Neutralizing Antibodies to Human Immunodeficiency Virus Type-1 gp120 Induce Envelope Glycoprotein Subunit Dissociation

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Summary

The spectrum of the anti–human immunodeficiency virus (HIV) neutralizing immune response has been analyzed by the production and characterization of monoclonal antibodies (mAbs) to the viral envelope glycoproteins, gp41 and gp120. Little is known, however, about the neutralization mechanism of these antibodies. Here we show that the binding of a group of neutralizing mAbs that react with regions of the gp120 molecule associated with and including the V2 and V3 loops, the C4 domain and supporting structures, induce the dissociation of gp120 from gp41 on cells infected with the T cell line–adapted HIV-1 molecular clone Hx10. Similar to soluble receptor-induced dissociation of gp120 from gp41, the antibody-induced dissociation is dose- and time-dependent. By contrast, mAbs binding to discontinuous epitopes overlapping the CD4 binding site do not induce gp120 dissociation, implying that mAb-induced conformational changes in gp120 are epitope specific, and that HIV neutralization probably involves several mechanisms.

Neutralization of enveloped viruses by antibodies is likely to be mediated by at least two mechanisms, including the inhibition of virus binding to its cellular receptor and interference with viral capsid entry into the host cell (1). HIV-1 induces an antibody response in infected individuals and immunized animals, a component of which is neutralizing (2–5). This neutralizing activity appears to be directed predominantly at the surface (gp120) envelope glycoprotein (6), although neutralization can also be mediated by a transmembrane glycoprotein (gp41)-specific fraction of antibodies (7). More recently, the HIV-neutralizing response has been dissected by the preparation and characterization of mAbs of rodent and human origin, allowing the identification of a number of neutralization epitope clusters on gp120 and gp41 (8). The known gp120-specific neutralizing activity can be divided into responses directed to the hypervariable loops V1/V2 (9–14) and V3 (15–27), or to more conserved regions that may contain linear epitopes such as the C4 domain (28–31), or complex, discontinuous epitopes clustered around the base of the variable loops that may interfere (32–36) or not (37) with CD4 binding to gp120. Although considerable success has been achieved in characterizing the specificity of the neutralizing antibody response to HIV-1, little is understood of the mechanism by which antibodies neutralize this virus. The group of mAbs that interfere with the CD4-gp120 interaction known as "CD4 binding site (CD4/BS) specific," bind to epitopes on gp120 that overlap the CD4-binding domain (32–36). Binding of soluble, recombinant gp120 in a monomeric form to CD4 is competed by these antibodies (34, 36), implying that their mechanism of neutralization may be based, at least in part, on steric inhibition of virus–receptor binding. It is unclear, however, whether results obtained with monomeric gp120 are representative of the effect of mAb binding to functional, gp41-associated, oligomeric gp120, as found on virions and virus-infected cells (8). Thus, results from some laboratories suggest that such mAbs are poor competitors of virus binding and may act at a postbinding step (McInery, T. L., C. F. Barbas, III, D. R. Burton, and N. J. Dimmock, manuscript submitted for publication), as has been proposed for other enveloped viruses (1). Other mAbs, including those directed to the V1, V2, and V3 loops, do not inhibit the monomer gp120–CD4 interaction, but they interfere with later events that may include virus–cell membrane fusion or capsid entry into the cell cytoplasm (17, 38). A third group of antibodies to linear epitopes in the ectodomain (39, 40) or cytoplasmic tail (41) of gp41 are likely to inhibit virus–cell membrane fusion, although the nature of the virus–antibody interaction in the latter case is obscure (42). It therefore seems probable that there are various levels at which an antibody might inhibit HIV infection of a cell, implying the existence of diverse neutralization mechanisms.

The entry of HIV into CD4$^+$ cells is thought to take place by "receptor-mediated activation of virus fusion" (43, 44). Thus, the binding of HIV to cell-surface CD4 triggers

a series of conformational changes in the viral envelope glycoproteins that are believed to result in exposure of the fusion domain of gp41 and lead to virus–cell or cell–cell membrane coalescence (45, 46). Conformational changes have been demonstrated in model systems in which a soluble form of CD4 (sCD4)[1] has been substituted for the membrane-anchored molecule (47, 48). With T cell line–adapted isolates of HIV-1 (but not with HIV-2 or SIV; 48), sCD4 binding to HIV virions or virus-infected cells results in the dissociation of gp120 from gp41 (49–51).

Although envelope glycoprotein subunit dissociation is unlikely to play a role in HIV entry (52, 53), it probably contributes significantly to virus inactivation (54–56). The relative inability of sCD4 to neutralize isolates of HIV (known as primary isolates) that have been passaged only in activated PBMC probably results, at least in part, from the insensitivity of these viruses to sCD4-induced gp120–gp41 dissociation (8, 56, 57). It seemed reasonable to suppose that certain neutralizing mAbs may inactivate virion infectivity by a mechanism similar to that used by sCD4, and that a component of the recently reported resistance of primary isolate viruses to neutralization by antisera and mAbs (58) may be a reduced ability of these reagents to induce HIV glycoprotein subunit dissociation. In this report, we have investigated whether this is so.

The majority of well-characterized neutralizing mAbs that are currently available have been prepared to the gp120 glycoprotein of the cell line–adapted HIV-1 LAI isolate. For this reason, we have chosen to study neutralization in a system using the molecular clone Hx10, a clone of the LAI isolate. We have recently shown that the neutralization of Hx10 correlated broadly with mAb affinity for the oligomeric, gp41-associated form of gp120 (59). In this study, which uses the same model system, we demonstrate that a group of gp120-reactive neutralizing mAbs induces the dissociation of the envelope glycoproteins on Hx10-infected cells. Although it is unclear whether this activity represents the dominant pathway of HIV neutralization, we suggest that the induction of conformational changes in the HIV-1 envelope glycoproteins triggered by mAb binding contributes to the neutralization of T cell line–adapted viruses.

