Effects of Anti-gp120 Monoclonal Antibodies on CD4 Receptor Binding by the *env* Protein of Human Immunodeficiency Virus Type 1

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Monoclonal antibodies (MAbs) to defined peptide epitopes on gp120 from human immunodeficiency virus type 1 were used to investigate the involvement of their epitopes in gp120 binding to the CD4 receptor. Recombinant vaccinia viruses were constructed that expressed either full-length gp120 (v-ED6), or a truncated gp120 lacking 44 amino acids at the carboxyl terminus (v-ED4). Binding of these glycoproteins to the CD4 receptor was detected directly with metabolically labeled gp120 or indirectly with the gp120 MAbs. Truncated gp120 from v-ED4 bound to CD4-positive cells less than 1/12 as well as gp120 from v-ED6, indicating that the C-terminal region of gp120, which is conserved in numerous isolates of human immunodeficiency virus type 1, is critical for CD4 binding. However, MAb 110-1, which recognizes a peptide contained in the region deleted from v-ED4 (amino acids 489 through 511), did not inhibit binding of gp120 to CD4. MAb 110-1 also reacted with gp120 bound to the CD4 receptor, indicating that the epitope for this antibody does not directly interact with CD4. A second MAb, 110-4, which recognizes a peptide epitope located between amino acids 303 and 323 and has potent viral neutralizing activity, also bound to gp120 on the CD4 receptor. Furthermore, pretreatment of gp120 with MAb 110-4 at concentrations approximately 1,000-fold higher than those required for complete virus neutralization inhibited subsequent CD4 binding by only about 65%. Taken together, these data suggest that neutralization mediated by antibody 110-4 does not result from binding of this MAb to the CD4-binding site of gp120.

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (1, 23, 36). Current evidence indicates that the tropism of this virus is mediated by interaction of the HIV-1 envelope protein (*env*) with the CD4 antigen on T lymphocytes (4, 17, 28, 29). The *env* gene encodes the precursor protein gp160, which is cleaved to the mature proteins gp120 and gp41 (38, 43). After virus binding, viral entry into the cell is mediated by fusion of the viral membrane with the host cell surface membrane (42). Infected cells may also fuse with one another by a mechanism which also requires interaction of the *env* protein with CD4 (25, 41).

The *env* protein has attracted much attention as a target for vaccine strategies designed to combat HIV-1 infection. *env* proteins have been shown to induce humoral (11–14, 19, 20, 33, 37, 39) and T-cell (45) immunity in animals from several species. Since the goal of these studies is to generate protective immunity against HIV-1, much work has been devoted to the elucidation of neutralizing epitopes on the *env* protein (2, 6, 11, 12, 16, 34).

It is currently not known whether blocking of gp120 binding to CD4 is an important neutralizing mechanism for HIV-1. CD4 binding involves several discontinuous regions on gp120 (18, 21), a finding consistent with the conclusion that the tertiary structure of gp120 is necessary for binding (30). Neutralization by mechanisms other than inhibition of viral binding is fairly common with other viruses (reviewed in reference 5). One neutralizing epitope on gp120 has been identified which is not involved in viral binding (2, 11, 12).

In this study, we have investigated the effects of two monoclonal antibodies (MAbs) to defined epitopes of gp120 on CD4 binding by the *env* protein of HIV-1. One of these

MAbs (110-4) recognizes a strain-specific neutralizing epitope on gp120 (16, 34; E. Kinney-Thomas et al., manuscript in preparation). The other MAb (110-1) recognizes a major immunodominant epitope on gp120 in seropositive individuals (35) and has been reported to inhibit viral binding but not viral replication (16, 30). We also have investigated the CD4-binding properties of a gp120 protein lacking a structurally conserved region (32) at the C-terminal end.

MATERIALS AND METHODS

Construction of recombinant viruses. Fragments of HIV-1 (BRU strain) proviral genomic DNA (1) were inserted into recombination vector pGS20 (27) at the *Sma*I site downstream from vaccinia virus 7.5K promoter. Two plasmids were constructed: one (pv-ED4) contains HIV sequences from nucleotides 5763 to 7373 (38), the other (pv-ED6) contains sequences from nucleotides 5673 to 8179. The HIV sequences, together with their upstream 7.5K promoter, were inserted into the thymidine kinase gene of vaccinia virus v-NY (15), and the resulting recombinant viruses were selected and purified by the general scheme of Mackett et al. (27). Recombinant v-ED6 contains HIV sequences encoding the entire gp120 and the N-terminal 20 amino acids of gp41. Recombinant v-ED4 contains the entire gp120-encoding sequence with the exception of the C-terminal 44 amino acids. Both recombinants contain 76 base pairs of 5'-untranslated sequences upstream of the gp120 initiation codon.

