JOURNAL OF VIROLOGY, Feb. 1993, p. 913–926
0022-538X/93/020913-14$02.00/0

Vol. 67, No. 2

CD4 Molecules with a Diversity of Mutations Encompassing the CDR3 Region Efficiently Support Human Immunodeficiency Virus Type 1 Envelope Glycoprotein-Mediated Cell Fusion

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Received 18 August 1992/Accepted 4 November 1992

The third complementarity-determining region (CDR3) within domain 1 of the human CD4 molecule has been suggested to play a critical role in membrane fusion mediated by the interaction of CD4 with the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein. To analyze in detail the role of CDR3 and adjacent regions in the fusion process, we used cassette mutagenesis to construct a panel of 30 site-directed mutations between residues 79 and 96 of the full-length CD4 molecule. The mutant proteins were transiently expressed by using recombinant vaccinia virus vectors and were analyzed for cell surface expression, recombinant gp120-binding activity, and overall structural integrity as assessed by reactivity with a battery of anti-CD4 monoclonal antibodies. Cells expressing the CD4 mutants were assayed for their ability to form syncytia when mixed with cells expressing the HIV-1 envelope glycoprotein. Surprisingly in view of published data from others, most of the mutations had little effect on syncytium-forming activity. Normal fusion was observed in 21 mutants, including substitution of human residues 85 to 95 with the corresponding sequences from either chimpanzee, rhesus, or mouse CD4; a panel of Ser-Arg double insertions after each residue from 86 to 91; and a number of other charge, hydrophobic, and proline substitutions and insertions within this region. The nine mutants that showed impaired fusion all displayed defective gp120 binding and disruption of overall structural integrity. In further contrast with results of other workers, we observed that transformant human cell lines expressing native chimpanzee or rhesus CD4 efficiently formed syncytia when mixed with cells expressing the HIV-1 envelope glycoprotein. These data refute the conclusion that certain mutations in the CDR3 region of CD4 abolish cell fusion activity, and they suggest that a wide variety of sequences can be functionally tolerated in this region, including those from highly divergent mammalian species. Syncytium formation mediated by several of the CDR3 mutants was partially or completely resistant to inhibition by the CDR3-directed monoclonal antibody L71, suggesting that the corresponding epitope is not directly involved in the fusion process. We observed that CDR3 synthetic peptide derivatives inhibited syncytium formation mediated by the mutant CD4 molecules with the same potency as for wild-type CD4. In contrast with our earlier findings with such peptides, we observed that mutation of the CDR3 region (E87G) in the soluble CD4 protein had no effect on its ability to inhibit syncytium formation or to stimulate gp120 release from the HIV-1 envelope glycoprotein. These results suggest that the previously described fusion-related activities of the CDR3 peptide derivatives are not due to their ability to compete for or to mimic the function of the corresponding region in the native CD4 protein. Taken together, the present findings challenge the prevailing notion that the CDR3 region of CD4 plays a critical role in HIV-1 envelope glycoprotein-mediated membrane fusion.

The primary route of human immunodeficiency virus (HIV) infection of helper T lymphocytes and macrophages is initiated by the binding of the external subunit of the HIV envelope glycoprotein (gp120) to CD4 molecules on the target cell surface (reviewed in references 12, 23, and 30). Following specific binding there is a direct, pH-independent fusion event between the HIV virion and the plasma membrane of the target cell (28, 29, 44, 45). By an analogous mechanism, HIV-infected cells expressing the viral envelope glycoprotein readily fuse with CD4-bearing cells, resulting in the formation of multinucleated giant cells (syncytia). The fusion event may be mediated by specific CD4-induced changes in envelope glycoprotein structure which activate its fusogenic property, possibly by exposing the hydrophobic amino terminus of the transmembrane subunit (gp41). The molecular interactions underlying CD4-envelope glycoprotein-mediated membrane fusion remain obscure (see references 1, 16, and 34 for reviews and discussions of various models).

Domain 1 of CD4 contains all the determinants essential for high-affinity gp120 binding (2, 13). This domain shares sequence (27) and structural (27, 39, 48) features with the immunoglobulin light-chain variable region, including a framework structure with two β sheets, as well as three loops which are analogous to the complementarity-determining regions (CDRs) of the immunoglobulin molecule. Several independent mutagenesis studies have identified the stretch of amino acid residues 40 to 55, including the region corresponding to CDR2, as critical for the binding of gp120 (12, 23, 30). In addition, the regions around CDR3 have been proposed to be critical for membrane fusion mediated by the CD4-envelope glycoprotein interaction, possibly independent from any involvement in gp120 binding. The initial evidence came from studies showing that synthetic peptide

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derivatives overlapping this region inhibit HIV infection and syncytium formation (7, 20, 22, 25, 26, 36, 43a). The second line of evidence involved genetic studies (11, 21), including analysis of CD4 molecules with different sequences in the CDR3 region (11). Rhesus and chimpanzee CD4 molecules expressed on the surface of transformant human cell lines were reported to be infectible with HIV-1 but incapable of supporting syncytium formation when mixed with cells expressing the HIV-1 envelope glycoprotein; direct-binding studies indicated that gp120 bound identically to human and chimpanzee CD4 (11). Site-directed mutagenesis studies suggested that the inability of chimpanzee CD4 to mediate cell fusion could be attributed to a single amino acid difference at position 87 in the CDR3 region: glutamic acid in human CD4 versus glycine in chimpanzee CD4. In transfection experiments, changing Glu to Gly at position 87 (E87G) in the human CD4 molecule reportedly abolished the ability to support syncytium formation, whereas the reciprocal substitution (G87E) reportedly rendered chimpanzee CD4 syncytium competent (11). These results suggested that the CDR3 region of CD4 participates in cell fusion independent of any role in gp120 binding (11). Interestingly, the E87G substitution has also been found to diminish the ability of synthetic peptide derivatives from this region to inhibit syncytium formation (7, 26). The third line of evidence derived from studies with monoclonal antibodies (MAbs) directed against the CDR3 region, which were reported to inhibit HIV infection and syncytium formation without disrupting CD4-gp120 binding (46).

A related series of findings concerns the ability of soluble forms of CD4 (sCD4) to induce changes in the structure of the HIV-1 envelope glycoprotein which may be related to activation of its fusogenic property. sCD4 has been shown to induce the release of gp120 from the gp120-gp41 complex expressed at the virion or cell surface (7, 19, 24, 32, 33). We (7) and others (41) have found that this effect can be achieved by using various types of peptide derivatives which overlap the CDR3 region. Consistent with the points noted above, we observed that the E87G substitution reduces the ability of the peptide derivatives to promote gp120 release (7). These results suggested that the CDR3 region might be involved in CD4-induced structural changes in the envelope glycoprotein related to membrane fusion.

Prompted by these findings, we conducted a detailed site-directed mutagenesis study of the full-length CD4 molecule to analyze the involvement of the region encompassing CDR3 in membrane fusion mediated by the HIV-1 envelope glycoprotein. The effects of mutation of the CDR3 region on sCD4 inhibition of syncytium formation and sCD4 stimulation of gp120 release were also examined. Our results challenge the notion that functional interaction between CD4 and the HIV-1 envelope glycoprotein requires highly specific sequences in the region encompassing CDR3.

(A preliminary report of this work has been presented previously [8].)

MATERIALS AND METHODS

Cell lines and culture conditions. Cell lines were obtained from the following sources. HeLa and BSC-1 cells were from the American Type Culture Collection, Rockville, Md.; A2.01 and 8E5 were from K. Clouse, Food and Drug Administration, Bethesda, Md.; H9/HTLV-IIIB was from M. Robert-Guroff, National Cancer Institute, Bethesda, Md.; and HeLa-Hs (expressing human CD4), HeLa-Pt (expressing chimpanzee CD4), and HeLa-Mm (expressing rhesus macaque CD4) were from B. Seed, Harvard Medical School, Boston, Mass. Adherent cell lines were maintained in Eagle's minimal essential medium (MEM) or Dulbecco modified Eagle's MEM (Quality Biologicals, Rockville, Md.) supplemented with 10% fetal bovine serum (FBS; Hazleton Laboratories), 2 mM L-glutamine, and antibiotics. Suspension cell medium contained RPMI 1640 (Quality Biologicals) supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and antibiotics. Cells were maintained at 37°C in 5% CO_2 atmosphere.

Reagents. Restriction endonucleases were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind., or Bethesda Research Laboratories, Gaithersburg, Md. The Klenow fragment of DNA polymerase I and T4 DNA ligase were from Bethesda Research Laboratories. *Taq* DNA polymerase was from Perkin Elmer Cetus, Norwalk, Conn. DNA-sequencing reagents were from United States Biochemical Corp., Cleveland, Ohio.

Murine anti-CD4 MAbs were obtained from the following sources. OKT4, OKT4A, OKT4D, OKT4E, and OKT4F were from P. Rao, Ortho Pharmaceuticals, Raritan, N.J.; Leu-3A was from Becton Dickinson; and L71 was from D. Buck, Becton Dickinson. Polyclonal rabbit antiserum to human CD4 was obtained from American Bio-Technologies, Cambridge, Mass. The murine anti-HIV gp120 MAb produced from hybridoma 902 was from B. Chesebro, National Institute of Allergy and Infectious Diseases, Hamilton, Mont., and was provided as a tissue culture supernatant by J. Yewdell, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin (Ig), goat anti-mouse IgG beads, and protein G-agarose were purchased from Calbiochem, La Jolla, Calif.