Materials and Methods

Antibodies. The anti-gp120/V3 mAbs were from the following sources: 110.I (60) from F. Traincard (Hybridolab, Pasteur Institute, Paris, France); 110.5 (19) from Genetic Systems (Seattle, WA); 9284 (18) was purchased from DuPont de Nemours (Les Ulis, France); BAT123 (22) was Tanox Biosystems Inc. (Houston, TX) and D. Ho (Aaron Diamond AIDS Research Center, New York). The anti-gp120/V2 mAbs BAT085, G3-136 (10), and G3-4 (9), as well as the anti-C4 mAbs G3-42, G3-519, G3-299, and G3-536 (28, 30) were also from Tanox and D. Ho. The human mAbs specific for complex epitopes 15e, 21h (31), 48d,

and 17b (37) were from J. Robinson (Department of Pediatrics, University of Connecticut, CT), and IgG1-b12 was from D. Burton (The Scripps Research Institute, La Jolla, CA) was selected from a recombinant Fab library (34) and subsequently engineered into an IgG molecule (35). The human anti-gp41 mAb 50-69 (61) was from S. Zolla-Pazner (New York University, New York).

Cell Culture and Virus Infection. H9 cells (from R. C. Gallo, NIH, Bethesda, MD) were cultured in growth medium (GM), RPMI/10% FCS in the presence of 5% CO_2. Infection of H9 cells with supernatant containing infectious virus of the Hx10 clone of HIV-1 (62, from A. Fisher (Hammersmith Hospital, London, UK) was as follows: 1 million cells in 1 ml were exposed to $\sim 10^4$ $TCID_{50}$ of virus for 2 h at 37°C. After washing, the cells were resuspended in GM and cultured for 6–8 d. At this time, 100% of the cells expressed large amounts of viral envelope glycoproteins, but no CD4, as detected by immunofluorescent staining and flow cytometry using mAbs directed to the gp120 V3 loop and domains 1 and 4 of CD4, respectively.

Measurement of mAb-induced gp120 Dissociation from Virus Particles. H9 cells infected with Hx10 were metabolically labeled on day 8 after infection, this time point having been previously shown to yield the highest amount of cell-free virus. Labeling was carried out as follows: the infected cells were starved in methionine- and cysteine-free RPMI medium for 30 min before being resuspended at a concentration of 3×10^6 cells/ml in the same medium containing 3% dialyzed FCS, [^{35}S]methionine/[^{35}S]cysteine mix (EXPRE $^{35}S^{35}S$ Protein Labeling Mix; DuPont de Nemours) with the addition of extra [^{35}S]cysteine (DuPont de Nemours) to give a final activity of 100 μCi for each amino acid. After 18 h of culture, the cells were pelleted, the supernatant was recovered and filtered through a 0.45-μm pore size cellulose acetate filter (Acrodisc; Gelman Sciences, Ann Arbor, MI), then centrifuged for 20 min at 70,000 rpm in a centrifuge (TL100; Beckman, Gagny, France). For the analysis of envelope glycoprotein subunit dissociation, the virus pellet was resuspended in RPMI/10% FCS containing mAb (40 μg/ml) or sCD4 (13.3 μg/ml) in a total volume of 100 μl, and incubated at 37°C for 2 h. The amount of gp120 released from the virion surface was measured essentially as reported by Willey et al. (63). Briefly, the mAb- or sCD4-treated samples were centrifuged in a microfuge at 13,000 rpm for 90 min at 4°C to gently pellet the virus. 90 μl of the supernatant was removed, and the virus pellet was washed with the addition of 40 μl RPMI, and recentrifuged as before for an additional 40 min. 40 μl of the supernatant was removed and pooled with the original 90 μl. The pelleted virus was resuspended in 120 μl of RPMI, and both the supernatant and virus were resuspended in 600 μl of lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% NP-40, and 0.15% SDS), and denatured by boiling for 5 min. The lysates were precleared overnight at 4°C with protein A–Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ), then viral proteins were immunoprecipitated for 2 h at 4°C with the same beads precoated with human HIV immune globulin (obtained from the NIH AIDS Reagent and Reference Program, Bethesda, MD; 64). Pelleted beads were washed three times with lysis buffer and boiled for 3 min in reducing sample buffer. Immunoprecipitated proteins were separated on an SDS 8% acrylamide gel. The gel was dried and analyzed by fluorography using a Fugix Bas 2000 Bio-image Analyzer (Fuji, Japan), and the intensity of the gp120 bands was quantified. The gel was then autoradiographed at −70°C.

Analysis of Antibody Binding by Flow Cytometry. The analysis was carried out essentially as previously described (59). Briefly,

H9 cells infected with Hx10 virus as described above were washed twice in RPMI/5% FCS, and were resuspended at a concentration of 5×10^7 cells/ml. 20 μl of mAb previously diluted in PBS/1% FCS (wash buffer [WB]) was added to 20 μl of cell suspension in a U-bottomed, 96-well microtiter plate, and was incubated with agitation at 37°C for 2 h. The cells were pelleted, and the supernatants were harvested for analysis of HIV-1–soluble gp120 (see below). The cells were washed three times in ice-cold WB, then fixed in WB + 0.5% formaldehyde overnight at 4°C. After two further washes in WB, the cells were divided into two equal samples: one sample was stained with 50 μl of biotinylated anti-gp41 mAb 50-69 at 10 μg/ml for 1 h at 4°C before washing. Subsequently, both samples were stained for 1 h at 4°C with the appropriate dilution of fluorochrome-conjugated second layer antibody; the first with streptavidin-PE, the second with anti–human or anti–mouse conjugated to PE (all obtained from Immunotech SA, Marseille, France). The cells were washed twice as before, then analyzed by flow cytometry using a FACScan® (Becton Dickinson & Co., Mountain View, CA) with Consort 30 software.

Kinetic analysis of mAb and sCD4 binding to HIV-infected H9 cells was carried out essentially as described above, except that the concentration of ligand was kept constant (67 nM), whereas the incubation time was varied. Briefly, 20 μl previously diluted mAb or sCD4 was added to 20 μl of cells at a concentration of 5×10^7/ml in a U-bottomed, 96-well microtiter plate, and incubated with agitation at 37°C. At the time points indicated, 10 μl of cell suspension was removed, then the cells were washed, fixed, stained, and analyzed as described above.

ELISA Assays for mAb-induced gp120 Dissociation. Release of gp120 from the surface of Hx10-infected, mAb-treated cells was determined as follows: 1 million cells in 40 μl previously incubated at 4 or 37°C for 2 h with mAb as described above for the mAb-binding studies were pelleted, and the supernatants were harvested and spun at 12,000 rpm for 2 min in microfilters (Spin-X; Costar, Brumath, France) to remove remaining cells and cell debris, and 4.5 μl of a 10% solution of NP-40 was added. To prevent interference with the gp120 detection, the samples were denatured to eliminate prebound mAb. Thus, SDS and dithiothreitol were added to 20 μl of supernatant to yield final concentrations of 1% and 50 mM, respectively. After boiling, the treated supernatants were then diluted in 180 μl of TBS/FCS (0.1%)/NP-40 (0.1%). 100 μl was then added to duplicate wells of an ELISA plate precoated with the gp120 capture antibody D7234. The detection and calibration of denatured gp120 in the supernatant was carried out as previously described (60), using the murine mAb B13 (from G. Lewis, University of Maryland Medical School, Baltimore, MD), which reacts preferentially with denatured gp120 (60).

