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Conformational Changes Induced in the Envelope Glycoproteins of the Human and Simian Immunodeficiency Viruses by Soluble Receptor Binding

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We have investigated the molecular basis of biological differences observed among cell line-adapted isolates of the human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) and the simian immunodeficiency virus (SIV) in response to receptor binding by using a soluble form of CD4 (sCD4) as a receptor mimic. We find that sCD4 binds to the envelope glycoproteins of all of the HIV-1 isolates tested with affinities within a threefold range, whereas those of the HIV-2 and SIV isolates have relative affinities for sCD4 two- to eightfold lower than those of HIV-1. Treatment of infected cells with sCD4 induced the dissociation of gp120 from gp41 and increased the exposure of a cryptic gp41 epitope on all of the HIV-1 isolates. By contrast, neither dissociation of the outer envelope glycoprotein nor increased exposure of the transmembrane glycoprotein was observed when sCD4 bound to HIV-2- or SIV-infected cells. Moreover, immunoprecipitation with sCD4 resulted in the coprecipitation of the surface and transmembrane glycoproteins from virions of the HIV-2 and SIV isolates, whereas the surface envelope glycoprotein alone was precipitated from HIV-1. However, treatment of HIV-1-, HIV-2-, and SIV-infected cells with sCD4 did result in an increase in exposure of their V2 and V3 loops, as detected by enhanced antibody reactivity. This demonstrates that receptor binding to the outer envelope glycoprotein induces certain conformational changes which are common to all of these viruses and others which are restricted to cell line-passaged isolates of HIV-1.

The first step in the infection of $CD4^+$ cells by the human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) and the simian immunodeficiency virus (SIV) is the high-affinity binding of CD4 to the surface envelope (SU) glycoproteins of these viruses (34, 38, 40, 45, 56). Events subsequent to this that result in the entry of the virus capsid into the cell cytoplasm are poorly defined. Recently, however, a number of studies using soluble CD4 (sCD4) to mimic cell-anchored CD4 have demonstrated that receptor binding induces conformational changes in the envelope glycoproteins of cell line-adapted isolates of HIV-1, events which may be precursors to membrane fusion. Such sCD4-induced conformational changes have been detected on virions and include the dissociation of the SU glycoprotein, gp120, from the transmembrane (TM) glycoprotein, gp41 (21, 30, 41–45, 47), the increased exposure of the gp120/V3 loop demonstrated by greater cleavage of this loop by an exogenous proteinase (57), and stronger staining of gp41 with a monoclonal antibody (MAb) (21). Similar results have been obtained with HIV-infected or transfected, *env*-expressing cells (7, 14, 15, 47, 57). Such molecular rearrangements are thought to be analogous to those occurring in certain other enveloped viruses, most notably influenza virus, in which conformational changes triggered by a pH change result in the exposure of the hydrophobic N-terminal fusion peptide of the TM glycoprotein, which induces fusion of the virus and cell membranes (8, 18, 32, 33, 42, 61, 67). Since HIV fusion is pH independent (8, 18, 33, 42), we (42, 46, 55) and others (1) have therefore proposed that entry of HIV into $CD4^+$ cells may be initiated by receptor-mediated activation of membrane fusion.

Although HIV-1, HIV-2, and SIV use CD4 as their primary receptor (2, 10, 56), there is evidence that binding and subsequent events may differ among these viruses. It has been reported that virions of HIV-2 and SIV are relatively resistant to neutralization by sCD4, compared with HIV-1 (11, 37, 60); indeed, infection and syncytium formation by HIV-2 and SIV can be enhanced by subinhibitory doses of sCD4 (2, 11, 60, 66) and their dependence on cellular CD4 for entry can be bypassed (11). Potentially relevant to this sCD4 neutralization resistance are reports that soluble forms of the SU glycoprotein of some isolates of HIV-2 have affinities for CD4 which are lower than those for HIV-1 (6, 26, 27, 40, 48, 49). Recent studies comparing the interaction of sCD4 with soluble or membrane-anchored glycoprotein molecules concluded that the binding affinity was influenced by the oligomeric presentation of the envelope glycoproteins (44, 49). Affinity measurements made with recombinant, soluble monomeric glycoproteins should therefore be treated with some caution.

In the present study, we have analyzed interactions taking place between soluble receptor molecules and functional virus envelope glycoprotein oligomers in an attempt to define some of the differences among the HIV-1, HIV-2, and SIV envelope glycoproteins which give rise to the divergent biological properties mentioned above and to increase our understanding of the virus-cell fusion reaction. We confirm the relative resistance of HIV-2 and SIV to inhibition of virus glycoprotein-induced membrane fusion by sCD4 and find that all of the cell line-adapted immunodeficiency viruses that we have tested bind sCD4 half-maximally within an eightfold sCD4 concentration range. Unlike HIV-1, the binding of sCD4 to the SU glycoproteins of HIV-2 and SIV induces neither dissociation of the envelope glycoprotein subunits nor increased exposure of

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TM glycoprotein epitopes. We demonstrate that this is a consequence of stronger bonding between the glycoprotein subunits of HIV-2 and SIV. As with HIV-1, however, sCD4 binding results in increased exposure of the V2 and V3 loops on HIV-2- and SIV-infected cells. We propose that (i) the complete dissociation of the outer envelope glycoprotein from the TM glycoprotein is not obligatory for CD4-activated HIV fusion to proceed, provided that cell-anchored CD4 and sCD4 behave equivalently in this system; and (ii) the lack of sCD4-induced envelope glycoprotein dissociation may explain at least in part the resistance of HIV-2 and SIV to sCD4 neutralization and inhibition of syncytium formation.

MATERIALS AND METHODS

Viruses. The virus isolates used in this study were obtained from the following sources: HIV-1 isolates IIIB, RF, and MN from R. C. Gallo, National Cancer Institute, Bethesda, Md.; HIV-1 isolate SF-2 from J. Levy, University of California at San Francisco, San Francisco; HIV-1 isolate CBL4 from R. A. Weiss, Chester Beatty Laboratories, London, United Kingdom; HIV-1 isolate NDK from J.-C. Chermann, Unité de Recherches sur les Rétrovirus et Maladies Associées, Marseille, France; HIV-2 isolate LAV-2_{ROD} from L. Montagnier, Pasteur Institute, Paris, France; HIV-2 isolate CBL20 from P. Clapham and R. A. Weiss, Chester Beatty Laboratories; and $SIV_{mac}251$ from the Medical Research Council AIDS Directed Programme, London, United Kingdom. All viruses were grown to high titers by the following protocol. H9 cells (10^7) were infected with each virus at a multiplicity of infection of approximately 0.001; then, when envelope expression was maximal at the surfaces of the infected cells (see below for measurement of envelope expression), the cells were pelleted, and the pellets were resuspended in growth medium (GM [RPMI 1640 supplemented with 10% fetal calf serum]) at a concentration of 10^7 cells per ml. After overnight culture, the cells were vortexed vigorously, and the supernatant was harvested, clarified by centrifugation at 1,600 × *g* for 20 min and filtration through a 0.45-μm-pore-size filter, and then aliquotted and stored at −80°C. The stocks were titrated by limiting dilution onto c8166 cells, and the endpoints were determined by evaluation of syncytium formation and intracellular p24 expression.