Virus infection. Confluent monolayers of African green monkey kidney (BSC-40) cells were infected with parental vaccinia virus, recombinant v-ED4, or v-ED6 (multiplicity of infection, 10). Six hours later, culture medium (Dulbecco modified Eagle medium with 10% fetal bovine serum) was removed and replaced with serum-free medium. Incubation was resumed for an additional 12 h before the culture
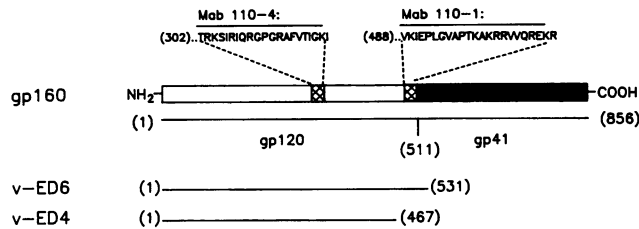
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FIG. 1. Schematic map of the HIV-1 *env* protein, indicating peptides containing epitopes for MAbs 110-1 and 110-4 as well as regions expressed by the recombinant vaccinia viruses v-ED4 and v-ED6. The numbering system used is based on the HXB2 strain as a reference (32), by using the initiating methionine as amino acid 1; for the corresponding amino acids of the BRU isolate, from which the *env* gene in recombinants v-ED4 and v-ED6 was derived, the reader is referred to reference 43. Symbols: □, region corresponding to gp120; ▨, epitopes for MAbs 110-1 and 110-4; ■, region corresponding to gp41.

medium was harvested and processed for the purification of HIV envelope glycoproteins as described below.

MAbs. Anti-CD4 MAbs OKT4 and OKT4A were purchased from Ortho Diagnostics, Inc. Leu-3A was from Becton Dickenson and Co. Other anti-CD4 antibodies (G17-2, G19-2) have been previously described (22), as have MAbs to antigens CD5 (10.2), CD2 (9.6), CD3 (G19-4), CD7 (G3-7), and CD6 (G3-5) (10).

MAbs to HIV-1 gp120 (110-1 and 110-4 [8]) were provided by M. Kathleen Shriver, Genetic Systems Corp., and were purified by three cycles of ammonium sulfate precipitation. Epitope mapping and neutralizing properties of these MAbs are described elsewhere (16; Kinney-Thomas et al., in preparation). Briefly, MAb 110-1 reacts with a peptide comprising amino acids 489 through 511 of the HIV-1 envelope protein (numbering according to reference 32), and does not neutralize HIV-1 strains LAV, ARV, or RF (16). MAb 110-4 reacts with a peptide comprising amino acids 303 through 323 (Kinney-Thomas et al., in preparation) and completely neutralizes HIV-1 (LAV and IIIB strains) at concentrations of approximately 25 ng/ml (16). Locations of the epitopes for these MAbs are shown in Fig. 1.

Metabolic labeling. (i) Glucosamine labeling. BSC-40 cells were infected with the indicated virus for 1.25 h, washed, and incubated in glucose-free Dulbecco modified Eagle medium containing 10% dialyzed fetal bovine serum (glucosamine starvation medium) for 3.25 h at 37°C. Cells were trypsinized, washed, and suspended in 1 ml of glucose-free RPMI medium containing 0.5 mCi of D-[1,6-^{3}H(N)]-glucosamine (10 to 30 Ci/mmol; New England Nuclear Corp.) and 10% dialyzed fetal bovine serum. Cells were incubated for 4 h at 37°C with rotation, diluted with 9 ml of glucosamine starvation medium, and added to a 10-cm plastic tissue culture dish for an additional 18.75 h at 37°C.

(ii) Methionine labeling. Monolayers of BSC-40 cells were infected with recombinant vaccinia viruses for 4 h at 37°C. Cells were then washed and incubated in methionine labeling medium (Dulbecco modified Eagle medium lacking methionine and containing 10% dialyzed fetal bovine serum) for an additional 4 h at 37°C. The medium was then replaced with 2 ml of methionine labeling medium containing 0.25 mCi of [^{35}S]methionine (>800 Ci/mmol; Amersham Corp.) per ml. After 4 h of labeling at 37°C, 8 ml of methionine labeling medium containing 10% of normal levels of methionine was added, and cells were incubated for an additional 11.5 h at 37°C.

Immunoprecipitation analysis. Cells were lysed in immunoprecipitation lysis buffer (150 mM NaCl, 50 mM sodium phosphate [pH 7.2], 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 5 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride) at 4 × 10^7 cells per ml. The lysate was sheared to reduce viscosity by repeated passage through an 18-gauge syringe needle. MAb (2 μg) was added, and immunoprecipitation analysis was conducted as described elsewhere (26a). Radiolabeled antigens were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10 to 20% acrylamide) and identified by autoradiography after fluorography with En^{3}Hance (New England Nuclear Corp.).