HIV Neutralization Assays. To analyze mAbs for neutralization activity, we used an assay based on infection of HeLa cells expressing human CD4 and the HIV LTR gene fused to lacZ (65), which we have previously desribed (59). Infection of these cells with HIV leads to the production of the viral TAT protein, which transactivates the transfected LTR and activates the *lacZ* gene; detergent lysis of the cells is then followed by addition of a soluble substrate. The advantage of this assay system is that infection of the cells can be detected within 24–36 h of infection (approximately one cycle of replication with the moi used), and reduction in infectivity can be quantitatively assessed by measurement of the OD. The assay was essentially the same as that previously described, with the exception of the modification for ELISA readout; the modification is as follows. 24–36 h after in-

fection, the medium was aspirated and the cells were lysed in a solution of PBS containing 0.5% NP-40. An equal volume of a solution containing 16 mM chlorophenol red β-D-galactopyranoside (CPRG; Boehringer Mannheim, Meylan, France) was added; after 30 min of incubation with the substrate at 37°C, the OD was read at OD_{550nm}. Percentage inhibition was calculated using the formula $100 - \{(t - c)/(m - c) \times 100\}$, where t represents the OD signal for the test sample, c represents the background signal in the absence of virus, and m represents the maximum signal obtained with virus but no inhibitor.

Results

mAb Epitope Specificity. The mAbs used in this study represent the dominant gp120-specific component of the currently recognized spectrum of HIV-neutralizing activity. As shown in Fig. 1, the mAbs can be divided into two groups: those recognizing essentially linear epitopes, comprising the variable loops V1/V2 and V3, and the relatively conserved C4 region, and those recognising complex, discontinuous epitopes that are overlapping or nonoverlapping for the CD4-binding site. The V1/V2 and V3 loop
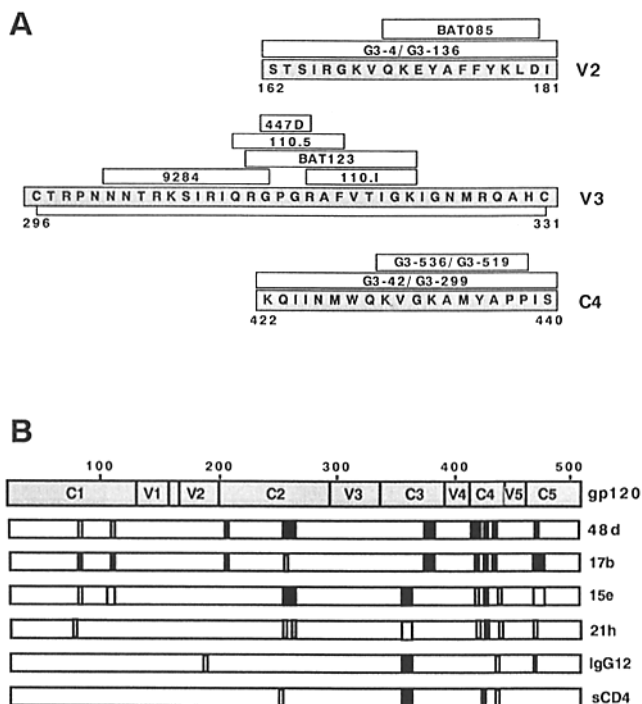

Figure 1. Epitopes of mAbs used in this study. The epitopes for the V2, V3, and C4 murine mAbs are represented as linear sequences, and were taken from the following sources: BAT085, G3-4, and G3-136 (11); 9284, 110.5, BAT123, and 110.1 (18, 29, 22, and 60 respectively); G3-42, G3-299, G3-536, G3-519 (28, 30). The discontinuous epitopes for the human mAbs were mapped by site-directed mutagenesis. A small open box represents an amino acid substitution that partially (50–95%) abrogates mAb binding; a small closed box represents an amino acid substitution that completely (>95%) abrogates binding; a large open box represents two or more amino acid substitutions that partially (50–95%) abrogate binding, and a large closed box represents two or more amino acid substitutions that completely (>95%) abrogate binding. Epitope mapping data were taken from the following sources: 48d and 17b (37); 15e, 21h, and sCD4 (33); IgG1 b12 (34).

mAbs used were chosen because they bind to epitopes exposed on oligomeric gp120; mAbs binding to flanking regions on these loops are either poorly exposed or not exposed on the glycoprotein oligomer (59, 60). Additionally, all of these mAbs have been shown to neutralize cell-free Hx10 virus (59). Experiments that we have recently carried out aimed at estimating mAb association and dissociation rates from oligomeric gp120 suggested that certain mAbs modulated gp120-gp41 dissociation (59). To investigate this more fully, we carried out studies to compare sCD4, which is known to disrupt efficiently the gp120–gp41 interaction with the anti-gp120 mAbs.

mAb-induced gp120 Dissociation from Hx10 Virions.   Metabolically labeled virus was concentrated by centrifugation, incubated with or without mAb or sCD4, pelleted, denatured, and the quantity of gp120 present in the supernatant or pellet was determined by immunoprecipitation, autoradiography and fluorography. The denaturation step was included to ensure that the Igs present in the reaction were dissociated from gp120 and inactivated, so as to avoid any potential interference with the precipitation step. In this experiment, we compared two mAbs, G3-4 and 9284, which bind to the V2 and V3 loops, respectively, with sCD4. As shown in Fig. 2 *A*, gp120 was found in both the supernatant and the viral pellet, and as expected, p24 and to a lesser extent other virion proteins were found in the virus pellet. Some p24 was also present in the supernatant, suggesting that soluble p24 is probably a contaminant in this system; similar observations have also been made by others (63). Levels of p24 remained constant in the samples, confirming that the observed changes in gp120 concentration were not a result of variation in the quantity of virus between samples. Visual inspection of the gp120 bands revealed that in the presence of sCD4 and the V3 mAb 9284, there was a striking diminution in gp120 signal in the virus pellet and a corresponding increase in the supernatant. To quantify this, the amount of gp120 in the supernatants and viral pellets was determined by phosphor imaging of the dried gel, and is represented in Fig. 2 *B*. The total amount of gp120 (virus + supernatant) remained constant under all conditions, except the 4°C incubation, in which it was higher; this may reflect a lower level of gp120 degradation at this temperature. Incubation of the virus alone at 4°C induced some spontaneous gp120 dissociation that increased subtly at 37°C, although the levels of gp120 recovered in the supernatant were similar. Preincubation of virus with 266 nM (13.3 µg/ml) of sCD4 for 2 h at 37°C increased by 41% the amount of soluble gp120 in the supernatant, and decreased by 32% virion-associated gp120. Similarly, preincubation with 266 nM (40 µg/ml) of mAb 9284 resulted in a 41% increase in supernatant gp120 and a 29% decrease in virion-associated gp120. By contrast, the V2 mAb G3-4 at the same concentration increased supernatant gp120 by only 16% and did not significantly alter gp120 dissociation from the virus compared to that lost spontaneously at 37°C.