The CD4 synthetic peptide derivatives used (donated by L. Eiden, National Institute of Mental Health, Bethesda, Md.) were as follows: 18 [$T_bYIC_bE_bVEDQK_{ac}EE$, CD4-(81–92), human sequence], 18C [$T_bYIC_bE_bVGDQK_{ac}EE$, CD4-(81–92), chimpanzee sequence], and 30* (acetyl-K_{ac} $EEIC_bE_bVEDQT_bY$-amide, scrambled human sequence) (7). The subscripts b and ac represent benzyl and acetyl derivatizations, respectively, of the preceding amino acid.

CD4 constructs and recombinant vaccinia viruses. An oligonucleotide cassette mutagenesis strategy was used to generate a large number of mutants in the desired region of the full-length CD4 molecule (see Fig. 1 and Table 1). Plasmid pCD4-GEM4 (obtained from A. Rabson, National Institute of Allergy and Infectious Diseases, Bethesda, Md.) has a 1.8-kb *Eco*RI-*Bam*HI insert containing the entire CD4 cDNA (27), cloned into the multiple cloning site of plasmid pGEM4 (Promega, Madison, Wis.). Numbering of the CD4 nucleotide sequence is as originally published (27) (GenBank accession no. M1 2807). Two new unique restriction endonuclease sites, *Nde*I and *Spe*I, were generated by introducing single-base changes at nucleotide positions 399 and 435, respectively, of the CD4 nucleotide sequence by using polymerase chain reaction technology (17). These alterations did not change the amino acid coding sequence of CD4. The resulting plasmid, designated pCD4-GEM4.NS.1, was used for oligonucleotide-directed mutagenesis. Most mutations were created by removing the *Nde*I-*Spe*I fragment from pCD4-GEM4.NS.1 and subcloning complementary synthetic oligonucleotide pairs containing the desired nucleotide substitutions plus overlapping 5′ ends corresponding to the *Nde*I and *Spe*I sites (see Fig. 1 and Table 1). In some cases, mutations were generated with longer oligonucleotide pairs

with overlapping ends corresponding to an existing upstream unique *Afl*II site at CD4 nucleotide 369 in conjunction with the *Spe*I site (see Fig. 1 and Table 1). The mutant constructs were verified by DNA sequence analysis (40) of a 200-bp region encompassing the substituted inserts.

Plasmid vectors were constructed for introduction of the mutated sequences into the CD4 cDNA and subsequent recombination into the vaccinia virus genome. For full-length CD4 (wild type and mutant), we constructed a series of plasmids in which the CD4 cDNAs were linked to the vaccinia virus compound P7.5 early/late promoter and flanked by vaccinia virus thymidine kinase sequences. An intermediate plasmid, pCB-3, was constructed by excising a 1.8-kb *Eco*RI-*Sal*I fragment from plasmid pEB-8 (4) and ligating it into pSC59 (see below) cut with *Eco*RI and *Stu*I. From this, a 1.8-kb *Eco*RI-*Spe*I fragment was excised and blunt-end cloned into the *Sma*I site of pSC11 to generate a plasmid designated pCB-7. To generate the plasmids used to construct the vaccinia virus recombinants for the wild-type CD4 and the mutants obtained by oligonucleotide mutagenesis, a 0.32-kb *Afl*II-*Nhe*I fragment from each of the CD4 plasmid constructs described above was excised and subcloned into the corresponding site in plasmid pCB-7. This set of plasmids was used to generate vaccinia virus recombinants vCB-100 (wild-type CD4) and vCB-100.1 to vCB-100.29 (mutant CD4s) (see Table 1). For the Ser-Arg insertions after positions 21, 48, and 91, a restriction fragment substitution method was used. A 0.6-kb *Eco*RI-*Sac*I fragment from the appropriate plasmid containing the mutation in full-length CD4 (31) was cloned into pCB-3. From these intermediate constructs, the entire CD4 cDNAs containing the mutations were excised with *Eco*RI and *Spe*I and blunt-end ligated into the *Sma*I site of pSC11. The resulting plasmids were used to generate vaccinia virus recombinants vCB-100.30 to vCB-100.32 encoding mutant CD4s (see Table 1).

For sCD4 (wild type and mutant), we constructed plasmids in which the cDNAs encoding the four extracellular domains of CD4 (372 amino acid residues) were linked to a newly developed vaccinia virus synthetic strong early/strong late promoter (12a), and flanked by vaccinia virus thymidine kinase sequences. Plasmid pCB-5 containing the wild-type sCD4 sequence was generated by inserting a 1.25-kb *Eco*RI-*Sal*I fragment from pCD4LTM1 (31) into the multiple cloning site of plasmid pSC59 (12a) cut with *Eco*RI and *Stu*I. Mutant sCD4 sequences were obtained by inserting the 0.32-kb *Afl*II-*Nhe*I fragments from the mutant plasmids obtained by oligonucleotide mutagenesis into the corresponding site in pCB-5. The resulting plasmids were used to generate vCB-200S encoding wild-type sCD4 and vCB-200.4S encoding sCD4 with the E87G substitution.

Vaccinia virus recombinants for the mutant CD4 constructs were generated by homologous recombination into the thymidine kinase locus of wild-type vaccinia virus WR. Recombinant plaques were obtained by thymidine kinase selection coupled with β-galactosidase screening (for viruses encoding full-length CD4) or slot-blot DNA hybridization (for viruses encoding sCD4), using standard methods (15).

Vaccinia virus recombinant vPE16 (14) encodes a full-length HIV-1 envelope glycoprotein, which is correctly processed to give a gp120-gp41 complex; expression is directed by the vaccinia virus P7.5 promoter. Vaccinia virus recombinant vPE6 (a gift from P. Earl, National Institute of Allergy and Infectious Diseases, Bethesda, Md.) encodes a truncated HIV-1 envelope glycoprotein representing the entire soluble gp120 molecule (rgp120). The gp120 sequence is under control of the bacteriophage T7 promoter; the secreted protein is expressed by coinfection with vPE6 and vaccinia virus recombinant vTF7-3, which encodes the bacteriophage T7 RNA polymerase directed by the vaccinia virus P11 promoter (18).

Expression of CD4 and HIV-1 envelope glycoprotein molecules. Full-length CD4 and HIV-1 envelope glycoprotein molecules were expressed on the cell types indicated for each experiment. Suspension cells were washed once with medium containing 2.5% FBS and suspended at 10^7 cells per ml, and recombinant vaccinia viruses were added at a multiplicity of 10 PFU per cell. After a 1-h adsorption period, cells were diluted to a density of 5×10^5/ml and placed in a 37°C CO_2 incubator for 10 to 15 h. Adherent cells were infected as a monolayer with recombinant vaccinia viruses at a multiplicity of 10 PFU per cell. After a 2-h incubation, the cells were trypsinized, washed, and incubated in suspension at a density of 5×10^5/ml for 10 to 15 h.

sCD4 proteins (wild-type or mutant forms) were obtained by infecting cells with the indicated vaccinia virus vector; as a negative control, cells were infected with vaccinia virus WR. Tissue culture flasks (150 cm^2) of confluent BSC-1 cells (approximately 1.5×10^7 cells per flask) were infected for 2 h at 37°C at a multiplicity of infection of 10 PFU per cell (5 ml per flask) in Eagle's MEM–2.5% FBS in a CO_2 incubator. Cells were washed twice with 15 ml of serum-free OPTI-MEM (reduced-protein medium; GIBCO, Gaithersburg, Md.) and then overlaid with 15 ml of OPTI-MEM. The infected cells were allowed to express protein for 24 h. The culture supernatants containing the secreted proteins were harvested, passed through a low-protein-binding 0.2-μm Acrodisc filter (Gelman Sciences), and concentrated 30-fold in a Centriprep concentrator with a 30-kDa cutoff (Amicon Corp., Beverly, Mass.). The amounts of sCD4 in the concentrated samples were quantified by Western immunoblot analysis of serial dilutions, using an sCD4 sample of known concentration (gift of S. Johnson, Upjohn Pharmaceuticals, Kalamazoo, Mich.) to generate a standard curve. Values were determined by laser densitometry of fluorograms with an LKB Ultroscan XL.

CD4 immunoprecipitation and CD4-rgp120 coprecipitation studies. Metabolic labeling of CD4 and HIV-1 envelope glycoprotein was performed with ^{35}S-labeled methionine (>800 Ci/mmol; Amersham) as described previously (6). Cell lysates were prepared by mixing the vaccinia virus-infected cells in lysis buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.5% [vol/vol] Triton X-100, 0.5% [vol/vol] Nonidet P-40, 0.4 mM phenylmethylsulfonyl fluoride, 0.02 mM N^{α}-tosyl-lysyl-chloromethyl ketone [Calbiochem], and 0.02 mM N^{α}-tosyl-phenylalanyl-chloromethyl ketone [Calbiochem]). Insoluble material was removed by centrifugation.

For MAb immunoprecipitation studies, aliquots of metabolically labeled CD4-containing cell lysates were mixed with 1 μg of each anti-CD4 MAb in lysis buffer. After rotation at 4°C overnight, the immune complexes were precipitated by the addition of 50 μl of a 20% suspension of goat anti-mouse IgG-agarose and washed twice with lysis buffer without protease inhibitors.

The binding and coprecipitation conditions of CD4 and rgp120 were essentially as described previously (31). Briefly, aliquots of metabolically labeled lysates were precleared and the supernatants were divided into two portions. The first portion was immunoprecipitated directly by addition of 1 μl of rabbit antiserum against human CD4 followed by 50 μl of a 20% suspension of protein G-agarose. To the second portion was added 200 μl of medium containing [^{35}S]methio-

nine-labeled rgp120. After preincubation for 8 h at 4°C, 300 μl of hybridoma 902 supernatant was added, and the samples were rotated overnight at 4°C. Immune complexes were precipitated by the addition of 50 μl of a 20% suspension of goat anti-mouse IgG-agarose. The agarose beads were washed twice with lysis buffer minus protease inhibitors, and proteins were solubilized by boiling in reducing sample buffer containing 8 M urea.