Purification of gp120. Culture medium from vaccinia virus-infected cells was collected 16 to 24 h postinfection. When unlabeled gp120 was to be purified, serum-free culture medium was used. The medium was centrifuged to remove cellular debris, treated with octyl glucoside (final concentration, 25 mM) to inactivate vaccinia virus, and added to packed Sepharose 4B-conjugated lentil lectin (Pharmacia Fine Chemicals) at a ratio of 1 ml of packed beads to 20 to 40 ml of medium. The mixture was incubated at 4°C for 16 h with rotation and then poured into a disposable syringe needle plugged with glass wool. The flowthrough was collected and reapplied to the resin. The resin was then washed sequentially with 3 to 10 volumes each of phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 8 mM sodium phosphate [pH 7.4]), phosphate-buffered saline containing 0.5% Nonidet P-40, phosphate-buffered saline containing 0.65 M NaCl, and 20 mM ammonium acetate. Bound gp120 was eluted with 3 to 10 column volumes of a solution containing 1 M α-methyl mannoside–25 mM EDTA–50 mM Tris (pH 8.0). The eluted gp120 was then dialyzed against 10 mM ammonium acetate and lyophilized before use. Solutions of purified gp120 were found to be stable in lyophilized form or in solution at 4°C but were unstable in frozen solution at −20°C.

Purification of gp120 from v-ED6-infected cells was monitored by using a double determinant immunoassay constructed as described previously (26) with monoclonal antibody 110-4 as a capture antibody and horseradish peroxidase-conjugated or ^{125}I-labeled monoclonal antibody 110-1 for detection. Final recoveries of purified gp120 from starting culture medium were estimated to be generally between 25 and 50%. Purification of gp120 from v-ED4-infected cells was monitored by immunoblotting (26).

Immunofluorescence assay for gp120 binding. Peripheral blood mononuclear cells were isolated by centrifugation on Ficoll (Litton Bionetics, Kensington, Md.). Cells (10^6/ml) were incubated with medium from recombinant or wild-type vaccinia virus-infected BSC-40 cells for 3 h at 37°C, washed, and incubated on ice for 30 min with MAb 110-1 or 110-4. Vaccinia virus in media used for binding assays was inactivated by UV light before the addition of cells. Where indicated, purified gp120 was used in place of medium. In some experiments, biotin-conjugated MAbs, prepared as described previously (7), were used. MAb binding was detected with a fluorescein isothiocyanate-conjugated goat anti-immunoglobulin second-step reagent (TAGO, Burlingame, Calif.), or with phycoerythrin-conjugated streptavidin (Becton Dickenson). Fluorescence was analyzed on a FACS IV fluorescence-activated cell sorter (Becton Dickenson).
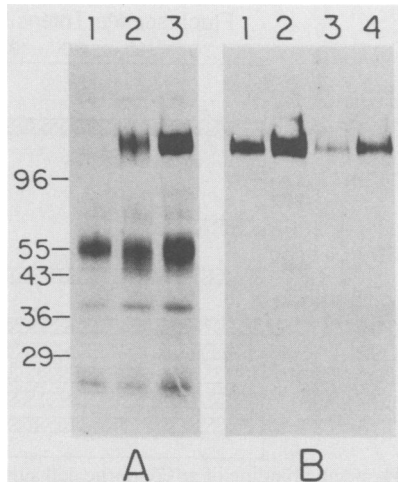
FIG. 2. Purification and binding of metabolically labeled gp120. (A) Monolayers of BSC-40 cells were infected with v-NY (lane 1), v-ED4 (lane 2), or v-ED6 (lane 3) and labeled with [^{35}S]methionine; gp120 was purified from the cell culture media by lentil lectin affinity chromatography as described in Materials and Methods. Samples of the lentil lectin eluates consisting of approximately 0.2% of the total preparation (1,000 to 2,500 cpm total; 50 to 360 cpm of immunoprecipitable gp120) were lyophilized and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Exposure time was 5 days. (B) Samples containing approximately 7,500 cpm of immunoprecipitable [^{35}S]methionine-labeled gp120 from v-ED6 infected cells (A) were lyophilized and suspended in 0.12 ml of binding buffer containing 4.3×10^7 CEM cells that had been briefly preincubated with 1 μg of MAb to the CD4 receptor. Binding of gp120 to CD4 was allowed to proceed for 2 h at 23°C, cells were collected by centrifugation, and cell-bound gp120 was isolated by immunoprecipitation with MAb 110-4 as described in Materials and Methods. Exposure time was 5 days. Lanes: 1, no antibody; 2, OKT4; 3, OKT4A; 4, G19-2.

