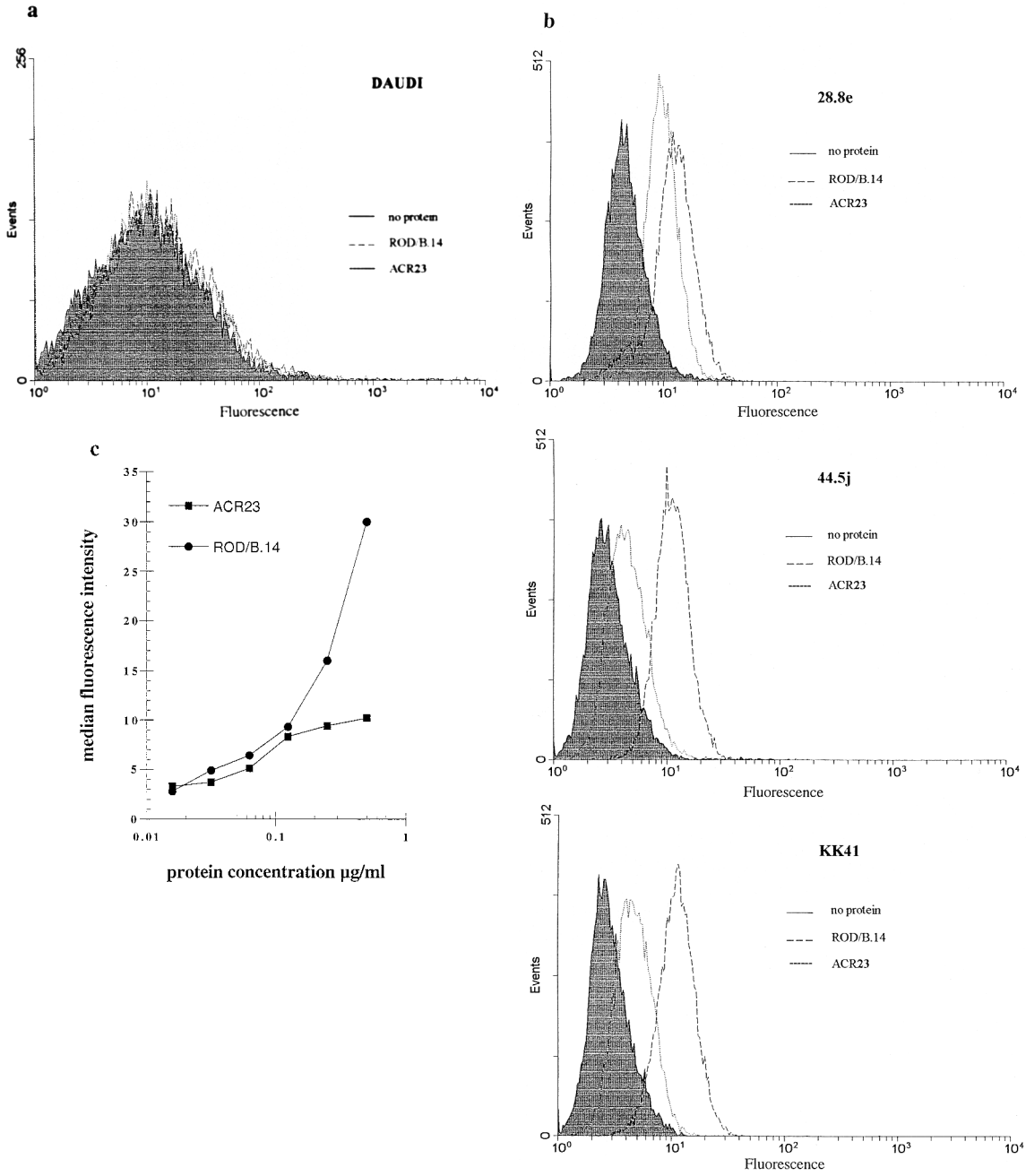


FIG. 8. Binding of recombinant envelope proteins to cell surfaces. (a) No significant binding of recombinant HIV-2 envelope proteins to the surface of Daudi cells. Daudi cells were incubated with recombinant envelope protein preparations followed by a pool of monoclonal antibodies to the HIV-2 envelope glycoprotein and quantitation by FACS analysis, as described in Materials and Methods. (b and c) The interaction of gp140 with membrane-anchored CD4 increases the binding of polyclonal anti-envelope sera and monoclonal antibodies to C1 and the transmembrane protein for the CD4-independent compared to the CD4-dependent envelope protein. (b) SupT1 cells were incubated with 0.5 μg of recombinant ROD/A or ROD/B gp140 per ml followed by either monoclonal antibody 28.8e to V3 (top panel), 44.5j to C1 (middle panel), or KK 41 to aa 570 to 588 in the TM protein (bottom panel), and binding was quantitated by flow cytometry. (c) SupT1 cells were incubated with increasing concentrations of recombinant ROD/A or ROD/B gp140, followed by an HIV-2-positive patient serum pool, and binding was quantitated by flow cytometry.