These results demonstrate that at the concentration tested, a V3 loop mAb was able to mimic sCD4 in its ability to induce gp120 dissociation from virion gp41 at 37°C.
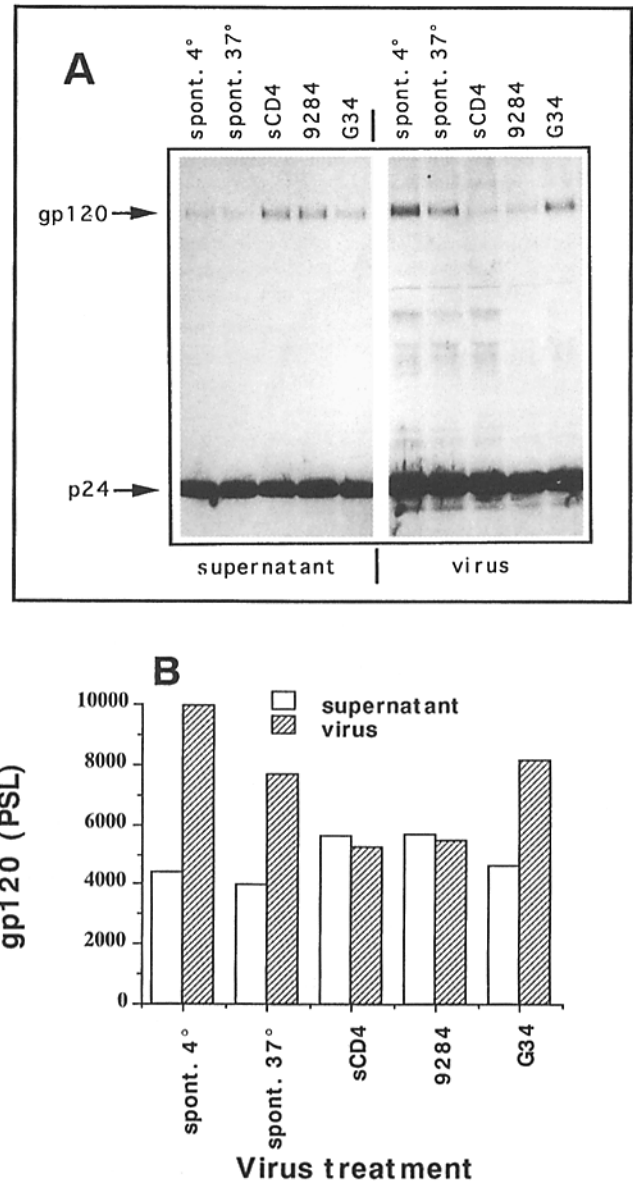

Figure 2.   mAb-induced gp120 dissociation from Hx10 virions. Metabolically labeled virus in supernatants from Hx10-infected H9 cells was pelleted by ultracentrifugation, then resuspended in the presence or absence of sCD4 or mAbs. After incubation for 2 h at 4°C or 37°C, the supernatant was separated from the viral pellet. Protein in the pellet and supernatant was denatured to destroy mAb or sCD4 interactions with gp120, and the gp120 was precipitated with a purified polyclonal anti–HIV-1 antiserum. After denaturing SDS-PAGE, the gel was dried and autoradiographed. (*A*) The five lanes on the left show viral proteins precipitated from the supernatant, and the five lanes on the right show proteins from the viral pellet. (*B*) The quantification of mAb- and sCD4-induced gp120 dissociation from Hx10 virions. The gp120 bands shown in the autoradiograph in *A* were quantified by phosphor imaging, and displayed as units of phosphor-stimulated luminescence (*PSL*).

It was important at this point to determine the epitope specificity intrinsic to the mAb-induced gp120 dissociation and to investigate whether (*a*) mAb-induced gp120 dissociation was dose and time dependent; and (*b*) mAb binding induced exposure of previously masked gp41 epitopes. To

investigate these parameters, we decided to study virus-mAb interactions using infected cells, since the analysis of mAb binding and ligand-induced conformational changes in multiple samples is facilitated in such a system (42, 47–50, 59).