Culture and infection of cells and measurement of envelope expression. H9 cells (10^7) grown in GM (GIBCO Laboratories, Grand Island, N.Y.) were infected with different HIV or SIV isolates at a multiplicity of infection of 0.01 by the addition of an appropriately diluted virus stock to the cells, resulting in a final volume of 10 ml. After overnight incubation at 37°C, the cells were washed and resuspended in 50 ml of GM. The cultures were monitored for syncytium formation and tested for expression of HIV envelope and cell surface CD4 at various times after infection. CD4 expression was undetectable by day 5 on cells infected with all viruses except for SF-2, CBL20, and $SIV_{mac}251$, which lost expression on day 7. Maximum envelope expression, detected by staining the cells with biotinylated sCD4 and then with streptavidin-coupled phycoerythrin (Immunotech, Marseille-Luminy, France) and analyzed by flow cytometry (see below for details), was found to be between days 5 and 14 for HIV-1 isolates, and days 7 and 21 for HIV-2 and SIV isolates. Therefore, all of the experiments described were performed between days 7 and 14.

Inhibition of syncytium formation by sCD4 and CD4 MAb. To measure sCD4 inhibition of syncytium formation, threefold dilutions of sCD4 (from 300 to 0.01 μg/ml) were made in volumes of 50 μl of GM in 96-well flat-bottom microtiter plates. A 50-μl volume of GM containing 5×10^4 HIV- or SIV-infected cells was added to each well, and the plates were incubated for 2 h at 37°C. Fifty microliters of GM containing 1.5×10^5 c8166 cells was added to each well, and the plates were incubated overnight. Syncytium formation was scored on the following day as previously described (22). Inhibition of syncytia by CD4 MAb Q4120 was carried out as previously described (22).

Measurement of binding of sCD4 to virus-infected cells. Assays to measure binding of sCD4 to virus-infected cells were performed as previously described (57). Briefly, virus-infected H9 cells strongly expressing viral envelope glycoprotein were incubated with various concentrations of sCD4 for 2 h at 37°C or on ice. After washing, cell-bound sCD4 (kindly donated by R. Sweet, Smith, Kline, and Beecham, Philadelphia, Pa. [13]) was detected by staining the cells with 10 μg of MAb L120 specific for domain 4 of CD4 per ml (22) and then with 1/200 anti-mouse phycoerythrin (Immunotech). After a further wash, the cells were fixed overnight in phosphate-buffered saline (PBS)–2% formaldehyde and then analyzed by flow cytometric analysis on a Becton Dickinson FACScan (BDIF, San Jose, Calif.). All MAb staining and washing steps were done on ice in prechilled PBS–1% fetal calf serum–0.02% sodium azide. When the results are expressed as the mean fluorescence intensity, the background staining (second layer only) has been subtracted.

Detection of gp41 exposure in sCD4-treated, virus-infected cells. Virus-infected cells treated with sCD4 as described above were stained with MAbs to the TM glycoprotein and were analyzed as described previously (57). The antibodies used were as follows: 50-69, a human MAb which reacts with a peptide synthesized from amino acid residues 579 to 613 (the immunodominant, conserved region of gp41) and containing a disulfide link between the two intrachain cysteines (19, 68); KK20 and KK53, mouse MAbs which bind to a peptide from residues 594 to 616 of $SIV_{mac}251$ corresponding to the immunodominant, conserved region which cross-react with the homologous region on HIV-2 (28, 29); KK7, a mouse MAb which is reactive with an unmapped region distinct from the immunodominant domain of the $SIV_{mac}251$ TM envelope glycoprotein and which cross-reacts with HIV-2; 36C, a mouse MAb derived from immunization with recombinant HIV-2 gp140 (Transgene, Strasbourg, France); and 36D, a mouse MAb derived from immunization with inactivated HIV-2 virions. Both of the last two MAbs react with a peptide spanning residues 578 to 603 of the HIV-2 TM glycoprotein sequence and cross-react with $SIV_{mac}251$ by Western blotting (63a). The exposure of the epitope to which MAb 50-69 binds is increased by sCD4 binding to gp120 on HIV-1-infected cells (57). After 2 h of incubation of the HIV-infected cells with sCD4, the supernatants were collected and centrifuged through 0.45-μm-pore-size Spin-X filters (Costar, Cambridge, Mass.) for assaying soluble gp120, and the cells were washed and stained with a saturating concentration of the relevant anti-TM glycoprotein MAb for 30 min. After a further wash, phycoerythrin-conjugated anti-mouse or anti-human antibody (Immunotech) was added, and the cells were again incubated for 30 min. After a final wash, the cells were fixed and analyzed by flow cytometry as described above and elsewhere (57).

Modulation of SU glycoprotein epitopes by sCD4 binding. H9 cells infected as described above were stained by a previously published method (57) by using the following murine MAbs: 9284, a neutralizing MAb which reacts with an epitope contained within residues 302 to 314 of the V3 loop of the IIIB isolate (Dupont, Wilmington, Del.); G3-4, a neutralizing MAb binding to an epitope contained within the V2 loop of IIIB

gp120 (23); KK46 (28, 29), which is reactive with a region of the $SIV_{mac}251$ V3 loop contained within residues 311 to 343 (kindly donated by K. Kent); and KK13 (28, 29) raised against $SIV_{mac}251$, which reacts with the V2 loop of $SIV_{mac}251$ and cross-reacts with the homologous region of $LAV\text{-}2_{ROD}$ (28). HIV-2 MAb 125-F was raised against purified $LAV\text{-}2_{ROD}$ virions and was mapped to the peptide sequence 322 to 332, corresponding to a region of the V3 loop.

Detection of soluble HIV-1, HIV-2, and SIV envelope glycoproteins by ELISA. We have used enzyme-linked immunosorbent assay (ELISA) systems described previously (40, 47) to detect the soluble SU glycoproteins of HIV-1, HIV-2, and SIV in the supernatant following the binding of sCD4 to the surfaces of infected cells. Modifications of this method used to detect HIV-2 soluble SU glycoprotein were as follows. HIV-1, HIV-2, and SIV SU glycoproteins were detected by capture from 1% Nonidet P-40 (NP-40)-inactivated culture supernatants onto a solid phase via antibodies D7324, D7335, or D7369 (Aalto Bioreagents Ltd., Dublin, Ireland) to their respective carboxy termini (40). Bound SU glycoprotein was detected with the appropriate human HIV-1- or HIV-2-positive serum or with a rhesus SIV_{mac}-positive serum sample (kindly supplied by J. Allan, Southwest Foundation, San Antonio, Tex.) and then with a goat anti-human alkaline phosphatase conjugate. Because we found that the reactivities of the SU glycoproteins of all HIV-2 isolates and some HIV-1 isolates were increased severalfold for their respective sera in the presence of sCD4, we added a saturating concentration (1 μg/ml) of sCD4 to all samples before assaying. This eliminated variation in the ELISA signal due to experimental manipulation of the sCD4 concentration in the supernatant. The increased reactivity of HIV-2 gp120 with HIV-2-positive sera in the presence of sCD4 (40a) probably reflects the increased exposure of SU glycoprotein epitopes after sCD4 binding, such as the V2 and V3 loops (see Results). HIV-1 assays were calibrated with CHO gp120 from the HIV-1 IIIB isolate. HIV-2 and SIV assays were calibrated against curves derived from lysates of HIV-2- or SIV-infected cells. We did this because neither HIV-2 nor SIV SU glycoprotein expressed in a mammalian system is available and because HIV-2 gp105 from baculovirus (American Biotechnology, Cambridge, Mass.) reacts poorly with human HIV-2-positive sera.