RESULTS

Purification and biological activity of gp120 produced by recombinant vaccinia virus v-ED6. Recombinant vaccinia viruses were constructed to express gp120 and its variant forms for binding studies (Fig. 1). Recombinant v-ED6 contains sequences encoding the entire gp120 and the N-terminal 20 amino acids of gp41, including the cleavage site(s) between the glycoproteins. Recombinant v-ED4 encodes amino acids 1 through 467 of the *env* protein but lacks the C-terminal 44 amino acids of mature gp120.

Upon infection of permissive cells, recombinants v-ED4 and v-ED6 synthesized a glycoprotein which was approximately 120,000 in molecular weight and was immunoreactive with a monoclonal antibody (110-4) directed against HIV gp120. The amount of gp120 released from v-ED6-infected cells was estimated by silver staining of the purified protein (see below) to be approximately 300 μg/liter. v-ED4-infected cells generally released lesser amounts of gp120 than did v-ED6-infected cells.

An example of the purification of metabolically labeled gp120 from v-ED4- and v-ED6-infected cells is shown in Fig. 2A. BSC-40 cells were infected with either parental vaccinia virus (v-NY), v-ED4, or v-ED6, and gp120 was metabolically labeled with [^{35}S]methionine and purified from culture medium by lentil lectin affinity chromatography as described in Materials and Methods. Affinity column eluates from v-ED4 and v-ED6 infected cells (lanes 2 and 3) but not v-NY (lane 1) contained a major labeled component of M_r 120,000.

This protein was identified by immunoprecipitation experiments as gp120 (Fig. 2B). All three eluates also contained other labeled proteins, most notably a diffuse component of M_r 55,000. The latter component was not a prominent component of gp120 preparations made from serum-free supernatants from unlabeled cells (see Fig. 6B, inset).

To demonstrate the ability of v-ED6-produced gp120 to bind the CD4 receptor, partially purified preparations of [^{35}S]methionine-labeled gp120 were incubated with CD4-positive CEM cells in the presence or absence of MAbs to CD4 (Fig. 2B). After binding, cells were separated from unbound gp120 and lysed, and the amount of cell-bound gp120 was then determined by immunoprecipitation analysis. Preincubation of cells with MAbs OKT4 and G19-2, which do not block binding of HIV-1 (30, 40), did not block binding of v-ED6-derived-gp120 (compare lanes 1 and 2; preincubation with antibody OKT4 actually stimulated binding of gp120 in this and two other identical experiments). In contrast, monoclonal antibody OKT4A inhibited gp120 binding to CD4, consistent with the ability of this antibody to block binding of HIV-1 (30, 40). These results demonstrate that gp120 produced by v-ED6-infected cells is biologically active and binds to CD4 positive cells with a specificity consistent with that of intact HIV-1.

Detection of gp120 binding to CD4 receptor with MAbs. To further characterize the biological activity of gp120 purified by v-ED6-infected cells, we tested whether binding of gp120 to CD4-positive cells could be detected with MAbs directed against gp120. Peripheral blood lymphocytes from normal healthy donors were incubated with culture medium from cells infected with v-ED6 as a source of gp120 (Fig. 3). Cells were then washed and incubated with either no MAb or MAb 110-1 or 110-4 (110-1 and 110-4 are directed against different sites on gp120; Fig. 1). Cell-bound antibody was then detected by addition of fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin.

Both MAbs detected a strongly fluorescent cell population, which was not seen when anti-gp120 MAbs were omitted from the binding reaction (Fig. 3). Binding was also observed when purified gp120 from v-ED6-infected cells was used (see Fig. 6A) but not when medium from v-NY-infected cells was used, demonstrating that the fluorescent signal was dependent on gp120 from the culture medium. (In other experiments, no difference was seen in fluorescence intensity between cells treated with medium from v-NY-infected cells, cells which received no MAb 110-1, or autofluorescence of unstained cells.) Approximately 50 to 60% of lymphocytes bound gp120, whereas the other cells were similar in fluorescence to background. The fluorescence distribution pattern of gp120 binding was very similar to the pattern of CD4-positive cells (data not shown). When MAbs 110-1 and 110-4 were used together, an additive pattern of fluorescence was seen (Fig. 3B).