Polyacrylamide gel electrophoresis and Western blotting. Protein samples were resolved by electrophoresis on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. Gels containing metabolically labeled material were treated with Fluoro-Hance (Research Products International Corp., Mount Prospect, Ill.) and visualized by fluorography. For Western blotting, proteins were electrophoretically transferred to nitrocellulose membranes and detected with rabbit anti-CD4 or anti-gp120 antiserum followed by [^{125}I]protein G (740 MBq/mg, 20 mCi/mg; Amersham). Proteins were visualized by autoradiography.

Flow cytometry. To measure cell surface expression of vaccinia virus-encoded CD4, cells were washed once with phosphate-buffered saline (PBS) containing 2.5% (vol/vol) FBS and 0.02% (wt/vol) NaN_3 and incubated for 1 h at 4°C with rabbit anti-CD4 antiserum. The cells were washed with PBS-FBS-NaN_3, stained with FITC-labeled goat Ig prepared from antisera against rabbit Ig, and fixed in PBS–2.0% paraformaldehyde. Background fluorescence was measured by staining cells infected with vaccinia virus WR. Surface fluorescence was quantitated by using an EPICS Profile flow cytometer.

Syncytium formation assay. HIV-1 chronically infected 8E5 or H9/HTLV-IIIB cells and cells infected with recombinant vaccinia viruses were washed twice in medium and suspended at 10^6 cells per ml in suspension cell medium. A 0.1-ml portion of a CD4-expressing cell suspension was mixed with a 0.1-ml portion of an HIV-1 envelope glycoprotein-expressing cell suspension in 96-well flat-bottom tissue culture plates (Costar, Cambridge, Mass.) and incubated at 37°C in a 5% CO_2 atmosphere. Syncytium formation was assessed hourly by microscopically counting the number of giant cells per random field (at ×200 magnification) with an inverted microscope. Quantitative data represent the means of quadruplicate samples. In some experiments adherent cell lines infected with vaccinia virus recombinants encoding CD4 were trypsinized, washed, and incubated in suspension in medium at 5×10^5 cells per ml to allow CD4 expression. The cells were then washed and used in the syncytium formation assays as above.

For fusion inhibition studies we used the following methods. For inhibition by MAb OKT4A or MAb L71, the CD4-bearing cells were preincubated with 1 μg of MAb OKT4A per ml or 5 μg of MAb L71 per ml for 30 min at 37°C in a 5% CO_2 atmosphere, prior to the addition of BSC-1 cells expressing HIV-1 envelope glycoprotein encoded by vaccinia virus recombinant vPE16. For inhibition by sCD4, the envelope glycoprotein-expressing cells were preincubated with the sCD4 proteins over a range of concentrations (0 to 400 nM) for 30 min at 37°C in a 5% CO_2 atmosphere and then mixed with HeLa cells expressing CD4 encoded by vaccinia virus vCB-100. For inhibition by the CD4 synthetic peptide derivatives 18, 18C, and 30*, the envelope glycoprotein-expressing cells were preincubated over a range of concentrations (0 to 200 μM) for 2 h at 37°C in a 5% CO_2 atmosphere and then mixed with HeLa cells expressing CD4 encoded by vaccinia virus vCB-100.

gp120 release assay. BSC-1 cells expressing the recombinant HIV-1 envelope glycoprotein encoded by vPE16 were incubated with sCD4 at a range of concentrations (0 to 500 nM) for 3 h at 37°C in a 5% CO_2 atmosphere. Release of gp120 was assessed by Western blot analysis of the cell supernatant and lysate fractions with polyclonal rabbit anti-gp120 antiserum as previously described (7). The conditioned media containing wild-type and mutant sCD4 encoded by vaccinia virus recombinants were concentrated and analyzed as described above; concentrated conditioned medium from cells infected with vaccinia virus WR was used to determine the background level of gp120 release.

RESULTS

Construction of vaccinia virus recombinants encoding mutant CD4 sequences. The goals of this study were to expand on previously reported mutagenesis experiments which suggested a critical role for the CDR3 region of CD4 in membrane fusion, distinct from an involvement in gp120 binding (11). To this end, we used cassette mutagenesis to construct a large number of plasmids containing specific mutations in this region of the full-length human CD4 cDNA. The mutant CD4 plasmid constructs were used to generate a complementary set of recombinant vaccinia viruses. By a variety of criteria (summarized in reference 7), CD4-HIV envelope glycoprotein-mediated cell fusion with the vaccinia virus-based expression system faithfully reproduces the specificity observed with HIV-infected cells. By infecting cells at a high multiplicity of the vector, the desired proteins are readily uniformly expressed, thereby circumventing problems often associated with less efficient transfection methods.

A total of 30 CD4 constructs with mutations in the CDR3 region were generated by substituting the appropriate oligonucleotide pairs or restriction fragments (Fig. 1; Table 1), as described in Materials and Methods. These mutants could be categorized into two sets. The first set consisted of mutants with a series of single and multiple amino acid substitutions around the CDR3 region, including replacement of the sequence from residues 85 to 95 of human CD4 with the corresponding sequences of mouse, rhesus, and chimpanzee CD4. The second set consisted of mutants with insertion mutations in this region, including a series of Ser-Arg double amino acid insertions after each residue from 79 through 91; the latter mutants complemented a series of previously described mutants with Ser-Arg insertion mutations in sCD4 (31). As controls we constructed a mutant with a Ser-Arg insertion after residue 48 in the CDR2 region and another mutant with a Ser-Arg insertion after residue 21. The former insertion has been previously shown to severely impair gp120 binding when introduced into sCD4, whereas the latter mutation has little effect (31). In the experiments below, detailed results are presented for a selected subset of mutants, and the data for all the mutants are summarized in Table 2.

Expression of mutant CD4 sequences. Expression of the mutant molecules was assessed after infection of human CD4-negative cells with the corresponding vaccinia virus recombinants. Western blot analysis of total cell lysates with rabbit polyclonal anti-CD4 antiserum indicated that all the mutant CD4 molecules were expressed at comparable levels. Figure 2 shows representative results with HeLa cells, and Table 2 summarizes the data for all the mutants expressed in A2.01 cells. Relative cell surface expression on A2.01 cells was examined by flow cytometry after staining with polyclonal rabbit antiserum to human CD4 followed by FITC-labeled anti-rabbit Ig. Representative flow cytometry pro-

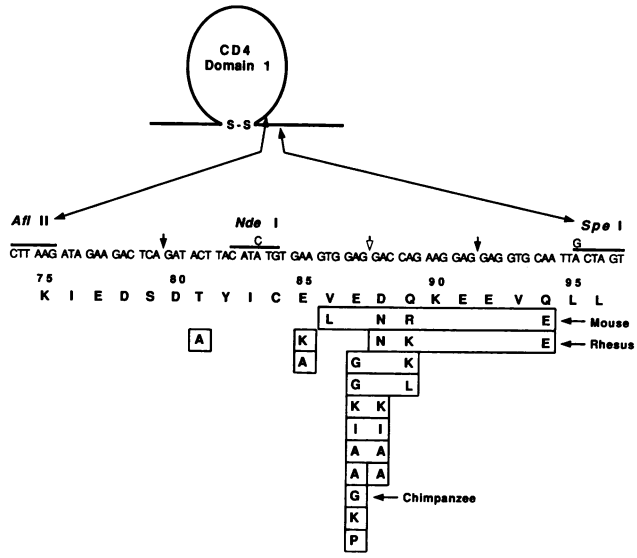

FIG. 1. Schematic diagram of the first Ig-like domain of human CD4, highlighting the strategy for mutagenesis of the sequence encompassing the CDR3 region. Two new unique restriction endonuclease sites, *Nde*I and *Spe*I, were generated by introducing single-base changes at nucleotide positions 399 (A to C) and 435 (A to G), respectively, of the CD4 cDNA nucleotide sequence, using polymerase chain reaction technology as described in Materials and Methods. The nucleotide and amino acid sequence of the region encompassing the CDR3 region is shown. Single and multiple amino acid substitution mutations are highlighted by the boxes; those corresponding to the mouse, rhesus, and chimpanzee CDR3 sequences are indicated. Vertical black arrows indicate the limits of the Ser-Arg double amino acid insertions after each residue between 79 and 91. The vertical open arrow indicates the position of individual proline and phenylalanine insertion mutations.

files are shown in Fig. 3, and the results are summarized in Table 2. Most mutant CD4 molecules were expressed on the surface at levels comparable to wild-type levels; the exceptions were the double insertion mutants i79SR to i83SR and the double substitution E87I/D88I, which were expressed at somewhat lower surface levels.

gp120-binding activity and overall structural integrity of the CD4 mutants. The mutant CD4 molecules were assessed for the ability to bind to rgp120 by coprecipitation analysis, using cell lysates prepared from [^{35}S]methionine metabolically labeled cells. For each mutant, the amount of labeled CD4 coprecipitated by addition of ^{35}S-labeled gp120 plus an anti-gp120 MAb was compared with the total amount of labeled CD4 directly immunoprecipitated with rabbit polyclonal anti-CD4 antiserum. Representative results are shown in Fig. 4, and data for all the mutants are tabulated in Table 2. As with the wild type, most of the CDR3 mutants gave efficient coprecipitation with gp120. The exceptions were one double amino acid substitution mutant (E87I/D88I) and five insertion mutants (i79SR to i83SR) which showed complete loss of gp120 binding and one double amino acid substitution mutant (E87K/D88K) and two insertion mutants (i84SR and i85SR) which showed significantly reduced binding. The control mutants gave the results expected on the basis of previous findings with soluble CD4 constructs (31): severely impaired binding was observed with the insertion mutation i48SR in the CDR2 region but not with the i21SR insertion.