Immunoprecipitation of the HIV envelope glycoproteins with sCD4. H9 cells infected with HIV-1 isolates IIIB, RF, and MN, HIV-2 isolates $LAV\text{-}2_{ROD}$ and CBL20, and $SIV_{mac}251$ were metabolically labelled on day 10 after infection, during the period of peak virus production. Approximately 1.6×10^7 infected cells (2×10^7 cells per ml) were suspended in methionine- and cysteine-free medium containing 100 μCi of [^{35}S]cysteine and 100 μCi of [^{35}S]methionine per ml. After 4 h, methionine- and cysteine free-medium containing 5% dialyzed fetal calf serum was added, and the cells were cultured for an additional 14 h at a concentration of 4×10^6 cells per ml. The labelled virions were then harvested by pelleting the cells at $1{,}500 \times g$ for 15 min, and the supernatants were subsequently concentrated approximately 10-fold with Centriprep concentrators (Amicon Corp., Beverly, Mass.) with a 30,000 cutoff. The virions were lysed by adding 20% (vol/vol) 5× lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2.5% NP-40 [pH 7.3]) containing the protease inhibitors phenylmethylsulfonyl fluoride (500 μg/ml), leupeptin (25 μg/ml), and aprotinin (10 μg/ml). The resulting samples were kept on ice for 30 min and then precipitated with 5 μg of biotinylated sCD4 and Immunopure-immobilized streptavidin (Pierce, Oud Beijerland, The Netherlands). Pelleted beads were washed four times with 1× lysis buffer and boiled for 3 min in reducing sample buffer. The samples were then divided in half and electrophoresed on two sodium dodecyl sulfate (SDS)-10% polyacrylamide gels. One gel was dried on filter paper and autoradiographed at −70°C. The other gel was analyzed for TM glycoproteins by Western blotting. Briefly, the gel was transferred to an Immobilon membrane (Millipore). The membrane was blocked with PBS–5% nonfat dry milk and then incubated for 2 h at room temperature either with the mouse MAb KK20 alone, which reacts with the immunodominant region of the TM glycoproteins of HIV-2 and SIV, or with a mixture of KK20 and KK12, which cross-reacts with the N termini of the HIV-2 and SIV SU glycoproteins (29). The membranes were subsequently washed extensively in PBS–0.1% Tween 20, then incubated for 2 h at room temperature with sheep anti-mouse immunoglobulin antibody conjugated to peroxidase, and washed in PBS–0.1% Tween 20, and the blots were developed with enhanced chemiluminescence immunodetection (Amersham France SA, Les Ulis, France).

RESULTS

Efficient inhibition of HIV-1 but not HIV-2 and SIV syncytium formation by sCD4. Our initial aim was to confirm earlier reports that HIV-2 and SIV are relatively resistant to sCD4 inhibition of syncytium formation in comparison with HIV-1. The efficiency of syncytium formation is influenced by the level of virus envelope expression at the cell surface, a factor which had not been considered in previous studies. Therefore, we measured gp120 expression on the cells infected with different isolates by indirect immunofluorescence staining with sCD4 and then by flow cytometry and used these cells in syncytium assays only when all expressed similar levels of gp120 (see Fig. 1 as an example of expression levels). The infected cells were preincubated at 37°C with a range of sCD4 concentrations before addition of $CD4^+$ target cells; if SU glycoprotein dissociation is a major component of inhibition of HIV fusion, then effective inhibition of syncytia should be achieved, since this treatment should remove a substantial proportion of SU glycoprotein sensitive to sCD4-induced shedding. All HIV-1-induced syncytia were abolished with sCD4 concentrations within a ninefold range (25 to 220 nM [Table 1]). By contrast, HIV-2- and SIV-induced syncytia were not affected by doses of sCD4 up to 2 μM, although this is 10 to 30 times the concentration required to saturate the SU glycoprotein binding sites on the surfaces of the infected cells (Fig. 1). By contrast with the results obtained with sCD4, syncytia induced by all viruses tested were completely inhibited by a saturating concentration of 5 μg of MAb Q4120 (22) per ml, which binds to an epitope on CD4 domain 1 and effectively competes with gp120 for CD4 binding (data not shown).

Binding of sCD4 to HIV-1-, HIV-2-, and SIV-infected cells. Since it has been proposed that the relative levels of resistance of HIV-2 and SIV to sCD4 neutralization might be a consequence of an SU glycoprotein affinity for sCD4 that is lower than that for HIV-1 (12), we measured sCD4 binding to HIV- and SIV-infected cells by immunofluorescence staining. This approach has the advantage that the soluble receptor molecules interact with natively folded glycoprotein molecules in their functional, oligomeric form. To detect the sCD4, we used a MAb to an epitope on CD4 domain 4 which is not affected by the gp120-CD4 interaction and then used flow cytometric analysis. Representative results for three of the six HIV-1 isolates tested are shown in Fig. 1; sCD4 binding is similar for all three. At 37°C, bound sCD4 was detectable at concentrations between 1.5 and 4.6 ng/ml (0.03 to 0.09 nM), and

TABLE 1. Interaction of soluble CD4 with HIV-1, HIV-2, and SIV envelope glycoproteins

Virus isolate	Syncytium inhibition (nM)[a]	sCD4 concentration (nM) required for the following half-maximal events:			gp120 dissociation index[b]
		sCD4 binding[c]	gp120 dissociation[d]	gp41 exposure[e]	
HIV-1					
MN	25	0.72	1.1	1.6	1.96
IIIB	25	1.24	2.3	2.0	2.75
SF-2	25	2.44	2.8	6.8	2.35
RF	74	1.40	2.0	3.6	2.22
CBL4	74	0.98	2.0	1.9	0.98
NDK	220	1.04	ND[f]	1.6	0.59
HIV-2					
CBL20	>2,000	5.50	>2,000	>2,000	0
$LAV\text{-}2_{ROD}$	>2,000	5.70	>2,000	>2,000	0
SIV					
$SIV_{mac}251$	2,000	5.00	>2,000	>2,000	0

[a] The concentration of sCD4 required to completely abolish syncytium formation in an overnight cocultivation assay.
[b] Values for the maximal concentrations of soluble gp120 detected in the supernatants of infected cells divided by the maximum signals obtained by sCD4 staining at 4°C to normalize results for total cell surface SU glycoprotein concentrations.
[c] Half-maximal binding constants of sCD4 for HIV- or SIV-infected cells measured by flow cytometric analysis at 37°C (means of at least three independent experiments).
[d] The concentrations of sCD4 required to induce half-maximal SU glycoprotein dissociation from HIV- or SIV-infected cells at 37°C, measured in the supernatants of the cells by gp120 ELISA.
[e] The concentrations of sCD4 required to induce half-maximal TM glycoprotein exposure from HIV- or SIV-infected cells at 37°C, as measured by gp41 MAb binding to the cells and flow cytometric analysis.
[f] ND, unable to calculate half-maximal gp120 release from available data, since saturation was not achieved.