located immediately upstream of a region containing a heptad repeat which has been shown to be involved in HIV-1 entry (24, 77). A similar region in the influenza virus hemagglutinin (HA) is involved in a drastic conformational change triggered by the low endosomal pH (7). This conformational change results in the formation of an extended coiled coil which projects the fusion domain of the HA2 subunit into the endosomal membrane (5, 7). In HIV-1, this coiled-coil domain may interact with another putative α-helical domain at the carboxy-terminal end of the extracellular part of gp41 (8).

The marked increase in sensitivity to sCD4, as measured in a cell-cell fusion assay, of the triple mutant with the L378F, K403R, and A526T mutations suggests that these mutations may lower the threshold for the triggering of conformational

changes required for envelope-mediated fusion. Either A526T or I528M alone results in a moderately increased fusogenicity in the presence of low sCD4 concentrations (data not shown). Our findings therefore suggest that subtle modifications of this region of the transmembrane protein facilitate the triggering of conformational changes in this region and that this is compounded by other mutations, in particular the pair L378F and K403R, in the vicinity of the V4 loop, as well as Q310K in V3. Because of our limited knowledge of the structure of retroviral envelope glycoproteins, it is difficult to envisage how these mutations affect the triggering of conformational changes. Changes in the C4 domain have been reported previously to influence CD4-dependent tropism. In both HIV-1 and HIV-2, mutation of the Ile residue immediately preceding Lys 403 interferes with replication in CD4-positive U937 or CD4-positive T-cell lines (16, 40). Mutation of the lysine residue in HIV-1 corresponding to Lys 403 to an arginine (i.e., the mutation observed in this study) has no effect on HIV-1 replication, whereas mutation to a threonine again interferes with replication in U937 cells (16). A K411R mutation (our numbering; K429R in the cited reference) allowed efficient infection by HIV-1 of Raji cells carrying a binding-deficient CD4 mutant (11); a change in aa 415 (our numbering; aa 448 in reference 54) affected the entry of SIV_{mac}. Several observations suggest a close proximity of this part of C4 with the ascending flank of V3 (53, 78). However, our R414I change in this region was not required for the CD4-independent phenotype and did not require compensatory changes in V3. In contrast, mutation K403R, 6 aa upstream of Trp 409 (our numbering), which is thought to be a crucial component of the CD4-binding pocket (56), played a role in the CD4-independent phenotype. The fact that both L378F and K403R are required for CD4-independent tropism could suggest an interaction between the beginning of C4 and regions immediately preceding the V4 loop or between one of these and regions in TM. However, we prefer the interpretation that the effect of these mutations is indirect, since a combination of L378F, K403R, and A526T is required for the CD4-independent phenotype and since we have no evidence that mutations in one region could compensate for changes in other regions. gp120 regions identified so far as interacting with gp41 include C1 and C5 (35).