The specificity of blocking the gp120-binding reaction for detection of gp120 binding by different MAbs was determined (Fig. 4). Different MAbs were preincubated with CD4-positive cells before the addition of gp120 from v-ED6-infected cells. Binding was then detected by using biotinylated monoclonal antibody 110-4 followed by phycoerythrin-conjugated streptavidin. gp120 binding was completely blocked (more than 90% inhibition) by MAbs Leu-3A and OKT4A (Fig. 4), which block binding of the intact HIV-1 virus (30, 40). In addition, the anti-CD4 MAb G17-2 blocked gp120 binding. In contrast, MAb OKT4 did not block gp120 binding. MAb G19-2, which shows variable ability to block HIV-1-induced syncytium formation depending on the strain
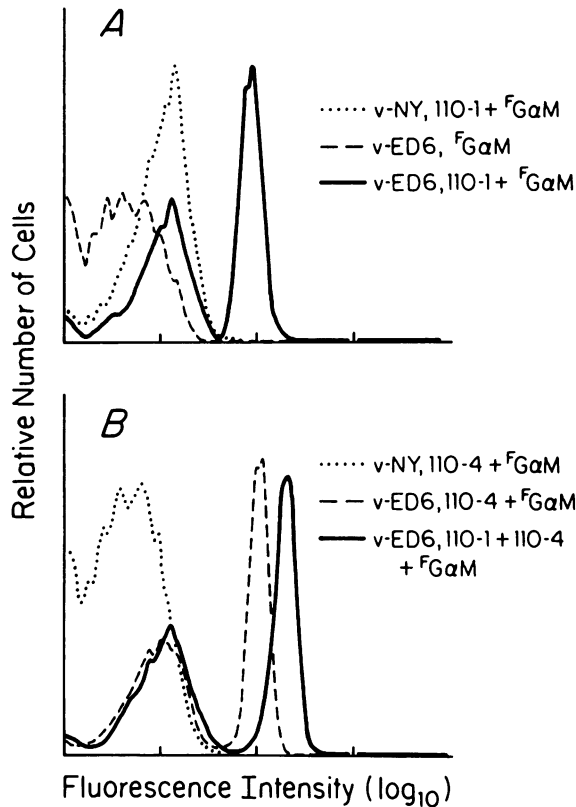
FIG. 3. Detection of gp120 bound to the cell surface by immunofluorescence. Culture medium from cells infected with wild-type (v-NY) or recombinant (v-ED6) vaccinia virus-infected cells was incubated with peripheral blood mononuclear cells for 3 h at 37°C. Cells were washed, and gp120 binding was detected in a two-step procedure by using MAb 110-1 (A) or MAb 110-4 (B) at 10 μg/ml, followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (^{F}GαM). Binding was also detected by a combination of MAbs 110-1 and 110-4.
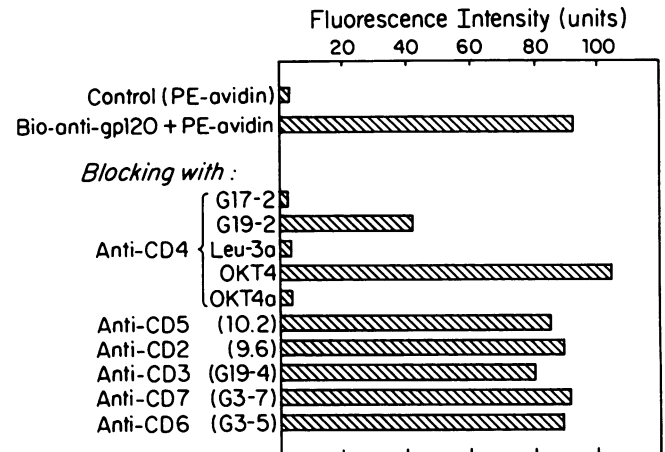

FIG. 4. CD4-specific binding of gp120 to the cell surface. Peripheral blood mononuclear cells were incubated with the indicated MAbs (10 μg/ml) for 15 min at 23°C. Cells were then incubated with culture medium from v-ED6-infected cells for 3 h at 37°C. gp120 binding was detected with biotin-conjugated MAb 110-4 followed by phycoerythrin conjugated-streptavidin. Fluorescence intensity is expressed in linear units.

of HIV-1 used (40), blocked gp120 binding only partially. MAbs to other T-cell antigens unrelated to CD4 also did not block gp120 binding. Thus, the specificity of antibody inhibition on gp120 binding was quite similar to the specificity of antibody inhibition of HIV-1 binding and syncytium formation, suggesting that the binding of recombinant gp120 mimics binding of the virus.

Since cell-bound gp120 was detectable with MAbs 110-1 and 110-4, the epitopes for both MAbs must be accessible to antibody binding once gp120 is bound to the CD4 receptor. We also tested the effects of preincubation of gp120 produced by v-ED6-infected cells with MAbs 110-1 and 110-4 on subsequent binding to CD4 (Fig. 5). Antibody 110-1 did not block binding of gp120 and at higher concentrations augmented binding. This could have resulted from either enhanced binding to CD4 by MAb 110-1-gp120 complexes or from enhanced detection of gp120 binding after preincubation with MAb 110-1. However, since augmentation of gp120 binding by MAb 110-1 was also seen in immunoprecipitation analysis (Fig. 5B), the possibility of enhanced detection of bound gp120 after MAb preincubation is unlikely. Preincubation with antibody 110-4 partially blocked gp120 binding, but only at higher concentrations (half-maximal effect at 3 μg/ml). Identical results were obtained in two other experiments: antibody 110-1 stimulated gp120 binding by 150 ±

20% (mean ± standard deviation), and antibody 110-4 inhibited binding by 63 ± 5%. Increasing the concentration of antibody 110-4 to 40 μg/ml did not result in significantly greater inhibition of binding (data not shown).