maximum values were obtained between 0.4 and 3.3 μg/ml (8 to 66 nM). At 4°C, the binding affinity was apparently reduced; however, saturation staining was higher than at 37°C, suggesting that at the higher temperature there is loss of gp120 by dissociation. Since there is little or no gp120 dissociation from cell line-adapted HIV-1 virions or infected cells below about 20°C (14, 31, 43, 45, 47, 57) (see Fig. 2), saturation of sites at 4°C results in a higher signal than at 37°C. The lower limit for detection of sCD4 bound to HIV-2- and SIV-infected cells at 37°C was between 4.6 and 14 ng/ml (0.09 to 0.28 nM), and maximum binding occurred between 3.3 and 10 μg/ml (66 to 200 nM). Unlike with the HIV-1 isolates, there was minimal truncation of the sCD4-binding curve at 37°C, resulting in similar saturation values at 37 and 4°C. One interpretation of such data is that little sCD4 is lost from the cell surface of HIV-2- and SIV-infected cells by dissociation of the sCD4-SU glycoprotein complex at either temperature.

To estimate the relative affinities of the SU glycoproteins of the different viruses, half-maximal binding values at 37°C were calculated for each isolate (Table 1). To minimize the effect of gp120 shedding on the sCD4 binding curves, saturation binding values were derived from infected cells stained with various concentrations of sCD4 at 4°C. Despite this, these values may be somewhat lower than the actual ones because of low-level SU dissociation at 4°C (see Fig. 2), and thus they should be taken as estimates only. Nevertheless, the results for HIV isolates IIIB and $LAV\text{-}2_{ROD}$ are similar to a number of those published previously (5, 6, 14, 15, 26, 27, 34, 49, 63). We found about a fivefold difference in half-maximal binding of sCD4 to IIIB and $LAV\text{-}2_{ROD}$ SU glycoproteins and a maximum difference of only eightfold between the HIV-1 isolate with the highest affinity (MN) and $LAV\text{-}2_{ROD}$.

sCD4-induced dissociation of the envelope glycoprotein subunits. Since it seemed unlikely that the relatively modest differences that we observed among the affinities of the HIV-1, HIV-2, and SIV envelope glycoproteins would be directly responsible for the substantial differences in susceptibility to sCD4 inhibition of fusion, we studied another factor which might influence this: sCD4-induced SU glycoprotein shedding. The sCD4-catalyzed dissociation of gp120 from gp41 on the surfaces of HIV-1 virions and infected cells is well established (7, 14, 15, 21, 30, 31, 41–45, 47, 57). Since the binding curves of sCD4 to HIV-2- and SIV-infected cells at 37°C suggested that there was little SU glycoprotein lost by comparison with HIV-1, we decided to measure directly the concentration of soluble SU glycoprotein-sCD4 complex released into the supernatant from the infected cells as a function of sCD4 concentration. ELISAs for detection of soluble HIV-2 and SIV SU glycoprotein were calibrated against lysates of cells infected with these viruses and were sensitive enough to detect low concentrations of these molecules. As shown in Fig. 2A, supernatants from cells infected with HIV-1 isolates IIIB, SF-2, CBL4, and NDK all showed increases in soluble gp120 after sCD4 treatment at 37°C, although the concentration of sCD4 required to induce a signal varied among isolates. In this respect, the two American isolates IIIB and SF-2 released detectable gp120 at 14 ng of sCD4 per ml (0.3 nM), whereas the African isolates CBL4 and NDK required 0.37 μg/ml (7.4 nM) and 3 μg/ml (60 nM), respectively. By contrast, neither the HIV-2 nor the SIV isolates showed any increase in soluble SU glycoprotein following sCD4 treatment at 37°C, indicating that sCD4 is unable to induce the dissociation of the envelope glycoprotein subunits in these viruses. These data are summarized in Table 1, in which the concentrations of sCD4 required to dissociate 50% of the gp120 and the maximal gp120 dissociation (normalized against the levels of envelope glycoprotein on the infected cells) are listed. It is interesting to note that the HIV-1 isolates which are relatively less susceptible to sCD4-induced gp120 dissociation (for example, CBL20 and NDK) require more sCD4 to block syncytium formation than the others (Table 1, columns 1 and 5), indicating a potential relationship between gp120 shedding and inhibition of HIV-1

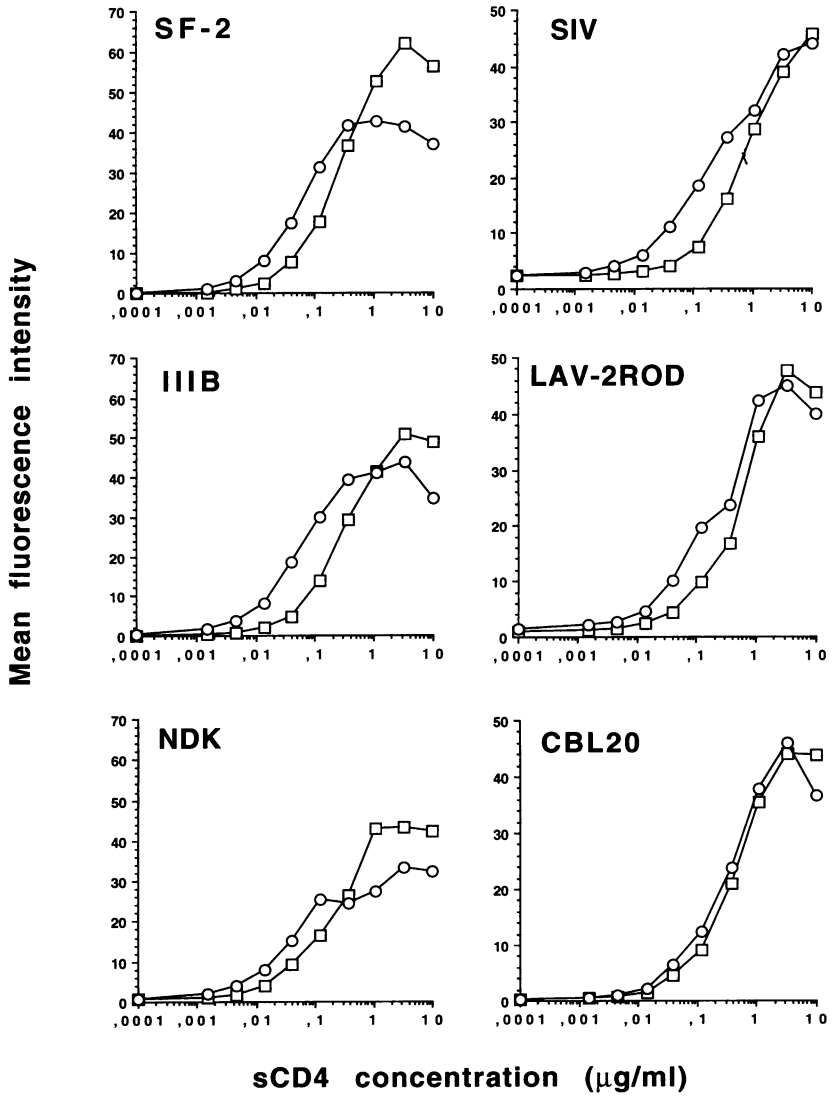

FIG. 1. Binding of sCD4 to virus-infected cells. sCD4 was titrated onto H9 cells infected with the indicated strains of virus for 2 h at either 4°C (□) or 37°C (○) and then detected by staining with CD4 MAb L120 reactive with domain 4 and then with anti-mouse phycoerythrin. Analysis was done with a FACSscan with Consort 30 software. Values shown are the mean fluorescence intensities for each concentration, with the background staining values (conjugate alone) subtracted. Each point represents 10,000 accumulated events.