Envelope protein mutants with a decreased activation threshold have been described for influenza virus and allow the fusion process to be triggered at a higher than usual pH (19). Mutations determining this phenotype were located in both HA1 and HA2 and fell into two groups. The first group involved the interfaces between subunits of the trimeric envelope protein complex, thus weakening the interactions between subunits, whereas the second group could destabilize the conformation which holds the fusion peptide in a hydrophobic crevice. Interesting analogies therefore exist between these influenza virus mutants adapted to fuse at higher pH and CD4-independent HIV-2 strains. Whether CD4-independent HIV-2 strains arise only in vitro as a result of selection for more fusogenic viruses or whether they exist in vivo is not known. However, all our ROD/B-derived molecular clones had a premature stop codon at position 731, as did the CD4-dependent molecular clone pACR23. Premature stop codons of this kind are the result of adaptation to in vitro culture and may contribute to increased fusogenicity (37, 42, 55). As the same stop codon was also present in the CD4-dependent clone pACR23, it is not the cause of CD4-independent tropism but may contribute by increasing fusogenicity. We have not investigated this possibility. However, whereas increased fusogenicity of a HIV-2/ST variant correlates with a markedly increased affinity for CD4 (37), we have shown that the CD4-independent envelope protein has only approximately two- to threefold-higher affinity for CD4 than does the CD4-dependent envelope protein. Increased affinity for CD4 is frequently observed in HIV-1 isolates which have been adapted to T-cell lines (52), and it may reflect a more open configuration of the native envelope glycoprotein complex (3, 68), which in turn may explain their increased fusion potential (48). By measuring the exposure of several antibody epitopes, we could not detect any conformational differences between CD4-dependent and -independent envelope proteins prior to CD4 binding; however, we cannot exclude that such differences exist, since we only used a limited set of monoclonal antibodies. We show, however, that after binding of gp140 to cell surface-associated CD4, there is increased binding of polyclonal HIV-2 sera and monoclonal antibodies to two epitopes in C1 (44.5j [47]) and TM (aa 570 to 588; KK41 [41]) for the CD4-independent gp140 compared to the CD4-dependent gp140. We did not succeed in measuring precise affinity differences for the interaction between these antibodies and the envelope proteins after their binding to CD4, because of the two sequential binding steps involved in this experiment. Since we did not observe a comparative increase in binding for a monoclonal antibody to V3, this may reflect increased exposure of the C1 and TM epitope. However, an increased binding of ROD/B gp140 to the cell surface or an increased aggregation of individual gp140 molecules as a result of conformational changes induced by contact with CD4 are also possible explanations. Both interpretations are compatible with the notion that conformational changes are induced more readily by membrane-anchored CD4 in the CD4-independent recombinant envelope protein than in its CD4-dependent counterpart. We also recognize the limitations of using baculovirus-expressed recombinant soluble gp140 envelope precursor proteins, which are only inefficiently processed into the outer and transmembrane glycoproteins. However, the fact that we observed differences in antibody binding after, but not before, interaction with CD4, as well as affinities for sCD4 in the expected range (51), strongly suggests a largely correct folding of these recombinant envelope glycoproteins and their susceptibility to CD4-induced conformational changes.

Because of its reduced activation threshold a CD4-independent HIV-2 strain with a suitable complement of mutations may utilize a surface structure for entry which, unlike CD4, is not capable of, or is much less efficient in, triggering conformational changes in the envelope protein (60). We have not succeeded in demonstrating the binding of recombinant envelope glycoproteins of either tropism to Daudi or RD/TE671 cells. The interaction between the HIV-2 envelope protein and this alternative receptor may thus be of much lower affinity than the interaction with CD4. However, it is also possible that this interaction involves carbohydrate-carbohydrate or protein-carbohydrate recognition, and a c-type membrane lectin binding to HIV-1 gp120 has previously been reported (17). In this case, our recombinant insect cell-derived envelope proteins, which contain mainly high-mannose carbohydrate modifications, may not be the ideal reagents to identify such a surface structure. We have recently reported that deglycosylation of several cell types increases their susceptibility to CD4-independent infection by ROD/B, suggesting that a membrane glycoprotein may be involved in CD4-independent entry or that deglycosylation improves the access of the virus to a membrane molecule involved in virus entry (72).

The glycolipid galactosyl ceramide mediates CD4-independent infection of some neural cell lines by certain HIV-1 isolates (32), and the V3 loop, as well as a region encompassing

V4 to V5, has been shown to confer this phenotype (33). HIV-1 variants capable of infecting in this CD4-independent manner bind to sulfogalactosyl ceramide, whereas those which are CD4 dependent do not (33). This is in contrast to the findings reported here for CD4-independent HIV-2. The V3 mutation, the mutations flanking V4, and a combination of these were all insufficient for CD4-independent entry, and we have no evidence that CD4-independent envelope proteins bind better to CD4-negative cell surfaces than do CD4-independent envelope proteins (but they may bind better to CD4-positive cell surfaces). CXCR4, the coreceptor for T-cell-line-tropic HIV-1 isolates (26), is also able to mediate the entry of some HIV-2 isolates that have acquired the ability to infect CD4-negative cell lines (36a, 58a). Whether CD4-independent envelope proteins interact primarily with CXCR4 or bind initially to another cell surface component is unknown.

In summary, we have provided evidence that the CD4-independent tropism of HIV-2 strains may depend on an envelope protein with an increased propensity to undergo the conformational changes required for the initiation of the fusion process. We have also identified amino acid residues in the envelope glycoprotein which are critical for this process. We suggest that CD4-independent HIV-2 variants may be conceptually similar to influenza virus variants capable of fusing at a higher pH. Two reports (64, 70) suggest that enhancement of infectivity by pretreatment with sCD4 also occurs in primary HIV-1 isolates. Our identification of amino acid residues in the HIV-2 envelope protein determining the increased sensitivity to sCD4 may therefore contribute to the understanding of this phenomenon and also of the antibody-mediated enhancement of infectivity, to which it is related (64, 70).

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