We also tested the ability of MAbs 110-1 and 110-4 to inhibit gp120 binding in immunoprecipitation analysis (Fig. 5B). In this case, MAbs and gp120 were added simultaneously to CD4-positive cells. The addition of MAb 110-1 stimulated binding of gp120 as measured by immunoprecipitation analysis, whereas the addition of MAb 110-4 resulted in a partial inhibition of binding. Densitometric analysis revealed that the stimulation of binding by MAb 110-1 was 2.2-fold, whereas MAb 110-4 inhibited binding by 30%. This experiment was repeated twice with similar results. MAb OKT4A was used as a specificity control for binding in all three experiments (Fig. 2) and in all cases inhibited binding to a greater extent than did MAb 110-4. These results confirm that MAb 110-4 partially inhibits binding of gp120 to CD4.

Comparison of binding of v-ED4- and v-ED6-produced gp120. The CD4-binding site(s) on gp120 was also studied by deletion mutation analysis. [^{3}H]glucosamine-labeled gp120 proteins produced by v-ED4- and v-ED6-infected cells were purified by lentil lectin affinity chromatography as described in Materials and Methods and tested for their abilities to bind to CEM cells. The dose-response analysis for ^{3}H-labeled v-ED4 and v-ED6 binding is shown in Fig. 6A. gp120 produced by v-ED4-infected cells bound much less readily than did gp120 from v-ED6-infected cells at all concentrations tested. By densitometric analysis of the lanes in Fig. 6A having detectable radioactivity (the three highest concentrations tested of each protein), the average ratio of bound (cell-associated) to free (in the supernatant) gp120 was determined to be 0.5 ± 0.1 for v-ED4 and 6 ± 2 for v-ED6. Thus, v-ED6 bound more than 12-fold more strongly to CD4 than did v-ED4.

We also tested the abilities of purified unlabeled gp120 proteins to bind CD4 by fluorescence-activated cell sorter analysis (Fig. 6B). Binding of purified gp120 from v-ED6 infected cells (>80% purity; see inset) bound in a dose-dependent manner to peripheral blood mononuclear cells. In
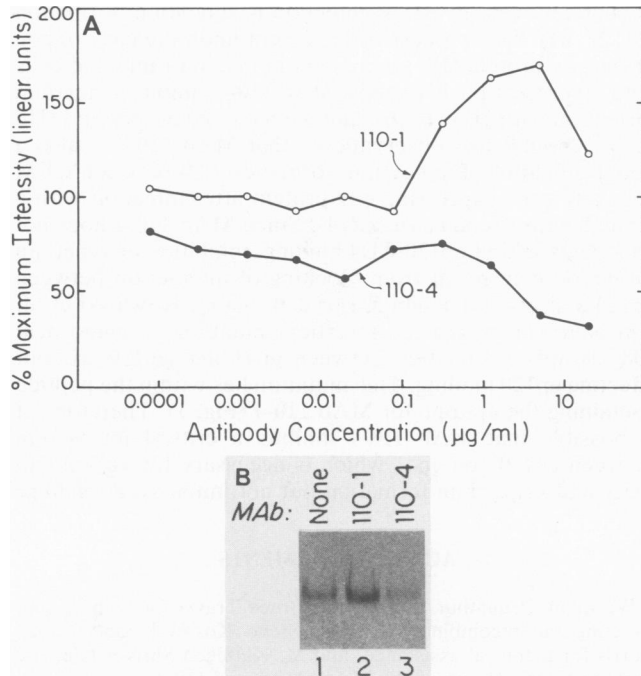
FIG. 5. Effects of pre-treatment with anti-gp120 MAbs on binding of gp120 produced by v-ED6. (A) Medium from v-ED6-infected cells was mixed with the indicated concentrations of MAbs, incubated for 15 min at 23°C, added to peripheral blood mononuclear cells, and tested for CD4 binding by fluorescent analysis by using for detection an excess (10 μg/ml) of the same antibody used for blocking. MAb binding was then detected with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin. Peak channels of fluorescence were determined, corrected for background (fluorescence was measured in the absence of gp120), and converted to linear fluorescence units. Fluorescence is expressed as the percentage of the intensity measured in the absence of MAb. (B) Samples of [^{35}S]methionine-labeled gp120 from v-ED6-infected cells (approximately 7,500 cpm of immunoprecipitable gp120) were lyophilized and added to 0.1 ml of CEM cells (3.6×10^{7} cells) that had been mixed with no MAb (lane 1) or 1 μg of MAb 110-1 (lane 2) or 110-4 (lane 3). Binding and immunoprecipitation analyses (with MAb 110-4) were carried out as described in Materials and Methods.

contrast, significant binding of gp120 produced by v-ED4 was not detectable at any of the concentrations tested. These results indicate that deletion of the C-terminal 44 amino acids of gp120 greatly reduces its ability to bind the CD4 receptor.