fusion. Studies of the effect of sCD4 binding to primary isolates of HIV-1 have demonstrated that glycoprotein subunit dissociation may take place with these viruses more readily at 4 than at 37°C (41, 43, 44). Since it was possible that HIV-2 and SIV behave more like primary isolates in this respect, we decided to test for sCD4-induced dissociation at 4°C. As shown in Fig. 2B, little or no envelope glycoprotein dissociation takes place at 4°C with any of the viruses, in accord with previously published results (14, 15, 21, 31, 43, 45, 47, 57). As with the gp120 dissociation at 37°C, it is important to note that all cell line-adapted HIV-1 isolates do not behave in the same way at 4°C, and this is true both for sCD4-induced SU glycoprotein dissociation and for spontaneous dissociation (39). Thus, in response to sCD4 binding, some isolates (typified by IIIB) release readily detectable quantities of gp120 at 4°C, whereas others (such as NDK) do not. Results obtained from one HIV-1 isolate, such as IIIB, should therefore be extrapolated to describe other laboratory HIV-1 isolates with caution.

Coprecipitation of SU and TM glycoproteins from HIV-2- and SIV-infected cells. One explanation for the finding that HIV-2 and SIV are resistant to sCD4-induced SU glycoprotein shedding is that the bonds between the two glycoprotein subunits are much stronger than those in HIV-1. To investigate this possibility, we precipitated the SU glycoprotein from lysates of concentrated, metabolically labelled virus with biotinylated sCD4 and streptavidin-coated beads. The precipitates were split into two and were run on identical SDS gels under reducing conditions, and they were either autoradiographed directly or blotted onto membranes and Western blotted for TM and SU glycoproteins with MAbs specific for HIV-2 and SIV. If the SU-TM bonds are resistant to sCD4-induced dissociation and nonionic detergent, then the TM glycoprotein should be detected on the blot. As shown in Fig. 3A, the SU glycoproteins of the viruses tested are clearly visible on the autoradiograph and are present at similar concentrations in the precipitate. The lack of direct detection of the TM

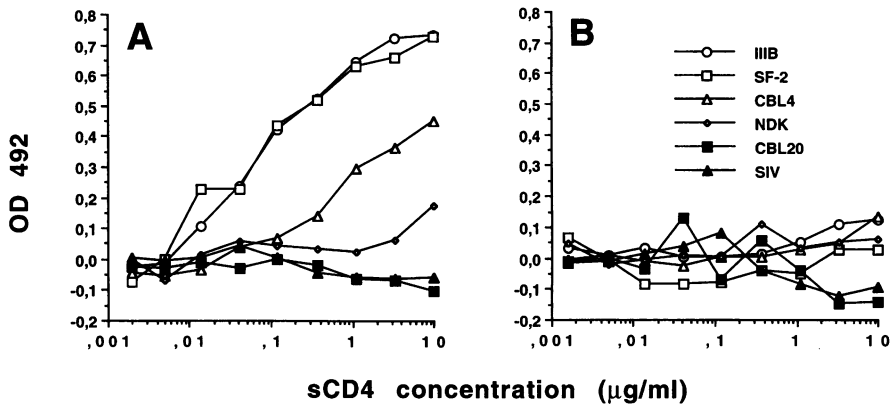

FIG. 2. Detection of soluble gp120 in the supernatant of sCD4-treated cells released at 37°C (A) and 4°C (B). Virus-infected H9 cells were treated with sCD4 as described in the legend to Fig. 1, and the supernatants were kept for analysis. After filtration to remove cellular debris and treatment with NP-40, sCD4 was added to each sample to complex all gp120 equally, and samples were assayed for gp120 by twin-site ELISA. Values shown are the signals for the test samples minus the background signals for the absence of sCD4 and are expressed as optical densities at 492 nm (OD 492).

glycoproteins after immunoprecipitation probably reflects poor labelling, yielding an insufficient signal for autoradiography, as has been described by others (4). Western blots of these precipitates revealed that the TM glycoproteins were coprecipitated with the SU glycoproteins from HIV-2 and SIV (Fig. 3B). Anti-TM glycoprotein MAbs alone detected bands only at 32, 36, and 41 kDa, confirming that the higher bands in the SU-TM blot were not a trimer or tetramer of TM subunits but were SU glycoproteins (results not shown). The use of different MAbs for the detection of the immunoblotted glycoproteins prevents accurate quantification of the relative amounts of TM and SU glycoproteins; however, our results strongly suggest that they are similar for LAV-2 and $SIV_{mac}251$. Interestingly,

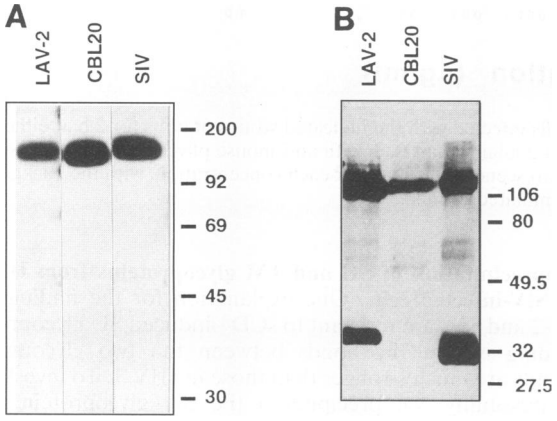

FIG. 3. (A) Autoradiograph of sCD4-biotin-immunoprecipitated viral glycoproteins. Virus-containing supernatants metabolically labelled with [^{35}S]methionine and [^{35}S]cysteine were concentrated, and the virions were lysed in 0.5% NP-40. Immunoprecipitation was carried out with biotinylated sCD4 and streptavidin-coated beads, and the precipitate was run on two reducing SDS-polyacrylamide gels. After drying, one of the gels was autoradiographed for 15 days at 70°C. The other gel was electroblotted for the Western blot. (B) The transferred immunoprecipitates were blotted with the murine MAbs KK20 and KK12, which react with the immunodominant conserved region of the TM glycoproteins and the N termini of the SU glycoproteins, respectively, of the HIV-2 and SIV strains tested.

the CBL20 TM glycoprotein was poorly represented on the SU-TM blot; this is a reproducible finding, suggesting that the interaction between the glycoproteins of this virus may be less stable in nonionic detergent than those of the other two or that the detection MAb binds less well to the CBL20 TM glycoprotein in Western blots. We do not believe that CBL20 is susceptible to sCD4-mediated glycoprotein dissociation, since we have never detected the release of SU glycoprotein or exposure of TM glycoprotein in the presence of sCD4. By contrast with the data obtained with HIV-2 and SIV, we have been unable to demonstrate coprecipitation of gp120 with gp41 from a number of cell line-adapted HIV-1 isolates under the same conditions (results not shown). Thus, we have provided direct biochemical evidence for bonding between the glycoprotein subunits of HIV-2 and SIV which is substantially more stable than between those of HIV-1 and have provided an explanation for the lack of sCD4-induced SU glycoprotein dissociation in the first two viruses. This is in agreement with a recent study (4) which concludes, by the use of immunoprecipitation alone, that the bonds between the SU and TM glycoproteins of SIV_{agm} are more stable than those of the HIV-1 isolate IIIB.