The mutated CD4 molecules showing impaired gp120 binding were examined for overall structural integrity by direct immunoprecipitation with a panel of anti-CD4 MAbs known to react with epitopes in the first domain of CD4. As shown in Table 3, all of the CDR3 mutants with defective gp120 binding showed coordinate impairment of MAb binding. The most dramatic effects were observed with the one double amino acid substitution mutant (E87I/D88I) and five insertion mutants (i79SR to i83SR); in agreement with the complete loss of gp120 binding noted above, these mutants lost reactivity with all of the MAbs. We point out that these results are consistent with a previous alanine-scanning mutagenesis study, which reported that mutation of residues 79, 80, and 82 caused coordinate disruption of gp120 binding and overall structural integrity (3). By contrast, Table 3 shows that the i48SR insertion in the CDR2 region caused selective loss of binding of MAb OKT4D but had no effect on binding of the other MAbs, in agreement with our previous findings with an sCD4 construct containing the same insertion (31). Thus, loss of gp120 binding occurred only for mutations around the CDR3 region which severely disrupted the overall structure of the molecule.

Ability of the mutant CD4 molecules to mediate syncytium formation. Each vaccinia virus-encoded mutant CD4 was expressed on a human cell type and tested for syncytium formation upon mixing with cells expressing HIV-1 envelope glycoprotein. Cultures were examined microscopically throughout the first 8 h and also at 24 h. Cell-cell fusion was quantitated by counting the total number of syncytia per random field (in quadruplicate), as described in Materials and Methods.

Figures 5 and 6 show syncytium formation in mixtures of HIV-1 chronically infected 8E5 cells mixed with A2.01 cells expressing a representative set of CD4 mutants; the data for the entire set of mutants are summarized in Table 2. Similar results were obtained when the mutant CD4 molecules were expressed on HeLa cells (data not shown). With wild-type CD4, giant cells were evident as early as 1 to 2 h after cell mixing. Surprisingly in view of the published findings discussed above, no impairment of syncytium formation was observed with 21 of the CD4 mutants. These included substitution of the region encompassing CDR3 with the corresponding sequences from mouse, rhesus, or chimpanzee CD4, as well as numerous single and double amino acid substitutions and insertions in this region. In addition to the E87G substitution corresponding to the chimpanzee sequence, no impairment was observed with a double mutant also containing a Q89K substitution, which has been reported to cause some inhibition of syncytium formation (11). Some impairment of syncytium formation was observed in nine of the CDR3 mutants; in four cases the defect was only partial, and in five it was complete. Thus, three mutants (i84SR, i85SR, and E87K/D88K) showed a kinetic impairment, with greatly reduced syncytium formation through 4 h but normal numbers by 24 h; another mutant (E87I/D88I) showed greatly reduced syncytium formation throughout the entire time course (Fig. 6; Table 2). Interestingly, the mutants showing weak syncytium activity corresponded to those with reduced gp120-binding activity and severe disruption of overall structural integrity, on the basis of loss of reactivity with the panel of anti-CD4 MAbs. The only mutants which were completely negative for syncytia throughout the 24-h time course (i.e., the five insertions i79SR to i83SR) also showed complete loss of gp120 binding and overall structural integrity, as well as reduced surface expression. The expected results were obtained with the control mutations: the i48SR insertion in the CDR2 region

TABLE 1. Recombinant vaccinia viruses encoding mutated full-length CD4 molecules

Recombinant vaccinia virus[a]	Amino acid change(s)[b]	Nucleotide change(s)[c]	Method[d]	Restriction sites used[e]
vCB-100	None	None	NA	NA
vCB-100.1	T81A	A391G	O	*Nde*I-*Spe*I
vCB-100.2	E85A	A404C	O	*Nde*I-*Spe*I
vCB-100.3	E85K	G403A	O	*Nde*I-*Spe*I
vCB-100.4	E87G	A410G	O	*Nde*I-*Spe*I
vCB-100.5	E87K	G409A	O	*Nde*I-*Spe*I
vCB-100.6	E87A	A410C	O	*Nde*I-*Spe*I
vCB-100.7	E87P	G409C, A410C	O	*Nde*I-*Spe*I
vCB-100.8	D88A	A413C	O	*Nde*I-*Spe*I
vCB-100.9	E87G/Q89K	A410G/C415A	O	*Nde*I-*Spe*I
vCB-100.10	E87G/Q89L	A410G/A416T	O	*Nde*I-*Spe*I
vCB-100.11	E87A/D88A	A410C/A413C	O	*Nde*I-*Spe*I
vCB-100.12	E87K/D88K	G409A/G412A, C414A	O	*Nde*I-*Spe*I
vCB-100.13	E87I/D88I	G409A, A410T, G411A/G412A, A413T, C414A	O	*Nde*I-*Spe*I
vCB-100.14	V86L/D88N/Q89R/Q94E	G406C/G412A/A416G/C430G	O	*Nde*I-*Spe*I
vCB-100.15	D88N/Q89K/Q94E	G412A/C415A/C430G	O	*Nde*I-*Spe*I
vCB-100.16	i87F	i411 TTT	O	*Nde*I-*Spe*I
vCB-100.17	i87P	i411 CCC	O	*Nde*I-*Spe*I
vCB-100.18	i79SR	i387 TCGCGA	O	*Afl*II-*Spe*I
vCB-100.19	i80SR	i390 TCGCGA	O	*Afl*II-*Spe*I
vCB-100.20	i81SR	i393 TCGCGA	O	*Afl*II-*Spe*I
vCB-100.21	i82SR	i396 TCGCGA	O	*Afl*II-*Spe*I
vCB-100.22	i83SR	i399 TCGCGA	O	*Afl*II-*Spe*I
vCB-100.23	i84SR	i402 TCGCGA	O	*Nde*I-*Spe*I
vCB-100.24	i85SR	i405 TCGCGA	O	*Nde*I-*Spe*I
vCB-100.25	i86SR	i408 TCGCGA	O	*Nde*I-*Spe*I
vCB-100.26	i87SR	i411 TCGCGA	O	*Nde*I-*Spe*I
vCB-100.27	i88SR	i414 TCGCGA	O	*Nde*I-*Spe*I
vCB-100.28	i89SR	i417 TCGCGA	O	*Nde*I-*Spe*I
vCB-100.29	i90SR	i420 TCGCGA	O	*Nde*I-*Spe*I
vCB-100.30	i91SR	i423 TCGCGA	R	*Eco*RI-*Sac*I
vCB-100.31	i21SR	i213 TCGCGA	R	*Eco*RI-*Sac*I
vCB-100.32	i48SR	i294 TCGCGA	R	*Eco*RI-*Sac*I

[a] Recombinant vaccinia viruses encode full-length wild-type or mutant CD4 molecules under control of the vaccinia virus P7.5 promoter. Vaccinia virus recombinant vCB-100 encodes wild-type CD4; vaccinia virus recombinants vCB-100.1 through vCB-100.32 encode mutant CD4s.

[b] Substitution mutations are designated as follows: wild-type amino acid, residue number, mutant amino acid. A slash is used to separate different amino acid substitutions in a given mutant. Insertion mutations are designated by the prefix i and then the position of the residue after which the indicated amino acids are inserted.

[c] Nucleotide changes in the CD4 coding sequence resulting in amino acid changes. For substitutions, changes are designated as follows: wild type nucleotide, nucleotide number, mutant nucleotide. A slash is used to separate nucleotide changes altering different amino acids in a given mutant. Insertion mutations are designated by the prefix i and then the position of the nucleotide after which the indicated nucleotides are inserted.

[d] Mutations generated by oligonucleotide-directed mutagenesis are designated by O. Mutations generated by restriction fragment substitution are designated by R. NA, not applicable.

[e] Mutations were introduced by cutting the parent plasmid with the indicated restriction enzymes and substituting either the corresponding complementary oligonucleotide pair or restriction fragment with the appropriate overhangs.

showed greatly weakened syncytium activity, consistent with its reduced gp120 binding, whereas the i21SR insertion behaved like the wild type. We conclude that membrane fusion was impaired only for those mutants with mutations encompassing the CDR3 region which were also disrupted in gp120 binding and overall structural integrity.

We also examined several modifications to the syncytium assay in an attempt to detect possible fusion impairments in the CDR3 mutants. These included reducing the incubation temperature to 30°C and lowering the expression level of the vaccinia virus-encoded CD4 and envelope glycoprotein by using cytosine arabinoside (35). These treatments lowered the rates of syncytium formation in all cases but failed to reveal any impairments of fusion efficiency beyond those noted above (data not shown). We also tested the activity of each mutant CD4 molecule against another HIV-1 chronically infected cell line, H9/HTLV-IIIB, as well as against cells expressing HIV-1 envelope glycoprotein encoded by vaccinia virus recombinant vPE16 (data not shown). Syncytium formation was more rapid and the giant cells were larger in these cases, but the effects of the CD4 mutations were qualitatively identical to those described above. In summary, impaired fusion was observed only for CDR3 mutants which displayed concomitant defects in gp120 binding and overall structural integrity.