DISCUSSION

In this report, we have demonstrated that CD4-specific binding of gp120 produced by a recombinant vaccinia virus (v-ED6) was dependent upon the presence of amino acids 468 through 511 at the carboxy terminus of gp120, indicating that the C-terminal portion of gp120 is essential for proper CD4 binding. This region of 44 amino acids comprises the C4 and a portion (three amino acids) of the V5 domains of gp120 (31). The three amino acids lacking from V5 are also conserved in numerous HIV-1 isolates, showing relatively few substitutions in 15 different isolates (32). Thus, the portion of gp120 deleted from v-ED4 gp120 is highly conserved, suggesting an important functional role for this region.

However, two observations suggest that at least part of the region deleted from v-ED4 is unlikely to interact directly
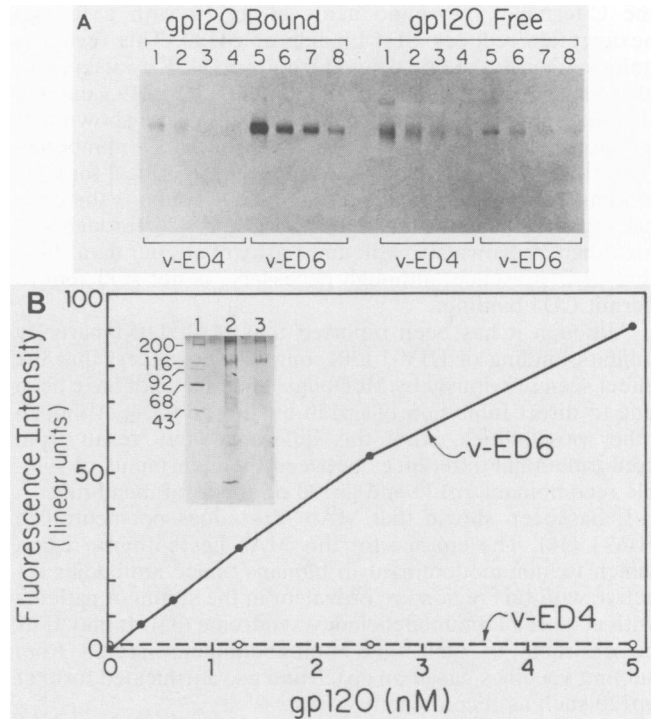

FIG. 6. Comparison of binding properties of env proteins produced by v-ED4 and v-ED6. (A) gp120 proteins produced by v-ED4- and v-ED6-infected cells were labeled with [^{3}H]glucosamine as described in Materials and Methods and tested for binding to CEM cells (4.3×10^{7}) as described in the legend to Fig. 2. After termination of the binding reaction, cells were pelleted, and the amounts of gp120 bound (in the cell pellet) and free (in the supernatant) were determined by immunoprecipitation analysis. Exposure time was 28 days. For v-ED4, cells were incubated with the following (lanes): 1, 28,800 cpm of immunoprecipitable gp120; 2, 14,400 cpm; 3, 7,200 cpm; 4, 3,600 cpm. For v-ED6, cells were incubated with the following (lanes): 5, 51,600 cpm; 6, 25,800 cpm; 7, 12,900 cpm; 8, 6,450 cpm. (B) Unlabeled gp120 proteins from v-ED4- and v-ED6-infected cells were purified by lentil lectin chromatography as described in Materials and Methods and tested for their abilities to bind to peripheral blood mononuclear cells by fluorescence-activated cell sorter analysis as described in the legend to Fig. 3. Concentrations of gp120 proteins were estimated from silver-stained gels of the purified preparations. Inset, Photograph of a silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel showing the following (lanes): 1, molecular weight markers; 2, v-ED4 gp120 (60 ng); 3, v-ED6 gp120 (30 ng).

with the CD4 receptor. First, MAb 110-1, which bound to an epitope included within the C-terminal 23 amino acids of gp120 (Fig. 1), was able to recognize gp120 bound to the cell surface (Fig. 3), showing that the epitope is not masked by its interaction with CD4. This would be unlikely if that region were to directly interact with CD4. Furthermore, preincubation of gp120 with MAb 110-1 did not inhibit gp120 binding (Fig. 5). These observations may indicate that the epitope for MAb 110-1 does not directly interact with CD4 but instead functions to maintain the structure of a binding site elsewhere in gp120. Alternatively, it is possible that the region deleted from v-ED4 which lies outside the epitope for MAb 110-1 contains a site that interacts directly with CD4.