Exposure of gp41 epitopes induced by sCD4 binding. Using immunofluorescence staining and flow cytometric analysis, we have previously demonstrated that the binding of sCD4 to the surfaces of HIV-1-infected cells results in an increase in the exposure of epitopes of gp41 (57) and have proposed that this may reflect receptor-induced conformational changes important in HIV fusion (18, 42, 46, 55, 57). Therefore, we decided to compare HIV-1 with the other primate immunodeficiency viruses to see whether they behave similarly in this respect. For the initial experiments to detect TM glycoprotein exposure, the following two MAbs were used: 50-69, a human MAb which recognizes the immunodominant epitope of HIV-1 gp41 defined by residues 579 to 613 (19, 68); and KK20, a murine MAb which reacts with the homologous region on the TM glycoproteins of $SIV_{mac}251$ and $LAV\text{-}2_{ROD}$ and which binds to a peptide synthesized from the $SIV_{mac}251$ sequence 594 to 616 (28, 29). Prior to the addition of sCD4, weak staining was seen with both 50-69 on the HIV-1-infected cells and KK20 on the HIV-2- and SIV-infected cells. Following a 2-h incubation with sCD4 at 37°C, we observed a substantial increase in staining of

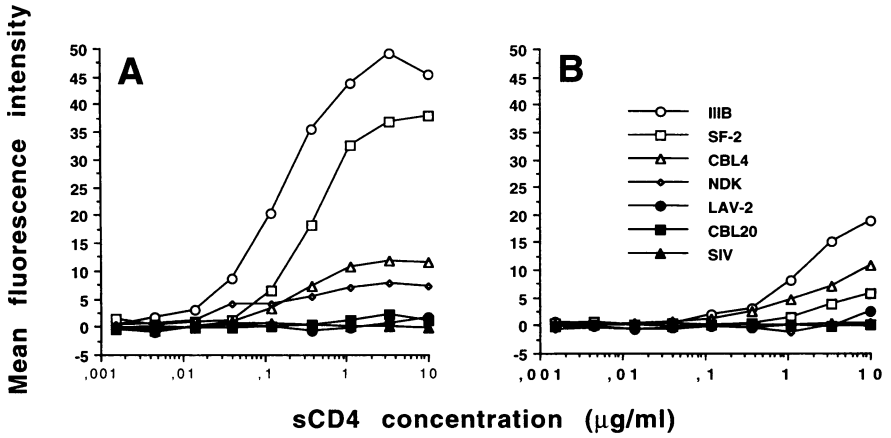

FIG. 4. Exposure of a cryptic gp41 epitope after sCD4 binding. Infected cells treated with sCD4 at 37°C (A) and at 4°C (B) as described in the legend to Fig. 1 were stained with MAbs directed to the conserved, immunodominant regions of the respective viruses by indirect immunofluorescence and were analyzed by flow cytometry. Values shown are the mean fluorescence intensities of the test samples minus the background staining values (conjugated antibody alone). Each point represents 10,000 accumulated events.

IIIB- and SF-2-infected cells with 50-69 at sCD4 concentrations between 0.014 and 0.12 μg/ml (0.28 and 2.4 nM, respectively) and CBL4- and NDK-infected cells at 0.37 to 1.11 μg/ml (7.4 to 22 nM, respectively [Fig. 4A]). Comparison of the gp120 dissociation and gp41 exposure columns in Table 1 suggests that there is a relationship between the half-maximal binding values of sCD4 for the different HIV-1 isolates and the concentrations of sCD4 required to induce half-maximal gp120 dissociation and gp41 exposure. Thus, the ability of sCD4 to induce conformational changes in the HIV-1 envelope glycoproteins may be dependent on the initial binding energy.

By contrast with HIV-1, no increased staining of HIV-2- or SIV-infected cells was seen at any sCD4 concentration with KK20, implying that in contrast to HIV-1, sCD4 does not induce the exposure of the immunodominant region of the HIV-2 or SIV TM glycoprotein. At 4°C, low-level gp41 exposure could be observed in IIIB, SF-2, and CBL4 at the highest sCD4 concentrations; however, as with the 37°C experiment, no HIV-2 or SIV TM glycoprotein was detected (Fig. 4B). We could not conclude unequivocally from this experiment that exposure of the TM glycoproteins of these viruses was not taking place, since we attempted to detect the exposure of only one epitope on the immunodominant, cysteine-linked conserved domain. Additionally, we had no direct evidence that this MAb recognizes the TM glycoproteins in their oligomeric, native form, since reactivity has been observed only in Western blots (28, 29) (Fig. 3B). For this reason, we decided to test a number of other TM glycoprotein-reactive MAbs for HIV-2 and SIV staining in the presence and absence of sCD4. Figure 5 shows the effect of increasing the sCD4 concentration on the exposure of LAV-2_{ROD} (Fig. 5A) and $SIV_{mac}251$ (Fig. 5B) TM glycoprotein epitopes. As we have previously noted from HIV-1 staining with TM-reactive MAbs (57), there is a background level of MAb reactivity with the HIV-2 and SIV TM glycoproteins. This may result from the expression of a small population of misfolded glycoprotein spikes in which the TM glycoprotein is exposed or may reflect deliberate low-level exposure of certain regions of gp41. In this respect, it is interesting to note that the epitope recognized by MAb 36D is relatively well exposed on the surface of HIV-2 and SIV compared with those of the other MAbs. Despite the ability of certain TM glycoprotein-specific MAbs to react with the

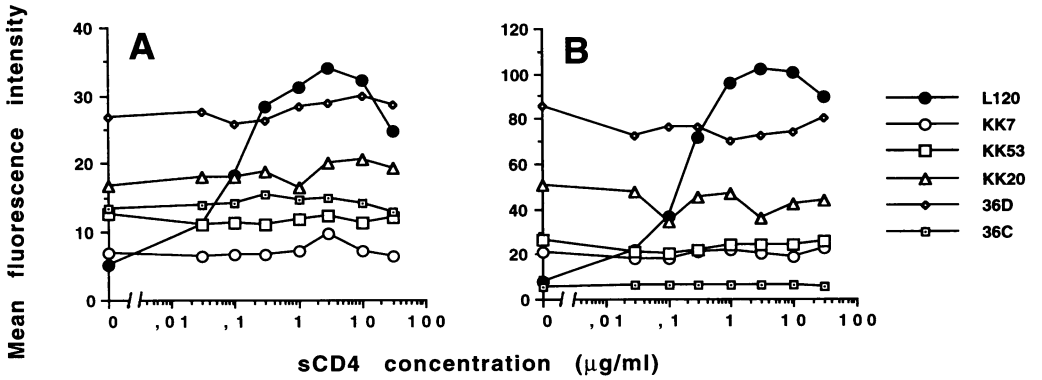

FIG. 5. TM glycoprotein MAb binding to sCD4-treated, HIV-2- or SIV-infected cells. H9 cells infected with LAV-2 (A) or $SIV_{mac}251$ (B) were treated with sCD4 for 2 h at 37°C before the addition of MAbs reactive with domain 4 of CD4 (L120) or the HIV-2 or SIV TM glycoproteins (KK7, KK20, KK53, 36C, and 36D). The cells were stained by indirect immunofluorescence with anti-mouse phycoerythrin and then were fixed and analyzed by flow cytometry as described in the legend to Fig. 1.