mAb-induced gp120 Dissociation from Hx10-infected Cells. We have previously shown that HIV-infected H9 cells express readily detectable gp120/gp41 complexes at the cell surface, the majority of which probably represent functional envelope glycoprotein oligomers on mature virus particles (47, 48, 59). To study in more detail the ability of mAbs to induce gp120 dissociation, Hx10-infected H9 cells were incubated with gp120 mAbs or sCD4, and the following parameters were measured: (*a*) binding of the mAbs to the virus-infected cell surface; (*b*) exposure of a gp41 epitope that is normally masked by gp120; and (*c*) accumulation of soluble gp120 in the supernatant. As in the previous experiment, we denature the supernatant proteins after treatment of the cells with sCD4 or mAbs to obviate any interference of gp120-bound ligand in the ELISA detection test. We have previously demonstrated that gp41 exposure correlates with gp120 dissociation (47, 48), and therefore have used this marker as further confirmation of the mAb-mediated conformational changes taking place in the viral envelope glycoproteins. Fig. 3 shows mAb binding to infected cells, gp41 exposure, and soluble gp120 released into the supernatant at one mAb–sCD4 concentration (67 nM). There was considerable variation in the fluorescence signals obtained after binding of the different mAbs. This reflects a combination of the differences in mAb association rate for oligomeric gp120 (59), the possibility that the B_{max} value is different for the different mAbs, and the fact that three different staining conjugates were used; anti–mouse PE, anti–human PE, and a two-layer detection for sCD4 consisting of anti-CD4 mAb L120 (47) followed by anti–mouse PE. A precise comparison of absolute numbers of mAb molecules bound per infected cell is therefore unfeasible under these conditions. Confirming our previous observations of relative binding activities (59), however, V3 loop mAbs bind well, mAbs to certain complex epitopes bind with intermediate efficiency, and certain mAbs to the V2 and C4 regions bind poorly under these conditions. Comparison of the values obtained for gp41 exposure and gp120 accumulation in the supernatant reveals a broad correlation, confirming that these two parameters are related. By contrast, a comparison of the values representing the total quantity of mAb bound with those representing gp41 exposure and dissociated gp120 does not reveal a simple relationship; certain mAbs such as 15e, 21h, and IgG1 b12, which bind relatively well to the cells at the concentration used, induce little or no gp41 exposure and gp120 dissociation, whereas others, such as 9284, BAT123, and 48d, induce strong gp41 exposure and gp120 release at 37°C. These experiments have been repeated at 4°C, and as with sCD4-induced conformational changes, little or no gp120 dissociation and gp41 exposure were seen (results not shown). To confirm the specificity of the mAb-induced conformational changes, we measured the effect of
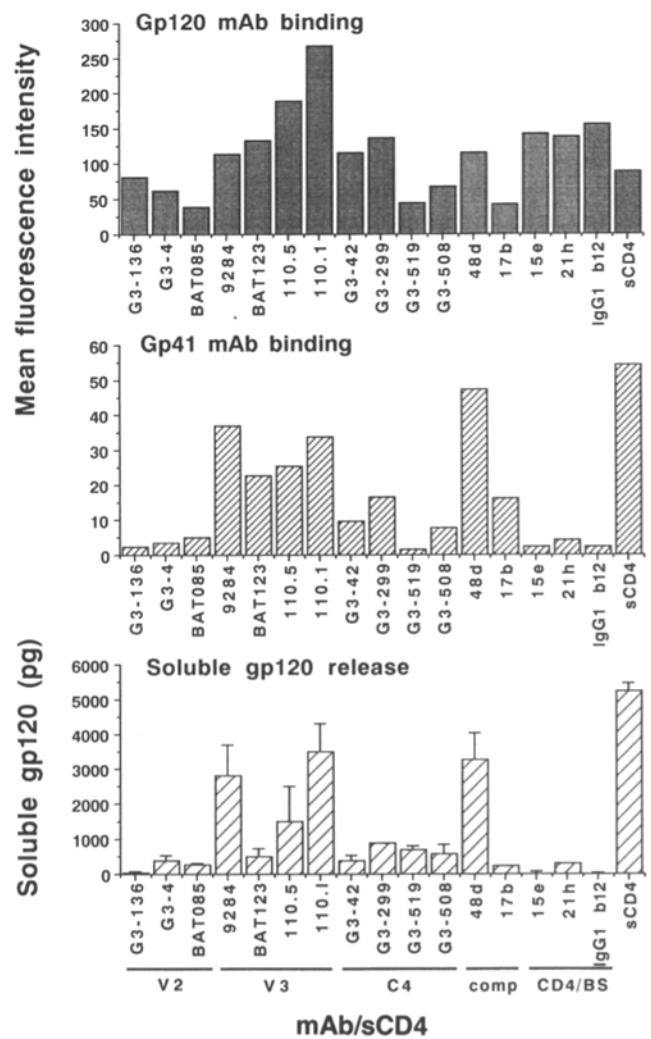

Figure 3. mAb binding and mAb-induced gp41 exposure and gp120 dissociation. (*A*) The binding to Hx10-infected cells of a panel of neutralizing gp120 mAbs, as was analyzed by indirect immunofluorescence and flow cytometry. Hx10-infected cells were incubated for 2 h at 37°C with mAbs or sCD4 at a concentration of 67 nM. Cells were pelleted, washed, fixed, and stained with anti–mouse or anti–human IgG coupled to PE. Each bar represents the mean fluorescence intensity for 10,000 accumulated events. (*B*) The binding of a biotinylated anti-gp41 mAb to the same Hx10-infected cells used in *A*. mAb-stained cells were fixed as described for *A*, then incubated for 1 h with 10 µg/ml of biotinylated gp41 mAb 50-69, which recognizes an epitope constitutively masked by gp120. Binding of the biotinylated gp41 mAb was revealed by incubation of the cells with streptavidin-conjugated PE, then analysis by flow cytometry as described above. (*C*) The release of soluble gp120 from the surface of the mAb-treated, Hx10-infected cells. After the mAb treatment as described for *A*, cells were pelleted and the supernatants were recovered. After centrifugation through Spin-X filters to remove cellular debris, supernatants were denatured, and the concentration of gp120 was determined by ELISA. Each bar represents the mean of three replicate wells, and the error bars represent ± 1 SD.

mAb concentration on gp120 dissociation and gp41 exposure at 37°C.

Dose Response of gp120 Dissociation from Hx10-infected Cells. mAbs or sCD4 were titrated from 30 or 10 µg/ml, respectively, (200 nM) to 0.014 or 0.004 µg/ml, respectively (~0.1 nM), and incubated with the infected cells as

in the previous experiment. As shown in Fig. 4, mAb binding was dose dependent, and saturation binding was achieved with mAbs specific for the V3 loop and some complex epitopes, in agreement with our previous report (59). By contrast, equilibrium-binding conditions were not attained with V2 mAbs, with C4 mAbs other than G3-42, and with the CD4/BS mAbs with the exception of IgG1 b12, in accord with the proposal that the majority of these mAbs associate relatively slowly with oligomeric gp120 (59). Gp41 exposure and gp120 dissociation were broadly related for all mAbs, although gp41 exposure appeared to