Syncytium formation with HeLa transformant cell lines expressing CD4 from different primate species. It has been reported that transformant cell lines (derived from HeLa cells or the JY B cell line) expressing native chimpanzee and rhesus CD4 are incapable of forming syncytia when mixed with cells expressing vaccinia virus-encoded HIV-1 envelope glycoprotein; chimpanzee and rhesus peripheral blood

TABLE 2. Expression and properties of the wild-type and mutant CD4 molecules

CD4 molecule	Expression		gp120 binding[a]	Syncytium score[b]
	Lysate[c]	Surface[d]		
WT	+	16	++++	+++++
T81A	+	16	+++	+++++
E85A	+	18	++++	+++++
E85K	+	19	+++	+++++
E87G (chimp)	+	17	++++	+++++
E87K	+	18	+++	+++++
E87A	+	18	++++	+++++
E87P	+	15	+++	+++++
E88A	+	16	++++	+++++
E87G/Q89K	+	14	++++	+++++
E87G/Q89L	+	16	++++	+++++
E87A/D88A	+	16	++++	+++++
E87K/D88K	+	18	++	+
E87I/D88I	+	9	−	(−)
Mouse 85–95	+	15	++++	+++++
Rhesus 85–95	+	14	++++	+++++
i87F	+	16	+++	+++++
i87P	+	15	++++	+++++
i79SR	+	9	−	−
i80SR	+	9	−	−
i81SR	+	6	−	−
i82SR	+	5	−	−
i83SR	+	6	−	−
i84SR	+	13	+	+
i85SR	+	21	+	++
i86SR	+	17	++	++++
i87SR	+	19	+++	+++++
i88SR	+	16	++++	+++++
i89SR	+	16	++++	+++++
i90SR	+	16	++++	+++++
i91SR	+	12	++++	+++++
i21SR	+	13	++++	++++
i48SR	+	20	+	(−)

[a] Binding of gp120 was determined for the wild type (WT) and each mutant by comparing the ratio of labeled CD4 coprecipitated with rgp120 plus anti-gp120 MAb 902 to labeled CD4 directly immunoprecipitated with rabbit polyclonal anti-CD4 antiserum. Symbols: ++++, >0.6; +++, 0.2 to 0.6; ++, 0.06 to 0.19; +, 0.02 to 0.05; −, <0.02.

[b] Syncytia were scored by counting the total number of giant cells per random field at ×200 magnification, in quadruplicate, after mixing equal numbers of 8E5 cells and A2.01 cells expressing vaccinia virus-encoded wild-type or mutant CD4. Scores were obtained 4 h after mixing. Symbols: +++++, >90; ++++, 70 to 90; +++, 50 to 69; ++, 20 to 49; +, 5 to 19. −, 0 at 4 and 24 h; (−), <5 at 4 h but more at 24 h.

[c] Expression in cell lysates of wild-type and mutant CD4 proteins encoded by recombinant vaccinia viruses was determined by Western blot analysis with rabbit polyclonal anti-CD4 antiserum. + represents the level of expression observed with wild-type CD4.

[d] Expression at the cell surface of mutant CD4 proteins encoded by recombinant vaccinia viruses was determined by flow cytometry after staining with polyclonal rabbit anti-CD4 antiserum followed by FITC-labeled anti-rabbit Ig. Results are expressed as the mean fluorescence intensity. The background mean fluorescence intensity was 1 and was determined by using cells infected with control vaccinia virus WR.

mononuclear cells were also reported to be negative for syncytium formation when mixed with envelope glycoprotein-expressing cells (11). The equivalent gp120 binding found for human and chimpanzee CD4 made these findings particularly noteworthy (11). In view of the unexpected fusion-positive phenotype noted above for most of the CD4 molecules containing CDR3 mutations (including substitutions corresponding to the chimpanzee and rhesus sequences

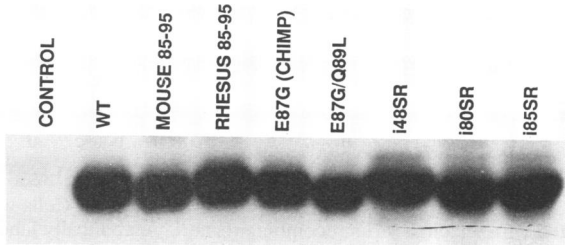

FIG. 2. Total expression of mutant CD4 molecules. Detergent lysates were prepared from HeLa cells infected with the recombinant vaccinia virus vectors and analyzed by Western blot with rabbit polyclonal antiserum to human CD4. The wild type and seven representative CD4 mutants are shown; only the CD4 band is indicated (55 kDa). The control lane represents a lysate prepared from HeLa cells infected with vaccinia virus WR as a control (top left panel).

in this region), it was essential to reexamine the fusion properties of transformant cell lines expressing native CD4 from these other species. Figure 7 shows the results obtained when HeLa transformants expressing CD4 of different spe-

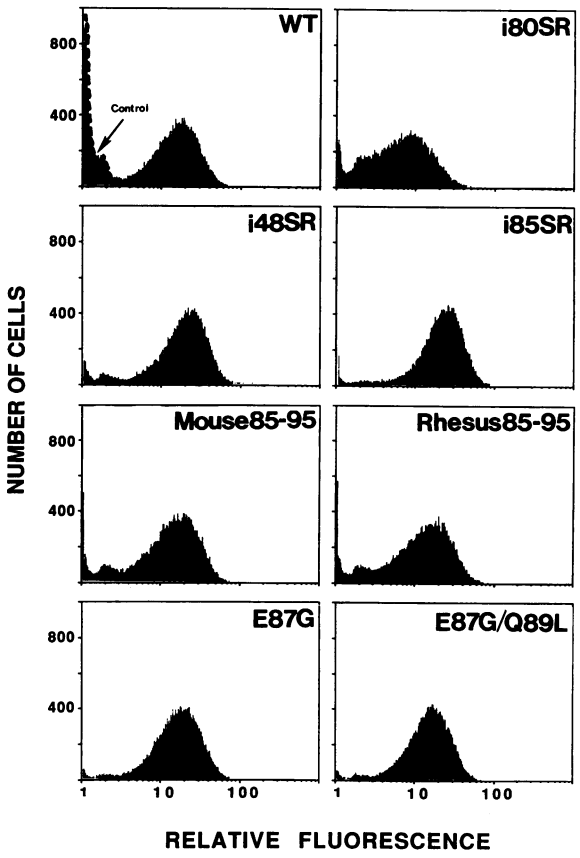

FIG. 3. Cell surface expression of mutant CD4 molecules. Human A2.01 lymphocytes were infected with vaccinia virus recombinants encoding the indicated mutant CD4 molecules. The infected cells were stained with an excess of rabbit polyclonal anti-CD4 antiserum followed by excess FITC-labeled anti-rabbit IgG antibodies and analyzed by flow cytometry. Histograms are shown for wild-type (WT) CD4 and seven representative mutants. Background fluorescence was determined with A2.01 cells infected with vaccinia virus WR as a control (top left panel).

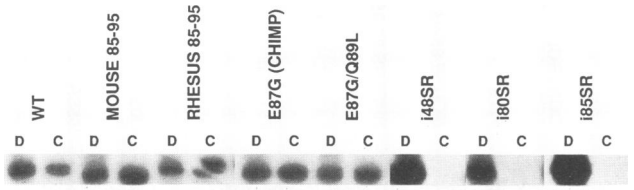

FIG. 4. Binding of mutant CD4 molecules to rgp120. Cells were infected with vaccinia virus recombinants and metabolically labeled with [^{35}S]methionine. Equivalent aliquots of each cell lysate were subjected to direct immunoprecipitation with rabbit polyclonal anti-CD4 antiserum (lanes D), or coprecipitated with rgp120 plus anti-gp120 MAb 902 (lanes C). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis, and labeled proteins were visualized by fluorography. Results are presented for the wild type (WT) and seven representative mutants; only the CD4 band is shown.

cies were mixed with A2.01 cells expressing HIV-1 envelope glycoprotein encoded by vaccinia virus recombinant vPE16. At 4 h, abundant syncytia were observed with HeLa cell lines expressing human, chimpanzee, or rhesus CD4 but not with control HeLa cells lacking CD4. No syncytia were observed when the fusion partner was A2.01 cells infected with control vaccinia virus WR. Syncytium formation mediated by human CD4 appeared somewhat more efficient than that mediated by the chimpanzee or rhesus CD4, as judged by the larger size of the giant cells (Fig. 7) and their slightly faster initial appearance (1 h for human CD4 versus 2 to 3 h for the chimpanzee and rhesus CD4 [data not shown]). Another experiment indicated that syncytium formation mediated by the nonhuman primate CD4 molecules did not require the high level of envelope glycoprotein expression on the partner cell as was obtained with the vaccinia virus vector. The HeLa transformant cells were mixed in equal numbers with the HIV-1 chronically infected H9/HTLV-IIIB cells (0.5×10^6 cells of each cell type), and syncytium formation was scored at 24 h. The mean numbers of syncytia per field at ×200 magnification were 86 ± 8 for human CD4, 43 ± 8 for chimpanzee CD4, and 38 ± 8 for rhesus CD4. No syncytia were observed in control mixtures in which the fusion partner consisted of uninfected H9 cells, which do not express the envelope glycoprotein. Thus, in an experimental system free of vaccinia virus-encoded proteins, the HeLa transformants expressing chimpanzee or rhesus CD4 were clearly capable of forming syncytia with cells expressing HIV-1 envelope glycoprotein, albeit at a somewhat lower efficiency than that of the HeLa transformant expressing human CD4.

In interpreting the lower syncytium-forming efficiency of

TABLE 3. Binding of anti-CD4 MAbs to mutant CD4 molecules which have impaired gp120-binding activity

CD4 molecule[a]	Binding of MAb[b]:				
	Leu3A	OKT4A	OKT4D	OKT4E	OKT4F
WT	+	+	+	+	+
E87K/D88K	+/−	+/−	+/−	+/−	+/−
E87I/D88I	−	−	−	−	−
i79SR	−	−	−	−	−
i80SR	−	−	−	−	−
i81SR	−	−	−	−	−
i82SR	−	−	−	−	−
i83SR	−	−	−	−	−
i84SR	+/−	+/−	+/−	−	+/−
i85SR	+/−	+/−	+/−	−	−
i48SR	+	+	−	+	+

[a] [^{35}S]methionine-labeled lysates from BCS-1 cells infected with recombinant vaccinia viruses expressing the mutated CD4 molecules were prepared as described in Materials and Methods. WT, wild type.