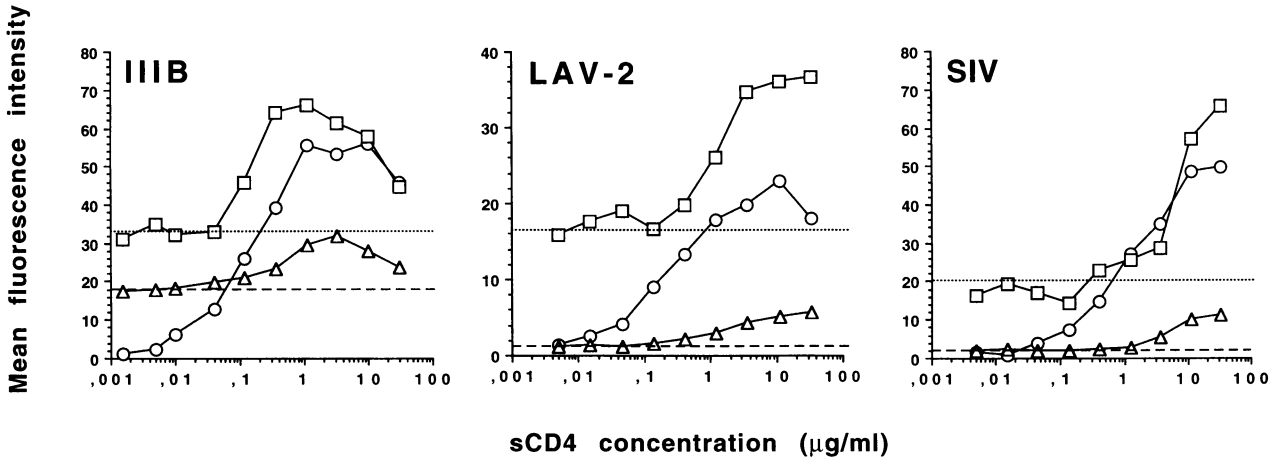

FIG. 6. Modulation of V2 and V3 loop epitopes by sCD4 binding. Infected cells treated with sCD4 at 4°C as described above were stained by indirect immunofluorescence with MAbs to the respective V2 and V3 loops and were analyzed by flow cytometry as described in the legend to Fig. 1. ○, CD4-reactive MAb L120; □, V3 loop-reactive MAbs (9284 for HIV-1 IIIB, 125-F for LAV-2_{ROD}, and KK46 for $SIV_{mac}251$); △, V2-reactive MAbs (G3-4 for HIV-1 IIIB and KK13 for LAV-2_{ROD} and $SIV_{mac}251$). Dotted line, V3 loop staining in the absence of sCD4; dashed line, V2 loop staining in the absence of sCD4. Values shown are the mean fluorescence intensities minus the background staining values. Each point represents 10,000 accumulated events.

surfaces of HIV-2- and SIV-infected cells, there is no increase in the exposure of any of the epitopes in response to sCD4 binding at either 37 or 4°C, even at the supersaturating sCD4 dose of 30 μg/ml. This strongly implies, therefore, that unlike HIV-1, HIV-2, and SIV TM glycoprotein exposure is not modulated by soluble receptor binding.

sCD4-induced enhancement of SU glycoprotein epitope exposure. In addition to the increased exposure of gp41, we have observed other indications of conformational change taking place in gp120 as a consequence of sCD4 binding, most notably increased exposure of the V3 loop (57). To examine whether HIV-1 differed from HIV-2 and SIV in all receptor binding-induced events, we tested the ability of sCD4 to modulate V2 and V3 loop exposure on HIV-1, HIV-2, and SIV, as detected by the binding of MAbs to the respective regions. As shown in Fig. 6, increasing concentrations of sCD4 increased both V2 and V3 loop exposure in HIV-1 IIIB, LAV-2_{ROD}, and $SIV_{mac}251$. The V3 loops of all of these viruses were well exposed on the surface before sCD4 binding. Increased V3 loop staining was first observed with all of the viruses when approximately 50% of the sCD4 binding sites were occupied, supporting the hypothesis that the functional unit of SU-TM glycoproteins is an oligomer and that conformational changes in these molecules are not triggered until more than one site in a glycoprotein spike is occupied by a receptor molecule. Maximal increases in V3 loop staining induced by the presence of sCD4 were 1.9-, 2.3-, and 3.2-fold for HIV-1 IIIB, LAV-2_{ROD}, and $SIV_{mac}251$, respectively, compared with the absence of sCD4. The actual value for IIIB is likely to be somewhat higher than this, since low-level gp120 dissociation reduces the MAb signal at higher sCD4 concentrations. The V2 loop epitope tested was less well exposed than the V3 loop on HIV-1 and was completely masked on the LAV-2 and SIV isolates. After sCD4 binding, there was a maximum increase of 1.8-fold for HIV-1 and the epitopes on HIV-2 and SIV became exposed, yielding mean fluorescence intensities of approximately 3- and 4-fold, respectively, over background staining.

DISCUSSION

In this study, we have compared cell line-adapted isolates of the related primate immunodeficiency viruses HIV-1, HIV-2, and SIV in terms of their interactions with sCD4 in an attempt (i) to define the mechanism underlying the differences in their relative susceptibilities to inhibition by soluble receptor of virus-induced membrane fusion and (ii) to gain further insight into the molecular mechanisms of receptor-induced activation of viral fusion.