be the more sensitive assay for mAb or sCD4-induced conformational changes. V2 mAbs induced little gp41 exposure <10 nM, and no detectable gp120 dissociation <100 nM; no obvious correlation could be established between the level of gp41 exposure/gp120 dissociation and the mAb-binding activity since the signals were generally too low to allow precise interpretation. By contrast, the V3 mAbs were more potent in all respects; binding was of high relative affinity, and gp41 exposure and gp120 dissociation were readily detectable at 0.3 nM and between 1 and 30 nM, respectively. The binding of the C4 mAbs was vari-
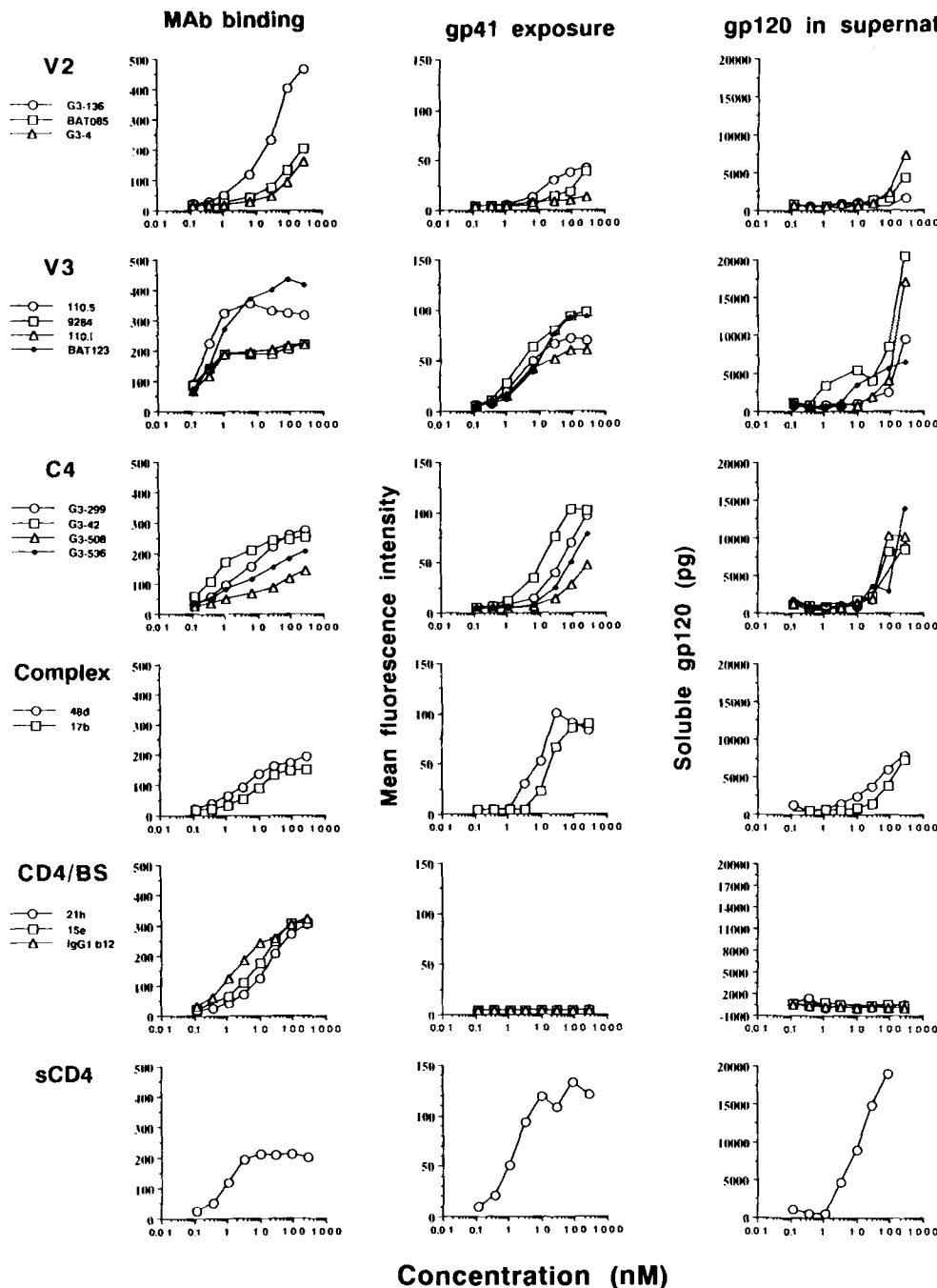

Figure 4. Dose dependence of gp41 exposure and gp120 dissociation. Hx10-infected cells were incubated with sCD4 or mAbs as described for Fig. 3, except that the ligands were titrated from 0.1 to 200 nM. The processing and analysis of the cells and supernatants were as described in Fig. 3. The left column represents mAb or sCD4 binding to the infected cells, the central column shows the binding of a biotinylated gp41 mAb to the infected cells, and the right column shows the amount of soluble gp120 detected in the supernatant.

able; G3-42 saturated at 30 nM, whereas the other mAbs did not achieve saturation under these conditions. Gp41 exposure correlated broadly with binding, and all C4 mAbs induced gp120 dissociation. The two mAbs reactive with complex epitopes nonoverlapping the CD4-binding site, 17b and 48d, bound with intermediate affinity to the infected cells, saturating at ∼30–100 nM. 48d was more potent at inducing gp41 exposure, and both mAbs induced gp120 dissociation. The CD4-binding site mAbs 15e and 21h bound with relatively low affinity compared to IgG1 b12, which saturated at ∼10 nM. By contrast with the other mAbs, none of the three CD4/BS mAbs was able to induce gp41 exposure and gp120 dissociation at any concentration tested. We tried concentrations of up to 600 nM, but saw no effect of these three mAbs on the destabilization of the gp120–gp41 interaction (data not shown). Finally, in confirmation of previous studies, soluble CD4 bound well to the Hx10-infected cells and induced gp41 exposure and gp120 dissociation. The extent of these effects appeared similar in magnitude to those induced by certain V3 and C4 mAbs. It should be noted that the induction of gp120 dissociation will alter mAb-binding curves as previously demonstrated for sCD4 (47–49, 54), preventing precise analysis of binding parameters. This source of inaccuracy should be taken into consideration when interpreting the half-maximal binding data for the gp120 mAbs taken from Fig. 4 and presented in Table 1. Inspection of the values in Table 1 reveals a broad relationship between the relative binding affinity of the gp120 mAbs (excluding the CD4/BS mAbs) and their ability to induce gp41 exposure. Those with the lowest half-maximal binding values for binding to gp120 also gave the lowest values for gp41 exposure, and thus the highest affinity mAbs induced gp41 exposure most efficiency.