[b] Anti-CD4 MAb binding was determined by comparative immunoprecipitation with wild-type CD4, using [^{35}S]CD4-containing cellular lysates. Precipitates were electrophoresed on SDS-containing gels under reducing conditions. Values were determined by densitometry of autoradiographs of the polyacrylamide gels. Symbols: +, >80% precipitated; ±, 1 to 10% precipitated; −, no detectable binding.

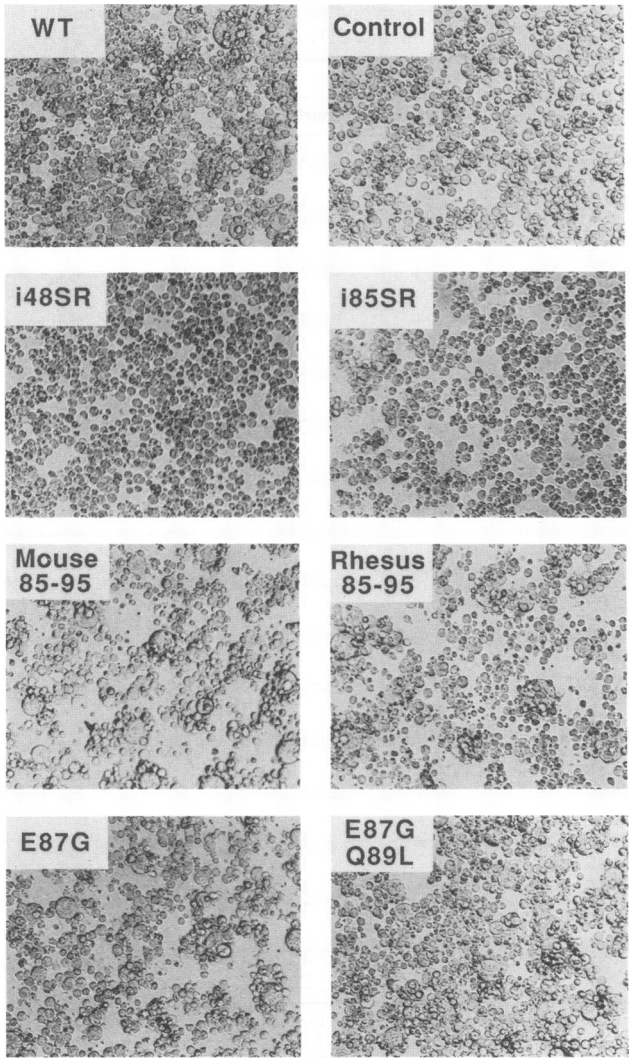

FIG. 5. Syncytium formation between cells expressing mutant CD4 molecules and cells expressing HIV-1 envelope glycoprotein. Human A2.01 lymphocytes infected with vaccinia virus recombinants encoding the mutant CD4 molecules were mixed with equal numbers of HIV-1 chronically infected 8E5 cells (total cell density, 10^6/ml in individual wells of 96-well flat-bottom tissue culture plates). The cells were incubated at 37°C, and syncytium formation was monitored microscopically. Results are shown 8 h after cell mixing for the wild type (WT) and seven representative mutants. The control represents 8E5 cells mixed with A2.01 cells infected with vaccinia virus WR.

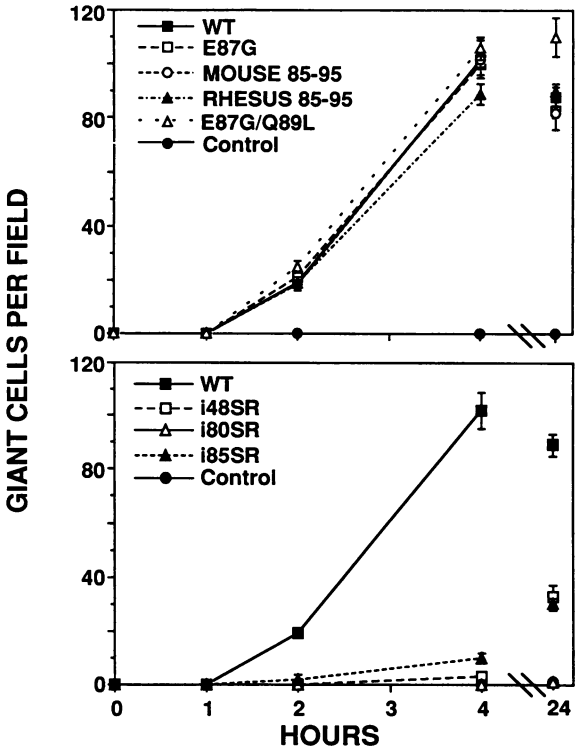

FIG. 6. Kinetics of syncytium formation after mixing cells expressing mutant CD4 molecules with cells expressing HIV-1 envelope glycoprotein. Cell mixtures were set up exactly as described in the legend to Fig. 5. Syncytium formation was assessed every hour by counting the total number of giant cells per random field (×200 magnification) with an inverted microscope. The results represent the mean of quadruplicate samples; error bars indicate standard deviations. Results are shown for the wild type (WT) and seven representative mutants (four substitution mutants in the top panel, three insertion mutants in the bottom panel). The control represents cells infected with vaccinia virus WR. The wild-type and control times courses are presented in both panels.

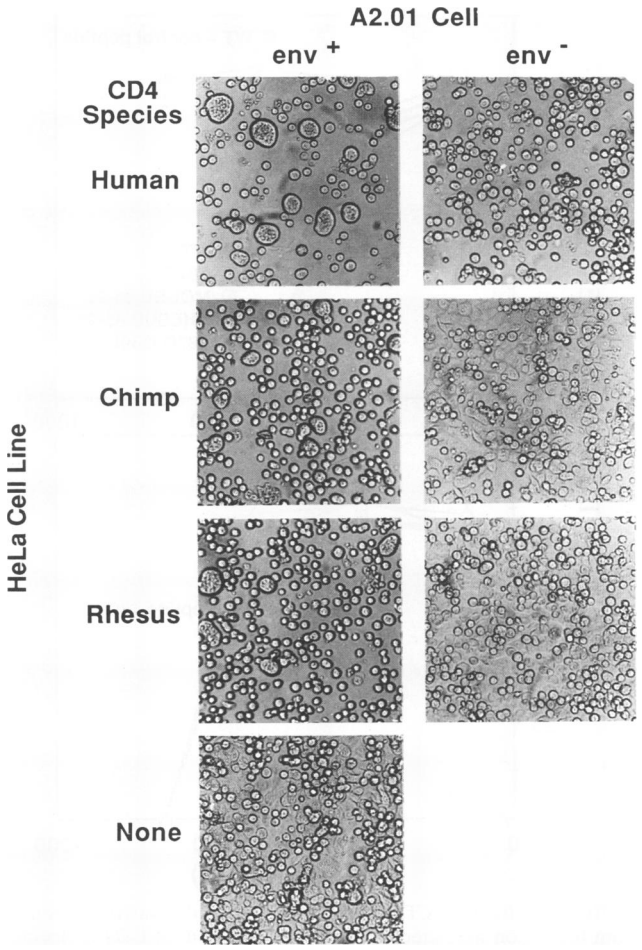

FIG. 7. Syncytium formation of transformant CD4-expressing HeLa cell lines with cells expressing HIV-1 envelope glycoprotein. HeLa transformants HeLa-Hs, HeLa-Pt, and HeLa-Mm encode human, chimpanzee, and rhesus CD4, respectively. Control HeLa cells did not express CD4 (None). The indicated HeLa cell lines were mixed with equal numbers of A2.01 cells infected with either vaccinia virus vPE16 encoding HIV-1 envelope glycoprotein (env^+) or WR (env^-); the total cell density was 10^6/ml. Cultures were incubated at 37°C, and photomicrographs were taken at 4 h.

the HeLa transformants expressing chimpanzee and rhesus CD4 molecules, several points should be noted. First, the level of surface CD4 staining with either polyclonal antibodies or MAbs against human CD4 was lower for the chimpanzee and rhesus CD4 transformants than for the human CD4 transformant (data not shown). We cannot ascertain at present whether this represents lower CD4 levels on the transformants expressing nonhuman CD4 or simply reduced reactivity of the nonhuman CD4 molecules with the heterologous antibodies raised against human CD4. However, these results make it unlikely that the fusion-competent phenotype of the HeLa transformants expressing chimpanzee or rhesus CD4 is due to higher expression levels of these CD4 molecules compared with the human CD4. Second, even if chimpanzee and rhesus CD4 molecules are somewhat less efficient than human CD4 in supporting syncytium formation, the effects may not be due strictly to differences in the CDR3 region. The sequences of human and chimpanzee CD4 differ at five residues; only one of these is within CDR3 (position 87), and three are at other residues within domain 1. The distinction between human and rhesus CD4 is even greater, with 34 different residues; 3 are in CDR3, and 9 are at other positions within domain 1 (11).