Measurement of the relative affinities of sCD4 for the functional, oligomeric forms of the envelope glycoproteins of these viruses reveals that HIV-2 and SIV have values two- to eightfold lower than those for HIV-1. Although this variation may subtly influence virus-receptor interactions, it is difficult to understand how, as in the cases of SF-2 and $SIV_{mac}251$, a difference of less than 2-fold in affinity can be directly responsible for an 80-fold difference in resistance to syncytium inhibition by sCD4 (affinity variation may, however, reflect different structural configurations of the glycoprotein oligomer which may influence SU-TM dissociation [62, 63; see below]). Moreover, it is not clear how variation in the affinity of the SU glycoprotein for soluble receptor can be translated into differential resistance to virus neutralization or syncytium formation by sCD4 without invoking the concept of variation between viruses in the context of differences between sCD4 and membrane CD4 interaction. Thus, if one virus binds strongly to membrane CD4 but weakly to sCD4, this virus will be less sensitive to sCD4 neutralization than a virus with the opposite set of characteristics, a possibility which we cannot rule out but for which there is currently no supporting evidence. A simpler concept is that sCD4-induced dissociation of the envelope glycoprotein subunits irreversibly inactivates the virus fusion mechanism, whereas in the absence of SU glycoprotein dissociation, sCD4 blocks fusion solely by competitive inhibition of receptor binding. This idea fits well with the data presented here, which show a relationship between resistance to SU glycoprotein shedding and resistance to inhibition of syncytium formation, and it is in agreement with the

conclusions drawn from studies of sCD4 interactions with primary isolates of HIV-1 (41, 44, 64). However, the explanation for HIV-2 and SIV resistance to sCD4-induced neutralization may be more complex than this, since sCD4 treatment of these viruses can increase infectivity and syncytium formation (2, 11, 60, 66) and allow HIV-2 to bypass the requirement for cellular CD4 (11). Thus, in addition to the lack of sCD4-induced SU glycoprotein shedding, another factor accounting for HIV-2 and SIV resistance to sCD4 may be that these viruses can use an alternative route of entry which is activated by sCD4 binding.

Our findings argue against the proposal that the resistance of HIV-2 and SIV to sCD4 neutralization is a result of the release of high concentrations of soluble SU glycoproteins by these viruses which compete with sCD4 for binding to the TM-associated SU glycoprotein (12), since we demonstrate, in agreement with Allan et al. (4), that the SU-TM association in these viruses is stabler than in HIV-1. Stabler bonding between envelope glycoprotein subunits in HIV-2 and SIV probably also explains the greater abundance of glycoprotein spikes on the surfaces of these viruses compared with HIV-1 (4, 24, 50, 51). The finding that the HIV-2 and SIV isolates that we tested all have somewhat lower affinities for sCD4 than the six HIV-1 isolates and are all resistant to SU glycoprotein dissociation suggests an association between affinity of the sCD4-SU glycoprotein interaction and shedding, in agreement with the conclusions of Thali et al., who found a similar correlation in a panel of mutants of an HIV-1 molecular clone (62). Our data showing that both the SU and the TM glycoprotein subunits can be precipitated from HIV-2 or SIV virions in a complex with sCD4 correlate with the inability of sCD4 to induce SU glycoprotein shedding and the lack of increased exposure of the immunodominant region of the TM glycoprotein in these viruses.

The half-maximal binding values that we have obtained for HIV-1 IIIB and RF and HIV-2 LAV-2_{ROD} are in relatively close agreement with those obtained in certain previous studies with soluble forms of gp120 (5, 6, 27, 34, 49) and with those measuring the binding of directly fluoresceinated sCD4 to IIIB-infected cells (26) or indirect staining of unlabelled sCD4 to *env*-expressing, recombinant vaccinia virus-infected cells (14) or to virions (45). Other estimates, particularly those for HIV-2, are less close; such discrepancies among results from different laboratories may reflect differences in technique, glycoprotein expression system, host cells, and/or kinetics of sCD4 binding under these different conditions (27, 40, 48). We suggest that by comparison with other systems, the measurement of soluble receptor binding to functional, multimeric envelope glycoprotein in the context of infectious virus production is of particular biological relevance. However, it should be remembered that the results presented here are derived from viruses isolated and extensively passaged in immortalized cell lines, culture conditions which are known to alter certain biological properties of HIV-1 virions (41, 44, 64).

The large differences that we have observed between HIV-1 and HIV-2/SIV in terms of the stability of the TM-SU association reflects different structural configurations either within single SU-TM pairs or within higher-order oligomeric forms. In this respect, both the SU and TM subunits of HIV-2 differ substantially from those of HIV-1 in their primary sequences (39% homology for the SU glycoprotein and 45% for the TM glycoprotein between HIV-1 LAV-1_{BRU} and HIV-2 LAV-2_{ROD} [20]) and in their predicted secondary structures (25, 36), although the functional consequences of these differences are not known. It has been reported that the SU-TM monomers of HIV-1, HIV-2, and SIV are stably associated into dimers (9, 16, 17, 52–54), and higher-order structures of both trimers (50, 53, 58) and tetramers (9, 53, 58, 65) have been reported. Since the TM glycoproteins of HIV-1 and SIV can form stable heterodimers (16), it seems unlikely that there are gross differences in the oligomeric arrangements of these molecules, and the same is likely to be true for HIV-1 and HIV-2. Although we have proposed that the envelope glycoproteins of HIV-1, HIV-2, and other retroviruses are linked in part by a common structural motif (59), the strength of the association is apparently highly variable.

It is thought that, by analogy with other fusogenic enveloped viruses such as influenza virus, HIV must expose the fusion peptide at the N terminus of the TM subunit for fusion to occur. How can we reconcile this hypothesis with our data which fail to show that several regions of the HIV-2 and SIV TM glycoproteins become more exposed following receptor binding? We suggest the following possibility as a working model. We propose that all of these viruses initially follow the same pathway of receptor-mediated activation of fusion but that the cell line-passaged HIV-1 isolates which have adapted to high cytopathicity in culture undergo more-extreme conformational changes, resulting in gp120 shedding. In this model, the minimum requirement for activation of fusion which takes place in HIV-2 and SIV virions is the receptor-triggered exposure of the fusion peptide in the absence of SU glycoprotein shedding and complete unmasking of the TM glycoprotein, such as may be the case with primary isolates of HIV-1. The extreme conformational changes that are induced by sCD4 in cell line-adapted HIV-1 isolates may reflect endpoint molecular rearrangements which are not required for but may facilitate fusion and which are selected against in vivo. Our observation that representatives of all types of virus tested showed increased exposure of their V2 and V3 loops after sCD4 binding supports the idea that there are common elements in the HIV-1, HIV-2, and SIV fusion pathways. The necessity to occupy more than one SU glycoprotein receptor-binding site within an oligomer to trigger conformational changes is also a shared feature of these viruses and has been previously proposed as an important factor in HIV infectivity (35). A preliminary report (2) described the V3 loop of SIV_{agm} as being cryptic but exposed only subsequent to sCD4 binding. Our data confirm the increased exposure of the SIV V3 loop but demonstrate that this domain is well exposed before sCD4 binding. Such differences may reflect the use of another strain of SIV or variation in the exposure of different V3 loop epitopes. The differences that we have observed between HIV-1 and HIV-2/SIV during the later stages of receptor-mediated activation might explain the phenomenon of sCD4 enhancement of infectivity which has been reported for HIV-2 and SIV but not for HIV-1 (1, 11, 60, 66). Thus, sCD4 treatment might prime the HIV-2/SIV envelope glycoproteins for fusion by exposing important functional cryptic epitopes, without the inactivating step of SU-TM dissociation which occurs with HIV-1, a concept we (42, 46, 55) and others (1, 2, 4) have previously proposed. An additional possibility is that HIV-1 and HIV-2/SIV have different requirements for molecules accessory to CD4 and hence expose different regions of their TM glycoproteins to allow for such interactions (11). Whether HIV-1, HIV-2, and SIV follow the same pathway for membrane fusion or not, it is clear from these and other studies that comparison of the interactions between their envelope glycoproteins and CD4 will lead to a greater understanding of virus-cell fusion mechanisms.

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