It is of interest to compare the region of gp120 which was deleted from the v-ED4 construct (amino acids 468 through 511) with regions shown by others to be involved in CD4 binding. Kowalski et al. (18) have shown that replacement of

the C-terminal 39 amino acids of gp120 with a foreign hexapeptide reduces CD4 binding of gp120. This region is quite similar to the one deleted from v-ED4. Kowalski et al. also identified mutations at amino acids 363, 419, and 473 that disrupt CD4 binding. Lasky et al. (21) have shown that a region comprising amino acids 426 through 437 (numbering from the HXB2 reference strain [32]) is also critical for CD4 binding. Since gp120 produced by v-ED4 contains the critical regions at amino acids 363, 419, and 426 through 437 identified by Kowalski et al. and Lasky et al., our data show that their presence, although necessary, is not sufficient to permit CD4 binding.

Although it has been reported that MAb 110-1 partially inhibits binding of HIV-1 (30), our results suggest that the effect seen previously by McDougal et al. may not have been due to direct inhibition of gp120 binding to CD4. Although other possibilities exist, this difference may result from conformational differences between the C terminus of soluble recombinant gp120 and gp120 on the viral membrane.

It has been shown that MAb 110-1 does not neutralize HIV-1 (16). The epitope for this MAb lies within a region which is immunodominant in humans, since antibodies reactive with this region are prevalent in the serum of patients with acquired immunodeficiency syndrome (35). It may thus be desirable to omit this immunodominant region from subunit vaccines based on gp120 and use a truncated form of gp120 such as recombinant v-ED4.

MAb 110-4 defines a strain-specific neutralizing epitope of gp120 (2, 12, 16, 34). Since gp120 bound to CD4 was detectable with MAb 110-4 (Fig. 3), the epitope for this antibody must be accessible to antibody after CD4 binding. This indicates that the epitope for MAb 110-4 either is not directly involved in CD4 binding or undergoes a conformational change to expose the epitope after the initial binding event.

Several observations favor the possibility that the epitope for this MAb is not directly involved in CD4 binding. First, although preincubation of gp120 with MAb 110-4 resulted in partial blocking of CD4 binding (Fig. 5), the inhibition required saturating antibody concentrations (>10 µg/ml) for maximal effect. In contrast, complete neutralization of HIV-1 is achieved by concentrations of MAb 110-4 of 25 ng/ml (16). Although virus neutralization assays and gp120-binding assays are conducted under different conditions and cannot be directly compared, the differences in antibody concentration required to inhibit virus replication and gp120 binding are large enough to suggest that direct inhibition of gp120 binding is not the mechanism of viral neutralization by MAb 110-4.

Second, the neutralizing activity of MAb 110-4 is strain specific (16), presumably as result of the fact that the epitope for this MAb lies largely within the V3 domain of gp120 (31), which is not well conserved between different viral isolates. It is unlikely that a region which directly interacts with the CD4 receptor could tolerate this degree of sequence divergence. Finally, the epitope for this MAb has not been implicated as being important for CD4 binding in any previous studies (18, 21). The available evidence strongly suggests that HIV-1 neutralization by MAb 110-4 does not result from binding by this MAb to the CD4-binding site of gp120.

If neutralization by MAb 110-4 does not result from inhibition of CD4 binding, the MAb may disrupt other events involving gp120 which occur after virus binding. Envelope proteins of other viruses have been shown to participate in viral penetration and cell fusion events which are distinct from initial binding of virus to the host cell (3). An example

of such an event in HIV-1 infection is syncytium formation (24, 25, 41). This process is dependent upon the gp41 region of the env protein (18) and results in fusion of infected cells with other CD4-positive cells. MAb 110-4 inhibits syncytium formation with HIV-1 (16), and we have recently found (Hu et al., unpublished observations) that MAb 110-4 is also a potent inhibitor of syncytium formation (24) between CEM cells and cells expressing env protein after infection with a recombinant vaccinia virus (14). Since MAb 110-4 does not effectively inhibit gp120-CD4 binding, inhibition of synctium formation may result from blocking of interaction between gp120 and gp41 or inhibition of cell fusion. Kowalski et al. (18) have shown that an insertion mutation at amino acid 308 disrupts association between gp41 and gp120 without affecting gp120 binding. This mutation lies within the peptide containing the epitope for MAb 110-4 (Fig. 1). Therefore, it is possible that MAb 110-4 inhibits a critical interaction between gp120 and gp41 which is necessary for viral infectivity and syncytium formation but not initial viral binding.

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