Inhibition of fusion mediated by mutant CD4 molecules, using CDR3 synthetic peptide derivatives. The notion that the CDR3 region plays a critical role in membrane fusion has evolved not only from examination of CD4 molecules with different sequences in this region but also from studies with CDR3 synthetic peptide derivatives which have been shown to inhibit HIV-1 infection and syncytium formation (7, 20, 22, 25, 26, 36, 43a). One possible interpretation of our finding that CD4 molecules with mutations in CDR3 efficiently mediate syncytium formation is that in the mutants, a different region of the CD4 molecule has taken over the function of CDR3, thereby circumventing the loss of CDR3 function. If so, the inhibitory effects of CDR3 synthetic peptide derivatives on syncytium formation might be expected to be lost with the mutant CD4 molecules. In the experiment whose results are shown in Fig. 8, this notion is tested for several of the syncytium-competent CDR3 mutants. Cells expressing vaccinia virus-encoded envelope glycoprotein were preincubated with the indicated peptide derivatives and then mixed with cells expressing the CDR3

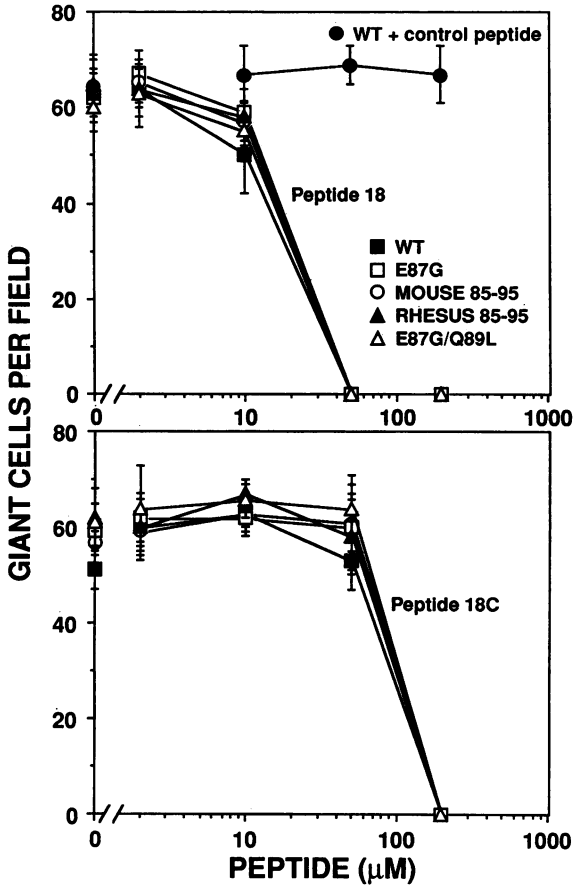

FIG. 8. Effects of CDR3 synthetic peptide derivatives on syncytium formation mediated by wild-type and mutant CD4 molecules. BSC-1 cells expressing HIV-1 envelope glycoprotein encoded by vPE16 were preincubated for 1 h at 37°C with the indicated concentrations of the peptide derivative 18 (top) or 18C (bottom). The cells were then mixed with HeLa cells expressing the indicated vaccinia virus-encoded wild-type (WT) or mutant CD4 molecule. Also shown in the upper panel are envelope-expressing cells preincubated with the indicated concentrations of control peptide 30* and mixed with HeLa cells expressing wild-type CD4. Syncytium formation was assessed at 8 h by counting the total number of giant cells per random field at ×200 magnification. The results represent the means of quadruplicate fields; the error bars indicate standard deviations.

mutant CD4 molecules. As we previously found with wild-type CD4 (7), the derivatized synthetic peptide 18 (corresponding to residues 81 to 92 of human CD4) showed a dose-dependent inhibition of syncytium formation; the control peptide 30* (containing the same amino acid derivatives in a scrambled sequence) had no effect (Fig. 8, top). When syncytium formation mediated by the mutant CD4 molecules was examined, peptide 18 inhibited with the same dose dependence. We also tested the effects of an analogous peptide derivative, 18C, which contains the E87G substitution (Fig. 8, bottom). With wild-type CD4, peptide 18C was severalfold weaker than peptide 18 for syncytium inhibition, consistent with previous results (7, 26). For this peptide derivative, too, the dose-response curves for syncytium inhibition by the mutant CD4 molecules were identical to that observed for wild-type CD4 (Fig. 8, bottom).

Inhibition of fusion mediated by mutant CD4 molecules, using an anti-CDR3 MAb. An additional line of evidence for the participation of the CDR3 region in membrane fusion is based on the effects of MAbs whose epitopes have been mapped to the CDR3 region. A prototype example is MAb L71, which potently inhibits HIV infection and associated syncytium formation; binding of this MAb to CD4 is impaired by amino acid substitutions between residues 88 and 96 (46). Table 4 shows that in the vaccinia virus-based expression system, L71 inhibited syncytium formation mediated by wild-type CD4. Syncytium formation by CD4 molecules containing amino acid substitutions in the region previously defined as the L71 epitope was completely or partially resistant to the inhibitory effects of this MAb. These results indicate that the amino acid sequence defining the epitope of this fusion-inhibiting MAb is not itself essential for the fusion process. The data also provide functional mapping of the L71 epitope to complement the previously reported mapping studies based on antibody-CD4 binding (46).

TABLE 4. Inhibitory effects of the anti-CD4 MAb L71 on the syncytium-forming activity of cells expressing mutant CD4 molecules

CD4 molecule[a]	% Inhibition of syncytium formation[b]
WT	100
T81A	100
E85A	100
E85K	100
E87G	30
E87A/D88A	63
E87G/Q89L	0
E87P	0
i87P	0
i87F	0
i87SR	0
i88SR	0
Mouse 85–95	0
Rhesus 85–95	0

[a] HeLa cells (10^5 cells) infected with the appropriate CD4-encoding recombinant vaccinia virus were preincubated for 30 min with MAb L71 (5 μg/ml) and then mixed with an equal number of BSC-1 cells expressing HIV-1 envelope glycoprotein encoded by vPE16. WT, wild type.

[b] Syncytium formation was scored at 8 h by counting the total number of giant cells per microscopic field at ×200 magnification. Percent inhibition was determined by comparison of the number of giant cells formed in wells containing MAb with the number of giant cells formed in the absence of MAb. Values are derived from the mean of quadruplicate scores.

Ability of sCD4 molecules with mutations in the CDR3 region to inhibit syncytium formation and to stimulate gp120 release. Recombinant sCD4 molecules not only inhibit HIV-1 infection and syncytium formation but also induce changes in envelope glycoprotein structure which may be related to activation of its fusogenic property. These include the release of gp120 from the gp120-gp41 complex (7, 19, 24, 32, 33) and exposure of cryptic epitopes on gp41 (19, 42). Recently, we (7) and others (41) found that synthetic peptide derivatives from the CDR3 region can induce such changes. Interestingly, the E87G substitution was also observed to impair the ability of a synthetic peptide derivative both to inhibit infection and syncytium formation (7, 26) and to stimulate gp120 release (7). These findings, coupled with the present observations of lack of effect of CDR3 mutations on the ability of cell-associated CD4 to mediate syncytium formation, suggested the importance of testing the effects of

mutating the CDR3 region on the activities of the sCD4 protein.

The vaccinia virus expression system was used to produce sCD4 molecules containing the entire four-domain extracellular region, with either the wild-type sequence or the E87G substitution. As shown in Fig. 9, the molecules had equivalent potency for both inhibition of syncytium formation (top) and stimulation of gp120 release (bottom). The inhibitory effect on these activities previously reported for the E87G substitution in the synthetic peptide derivatives is thus not observed with the same substitution in the sCD4 protein.

DISCUSSION

We have investigated the possible involvement of the CDR3 region of CD4 in membrane fusion mediated by the CD4–HIV-1 envelope glycoprotein interaction. Major evidence supporting a role for CDR3 in fusion has been based on the reported inability of CD4 molecules with different sequences in this region to support syncytium formation, despite normal gp120 binding (11). In an effort to explore this problem further, we conducted extensive mutagenesis of the CDR3 region. Using a vaccinia virus-based expression system, we examined the effects of mutating the region encompassing CDR3 on the ability of cell-associated CD4 to support syncytium formation and on the ability of sCD4 to inhibit fusion and to promote changes in envelope glycoprotein structure.

The findings presented herein refute the notion that specific mutations of CDR3 abolish syncytium formation. Of the 30 mutants with mutations encompassing the CDR3 region, 21 showed normal syncytium-forming activity after being mixed with the HIV-1 chronically infected 8E5 cell line (Fig. 5 and 6; Table 2). These mutations included the E87G substitution, which has been suggested to be the critical difference responsible for the reported inability of chimpanzee CD4 to support cell fusion (11). We observed no impairment with the E87G substitution alone or in combination with the Q89L substitution, which has also been reported to reduce syncytium-forming activity (11). Similarly, no impairment was detected when the sequences from rhesus or mouse CD4 were substituted in this region (three amino acid substitutions in rhesus CD4, four substitutions in mouse CD4). The partial or complete loss of syncytium formation which we observed in the remaining nine CDR3 mutants could be attributed to a coordinate disruption of overall structural integrity (Table 3) and impairment of gp120 binding (Fig. 4; Table 2). These conclusions held up even when the rates of syncytium formation were lowered, either by incubation at lower temperatures or by treatment with cytosine arabinoside to reduce the vaccinia virus-encoded CD4 expression level (data not shown).