Time Course of mAb-induced gp120 Dissociation. Hx10-infected H9 cells were incubated with a fixed concentration of mAb or sCD4 (67 nM) for different times at 37°C, the cells were fixed, and mAb binding and gp41 exposure were subsequently evaluated. Fig. 5 shows that the rate of association was variable, and it was ranked as follows with mAb 9284 having the most rapid kinetics; 9284 (V3) >sCD4 = IgG1 b12 (CD4/BS) = 48d (complex) >G3-42 (C4) >G3-136 (V2). With the exception of the CD4/BS mAb IgG1 b12, which did not significantly increase the gp41 exposure above background, the rate of gp41 exposure was similar to the mAb-binding pattern; sCD4 induced the most rapid response, then 9284 = 48d >G3-42 >G3-136. In general, mAb-induced gp41 exposure was somewhat slower than that obtained with sCD4, and it appeared to increase linearly with time, achieving a maximal extent by 160 min after addition of the ligand. The relationship between the time for the mAbs to achieve equilibrium binding and their ability to induce gp41 exposure suggests that the mAb association rate may be a determinant of mAb-induced conformational changes in the HIV-1 envelope glycoproteins.

mAb Neutralization of HIV-1 Hx10. We have analyzed previously (59) the neutralization activity for Hx10 of the majority of mAbs used in this study. Here we have repeated this analysis using the same system, but with an ELISA-based readout, and the data are summarized in Table 1. We found some differences between the two studies in the 50% inhibitory doses for certain mAbs; it is likely that this represents batch-to-batch variation in mAb stocks and a small difference in sensitivity between the assays. Despite these differences, we found essentially the same rank order in terms of the ability of the mAbs to neutralize Hx10.

To establish whether a relationship exists between the neutralization activity of a mAb and its ability to induce gp41 exposure and gp120 dissociation, we compared the concentrations required for half-maximal inhibition with those giving half-maximal gp41 exposure. We were unable to compare the same set of values for gp120 dissociation since maximal dissociation was rarely achieved under the conditions used; we thus make the assumption that in general terms, the level of gp41 exposure is indicative of, and correlated with gp120 dissociation. Within certain groups, there was a broad relationship between neutralization and gp41 exposure; the V3 loop mAbs, the C4-reactive mAbs

Table 1. *Comparison of mAb Binding, Neutralization, and gp41 Exposure Parameters*

Region	mAb	Half-maximal binding	Neutralization	Half-maximal gp41 exposure
nM		*nM*	*ID$_{50}$ in nM*	*nM*
V2	G3-136	20*	18.5	10
	G3-4	>70‡	20	>100§
	BAT085	>70	125	>100
V3	110.5	0.07	0.04	2
	9284	0.07	0.17	3
	BAT123	0.08	0.03	10
	110.I	0.45	1.3	3
C4	G3-42	0.5	0.5	10
	G3-299	3.0	2.5	40
	G3-519	>50	70	100
	G3-508	>50	37	>100
Complex	48d	2.5	0.3	8
	17b	10	0.8	20
CD4/BS	15e	10	0.8	–‖
	21h	7	8.3	–
	IgG1 b12	1	0.3	–
CD4	sCD4	0.8	0.2	1.5

*Results are the mean of two to five independent experiments for each parameter and each mAb.

‡Half-maximal binding values could be calculated for mAbs for which saturation binding was not achieved.

§Half-maximal gp41 exposure could not be calculated for mAbs for which saturation gp41 mAb binding was not achieved.

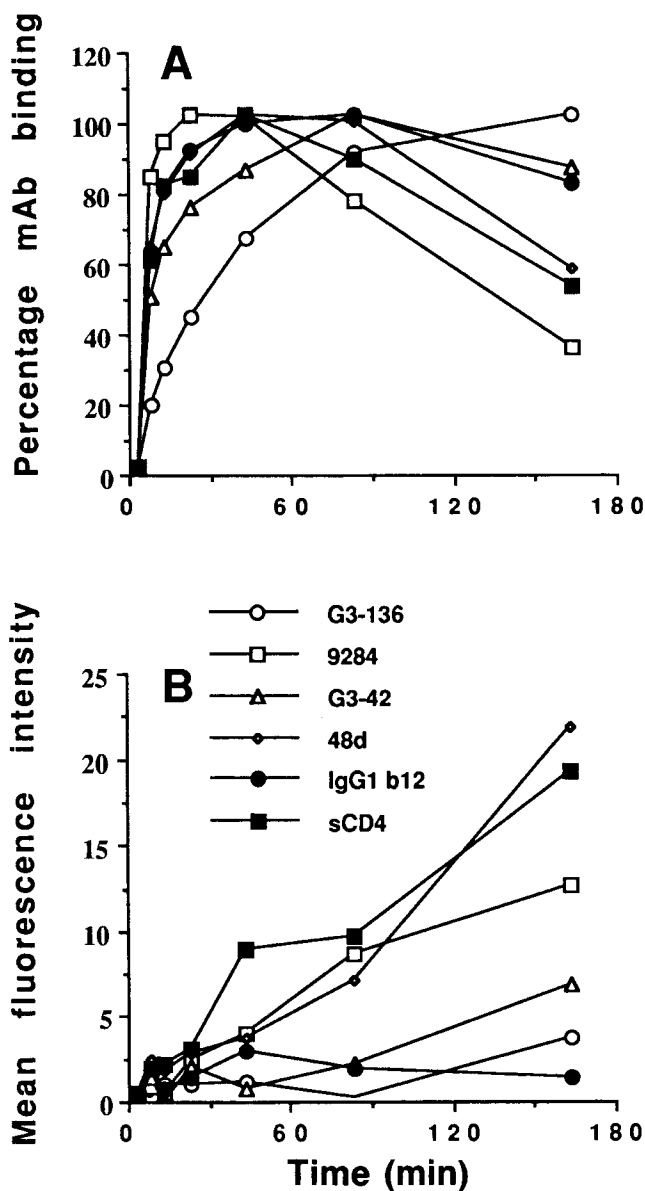‖No significant gp41 exposure was seen at any mAb concentration that was tested.

Figure 5. Time course of mAb binding and gp41 exposure. mAbs and sCD4 at a concentration of 67 nM were incubated with Hx10-infected H9 cells at 37°C. At the time points shown, aliquots of the samples were removed, washed once, and immediately fixed in 0.5% formaldehyde. The samples were washed and stained with the appropriate conjugated fluorochrome as described for Fig. 3. (*A*) Neutralizing mAb binding; (*B*) biotinylated gp41 mAb binding.

mAbs. The rate of association may, for example, influence the mAb neutralization efficiency (59).

Discussion

Our results demonstrate that the binding of neutralizing mAbs at 37°C to certain regions of HIV-1 gp120 induces the dissociation of gp120 from gp41, leading to the shedding of gp120–mAb complexes and the exposure of a previously masked epitope of gp41. The induction of such conformational changes in the viral envelope glycoproteins by soluble receptor molecules is well characterized (42–51), implying that the binding of mAbs to specific regions of gp120 may mimic, at least in part, the gp120–receptor interaction.