To test further the possibility that functional fusion defects in the CDR3 mutants were obscured by the relatively high expression levels achieved in the vaccinia virus-based system, we attempted to reproduce the earlier results that transformant cell lines expressing chimpanzee or rhesus CD4 are incapable of forming syncytia (11). We observed that chimpanzee and rhesus CD4 HeLa transformants readily formed syncytia after being mixed with cells expressing the HIV-1 envelope glycoprotein, either encoded by a vaccinia virus vector (Fig. 7) or expressed on the surface of the H9/HTLV-IIIB chronically infected cell line. These results directly contradict results of published experiments in which nearly identical experimental systems were used (11). We note that recent findings from several laboratories

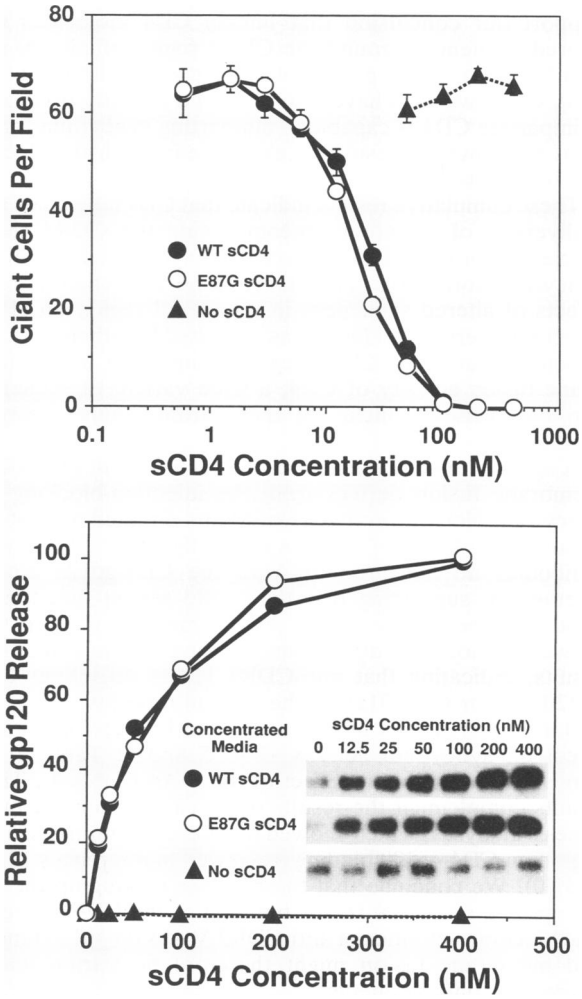

FIG. 9. Concentration dependence of sCD4 inhibition of syncytium formation and stimulation of gp120 release. Wild type (WT) or mutant (E87G) sCD4 was prepared as concentrated conditioned serum-free medium preparations from cell cultures infected with the appropriate recombinant vaccinia virus vectors. The sCD4 concentrations were determined by quantitative Western blot analysis of serially diluted samples, using a standard sCD4 preparation. Control conditioned medium (no sCD4) was prepared by infecting cells with vaccinia virus WR. (Upper panel) Inhibition of syncytium formation. BSC-1 cells expressing HIV-1 envelope glycoprotein encoded by vaccinia virus recombinant vPE16 were preincubated with increasing volumes of concentrated conditioned medium containing the indicated concentrations of either WT or the E87G mutant sCD4. Control cells were incubated with the corresponding volumes of control conditioned medium. Equal numbers of HeLa cells expressing wild-type CD4 were then added, and syncytium formation was monitored microscopically by counting the total number of giant cells per random field at ×200 magnification. The results represent the means of quadruplicate fields; the error bars indicate standard deviations. (Lower panel) Stimulation of gp120 release. BSC-1 cells expressing HIV-1 envelope glycoprotein encoded by vaccinia virus recombinant vPE16 were incubated at 37°C with increasing volumes of concentrated conditioned medium containing the indicated concentrations of either wild-type or E87G mutant sCD4. Control cells were incubated with the corresponding volumes of control conditioned medium. After 3 h, the medium fractions were isolated and analyzed for gp120 content by Western blot with rabbit polyclonal anti-gp120. Band intensities were quantitated by laser densitometry. Values are expressed relative to the amount of gp120 released with 500 nM wild-type sCD4, which was defined as 100. Fluorograms (inset) show only the relevant gp120 bands; each sample represents material derived from the same number of cells.

support our conclusion that human CD4 molecules with altered sequences around the CDR3 region efficiently support HIV-1 envelope glycoprotein-mediated cell fusion (36a, 43b). Other workers have also observed recently that native chimpanzee CD4 is capable of supporting syncytium formation, as shown by results of experiments with transfectant cell lines (40a).

These cumulative results indicate that CD4 molecules with a diversity of mutations encompassing the CDR3 region efficiently support HIV-1 envelope glycoprotein-mediated syncytium formation. Thus there is no discrepancy in the effects of altered sequences in the CDR3 region on HIV-1 infectivity versus cell fusion, as proposed by others (11). We conclude that if the CDR3 region is involved in the membrane fusion activity of CD4, a wide variety of sequences can be tolerated, including those from highly divergent species.

A second line of evidence for the involvement of CDR3 in membrane fusion derives from the infection-blocking and syncytium-blocking activities of MAbs directed against this region (e.g., L71 [46]). It was originally reported that such antibodies do not interfere with the CD4-gp120 binding interaction, suggesting that the CDR3 region functions in fusion independently of any involvement in binding (46). However, more recent experiments have contradicted these results, indicating that anti-CDR3 MAbs do inhibit CD4-gp120 binding (15a, 31a). In the present report we show that mutation of residues implicated in the L71 epitope has no effect on syncytium activity (Fig. 5 and 6; Table 2) but abolishes the inhibitory effect of the MAb (Table 4). These results complement the results of previous extensive mutagenesis analyses, which showed no effect of mutations in this region on CD4-gp120 binding (reviewed in references 12, 23, and 30). We conclude that the residues making up the L71 epitope are not essential for function of the CD4 molecule. The blocking activities of anti-CDR3 MAbs on gp120 binding and membrane fusion might therefore be attributable to indirect secondary effects.

The final major line of evidence implicating CDR3 in membrane fusion is based on the ability of synthetic peptide derivatives overlapping this region to inhibit HIV-1 infection and syncytium formation. Such results have been obtained with a variety of preparations of differing structures, including peptides representing residues 81 to 92 of human CD4 derivatized by benzylation and acetylation (7, 22, 25, 26, 36), as well as longer underivatized peptides with linear (20, 43a) or cyclized (41) structures. In related observations, both the peptide derivatives from residues 81 to 92 and the cyclized peptides have been shown to induce gp120 release (7, 41) and exposure of cryptic epitopes on gp41 (41), analogous to changes in envelope glycoprotein structure induced by sCD4 (7, 19, 24, 32, 33, 42). These findings raise the critical question of whether the activities of the peptide derivatives result from their ability to mimic the corresponding region in the intact CD4 protein, thereby competing or substituting for its function. Experiments presented herein argue against this interpretation. We show that cell fusion mediated by the CDR3 mutants is as sensitive as fusion mediated by wild-type CD4 to inhibition by the peptide derivatives from residues 81 to 92 (Fig. 8). It is difficult to reconcile this result with a model wherein the inhibitory effects of the peptides are attributed primarily to their ability to compete for a critical functional interaction of the corresponding region in the intact CD4 molecule. The present findings also raise questions about interpretation of experiments with peptide derivatives containing the E87G substitution. In addition to our results (discussed above) contradicting the original report that the E87G substitution abolishes the syncytium formation activity of cell-associated CD4, we observed that this mutation had no effect on the ability of sCD4 either to inhibit cell fusion (Fig. 9, top) or to stimulate gp120 release (Fig. 9, bottom). Other workers have recently made similar observations with sCD4 molecules containing this and other mutations (40a, 45a). These results with the sCD4 protein are in direct contrast to the findings that we and others previously obtained with the peptide derivatives from residues 81 to 92, showing that the E87G substitution reduced the activity for both syncytium inhibition (7, 26) and gp120 release (7). Both of these inhibitory effects of the E87G substitution on the peptide derivatives from residues 81 to 92 have recently been corroborated by workers in another laboratory (45a). We conclude that there is a major discrepancy between the functional consequences of the E87G substitution in the synthetic peptide derivatives and in the CD4 proteins (cell associated or soluble).

Several observations should be considered in attempting to resolve these discordant results. The ability of synthetic CDR3 peptide derivatives with different structures to stimulate gp120 release (7, 41) suggests that they might interact directly with the envelope glycoprotein. Indeed, direct binding of the peptide derivatives from residues 81 to 92 to gp120 has been demonstrated (5, 47). Results of competition experiments (5) suggest that at least some of this binding is to the V3 loop of gp120, the principal neutralizing determinant on the envelope glycoprotein (34, 37). However, it is presently unclear whether the observed CDR3 peptide-gp120 binding reflects an analogous interaction of the CDR3 region within the intact CD4 protein. Several groups have shown that the V3 loop, which is positively charged, is the target for fusion-inhibiting polyanionic compounds such as sulfated polysaccharides (5, 10, 43); such agents are also able to promote gp120 release (9). Thus the CDR3 peptide derivatives might exert their fusion-inhibiting effects through a nonspecific ionic interaction with the V3 region; according to this view, the E87G substitution diminishes the potency of the peptide derivative from residues 81 to 92 simply by reducing its negative charge. Additional arguments for nonspecific effects of the peptide derivatives from residues 81 to 92 have recently been presented, including their inhibitory effects on syncytium formation mediated by the unrelated virus human T-cell leukemia virus type I (38). The fact that activity of CDR3-based peptides requires appropriate derivatization (16) or conformational restraints (41) does not distinguish between specific and fortuitous nonspecific activities of these agents. However, a vigorous argument has been made for the specificity of the anti-HIV-1 activities of these peptide agents (reviewed in reference 16). Resolution of these complex issues will probably prove difficult. However, any model of the CD4–HIV-1 envelope glycoprotein interaction must take into account the present findings that the fusion-related activities of both cell-associated CD4 and sCD4 are unaffected by a wide range of sequence variations around the CDR3 region.

ACKNOWLEDGMENTS

This work was supported in part by the NIH Intramural AIDS Targeted Antiviral Program. C. C. Broder is a Research Associate of the National Research Council.

The excellent technical assistance of P. Kennedy is gratefully acknowledged.

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