In a previous study in which we analyzed the dissociation of gp120 induced by sCD4 (49), we were unable to demonstrate a similar activity for the V3 loop mAb 110.5, which in the present study induces gp120 dissociation. It is unclear why there is a discrepancy between these two data sets, although in the previous study, the gp120–mAb complex was not denatured as in the current study, thus the bound 110.5 might have interfered with the detection system.

We have been unable to demonstrate formally that the dissociation of gp120 is a dominant pathway of neutralization by non-CD4/BS mAbs; despite a broad correlation between gp41 exposure, gp120 dissociation and neutralization, we cannot rule out the possibility that other factors may play an equal or more important role. Evidence from studies with other reagents and viruses, however, lends support to the idea that gp120 dissociation is an important pathway of T cell line–adapted HIV-1 neutralization. HIV-1 inactivation by sCD4 has been shown to be comprized of two components; competition for membrane CD4 binding and gp120 dissociation. Although it is not certain that gp120 dissociation is the dominant pathway of virus inactivation by sCD4, the fact that it is irreversible argues strongly for an effect on infectivity that is more profound than that of competition for receptor binding (49, 54, 56). Support for this comes from studies showing that compared to HIV-1, sCD4 is poorly able to neutralize the related immunodeficiency viruses HIV-2 and SIV (48, 66–68) despite the fact that the relative affinities of sCD4 for HIV-1, HIV-2, and SIV oligomeric gp120 differ by less than one order of magnitude (48). One factor determining HIV-2 and SIV resistance to sCD4 neutralization may be that the association between their surface and transmembrane glycoproteins is strong, and unlike HIV-1, sCD4 binding does not lead to their dissociation (48, 69). Indeed, several studies have demonstrated that sCD4 binding to HIV-1 and SIV enhances virus infectivity and fusogenicity (69–72). Very recently, two studies have demonstrated that mAbs that are neutralizing for some primary isolates of HIV-1 are enhancing for others (73, 74). Thus, by analogy with sCD4 neutralization or enhancement of T cell line–adapted HIV-1 and HIV-2/SIV, respectively, primary isolates of HIV-1 may be neutralized or their infectivity may be enhanced,

G3-42 and G3-299, and complex epitope–reactive mAbs 48d and 17b were strong neutralizers and gave high gp41 exposure. The level of gp41 exposure, however, did not always correlate with neutralization potency. For example, the V3 loop mAbs BAT123 and 110.5 are the most potent neutralizers, but do not induce greater gp41 exposure than other V3 mAbs such as 110.I and 9284, which are more weakly neutralizing. Thus, factors other than the induction of conformational changes and gp120 dissociation may contribute to the neutralization efficiency of non-CD4/BS

depending on the conformation and stability of their envelope glycoproteins.

The regions of gp120 implicated in the mAb-induced conformational changes appear to be defined by three linear epitope clusters (V2, V3, and C4) and two related, discontinuous epitopes recognized by mAbs 17b and 48d. The binding of these latter two mAbs is reduced by the substitution of amino acids in several regions, including changes in and around the CD4-binding site (see Fig. 1), although the binding of these mAbs is not inhibited by preincubation of gp120 with sCD4 (37, 75). Neither the V1/V2 nor the V3 loops are required for binding of these two mAbs (75), although competition was seen between 48d and 17b and certain mAbs reactive with the V1/V2 and V3 loops and the C4 region (Moore, J., personal communication). Since both sCD4 binding to gp120 and removal of the V2 loop by mutagenesis increases the exposure of the 17b and 48d epitopes (75), and a substitution at the base of the V2 loop abolishes mAb binding (37), it seems likely that a component of their epitopes is located close to the base of the V2 loop. There is now a considerable body of evidence demonstrating physical interactions between the V2 and V3 loops (76), V2 and C4 (11), V3 and C4 domains (30, 77, 78), and all three domains (76). Moreover, the rearrangement of these domains is implicated in the conformational changes induced by sCD4 binding to gp120, and is therefore likely to be important in the HIV-cell fusion process (47, 48, 76). It seems reasonable to propose, therefore, that the V2/V3/C4 complex on gp120 is an element involved in HIV fusion, and that the binding of ligands to this complex may inactivate viral infectivity by inappropriate or incomplete triggering of virus fusion.

By contrast with the effect of mAb binding to the V2/V3/C4 domains, the interaction of mAbs with the CD4-binding site did not induce the dissociation of gp120 from gp41; we have therefore defined two functionally distinct mAb clusters. This implies that there are likely to be at least two different mechanisms of neutralization that are dependent on the epitope specificity of the neutralizing ligand. It is interesting to note that despite the similarity between CD4-binding site mAbs and CD4 in terms of contact residues on gp120, these mAbs do not induce the same conformational changes in oligomeric gp120 as does sCD4. Indeed, the effect of sCD4 is closer to that of the V2/V3/C4 complex mAbs, suggesting that the binding of sCD4 to gp120 may reflect a combination of the effects of both groups of mAbs. Since the V2 and V3 loops of gp120 appear to play a facilitating but not essential role in HIV binding and entry into CD4$^+$ cells (76, 79), it is unlikely that direct sCD4 contact with either of these loops is necessary to induce conformational changes in gp120. Perhaps more likely is the idea that the high affinity insertion of sCD4 within the gp120 binding pocket "opens" the V2/V3/C4 complex, resulting in an increase in the exposure of the V2 and V3 loops (47, 48) and the epitopes for the mAbs 48d and 17b (37, 75).

The experiments that we describe here were carried out on a molecular clone of the cell line adapted HIV-1 isolate, LAI, since the majority of neutralizing mAbs that are currently available react with this clone in a relatively type-specific manner. To establish whether the induction of conformational changes in the HIV envelope glycoproteins by neutralizing mAbs is a general phenomenon, such studies will need to be carried out on other virus isolates. In this respect, we have recently established that V3 mAbs and 48d induce gp41 exposure in other clones of the LAI isolate (HxB2, NL43, and LAI; our unpublished results). More importantly, the relative ability of neutralizing mAbs to induce such conformational changes in HIV-1 isolates that have been isolated and passaged in PBMCs (primary isolates) needs to be investigated. Such viruses are relatively resistant to antibody neutralization (58, 73, 74). An inability of mAbs reactive with the V2/V3/C4 cluster on primary isolate gp120 to induce gp120-gp41 dissociation may explain, at least in part, their resistance to neutralization.

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