A Rat CD4 Mutant Containing the gp120-binding Site Mediates Human Immunodeficiency Virus Type 1 Infection

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Summary

CD4 is the primary receptor for the human immunodeficiency virus type 1 (HIV-1). Early mutational studies implicated a number of residues of CD4, centered in the region 41–59, in binding to gp120. However, further mutational analyses, together with studies using inhibitory antibodies or CD4-derived peptides, have suggested that other regions of CD4 are also involved in binding or postbinding events during infection. To resolve these ambiguities, we used rat CD4 mutants in which particular regions were replaced with the corresponding sequence of human CD4. We have previously shown that some of these are able to bind HIV-1 gp120, and here we test their ability to act as functional receptors. We find that the presence of human CD4 residues 33–62 is enough to confer efficient receptor function to rat CD4, and we conclude that it is unlikely that regions of CD4 outside this sequence are involved in specific interactions with HIV-1 during either infection or syncytium formation.

The CD4 cell surface glycoprotein is the primary receptor for HIV-1, and its high affinity interaction with the viral glycoprotein gp120 is the initial step in HIV-1 infection of most susceptible cells (1, 2). The extracellular region of CD4 contains four Ig superfamily domains, the amino terminal of which closely resembles an Ig light chain variable domain (3, 4). The three-dimensional structure of the amino-terminal two domains has been confirmed by x-ray crystallography (5, 6). In vitro mutational analyses have identified residues 41–59 within the C'-C" ridge of CD4 domain 1 as the probable binding site for gp120 (5, 7–11).

The postbinding events that lead to fusion of the virus and cellular membranes are unclear. CD4 may induce conformational changes in gp120 that expose the fusogenic region of gp41 (12–15). It has been proposed that regions of CD4 outside the gp120-binding site might be involved in this process (16–18). For example, mAbs that bind the F-G turn (equivalent to the CDR3 loop of Igs) block syncytium formation and viral entry while only poorly inhibiting the binding of gp120 or HIV-1 (16). Derivatized peptides corresponding to residues 81–92 of human (h)[1] CD4, which encompass this loop, inhibit postbinding events (17). Another study implicated this region by demonstrating that chimpanzee

CD4 and hCD4 with a chimpanzee CDR3 loop were unable to mediate HIV-1-dependent syncytium formation, unlike wild-type hCD4 or chimpanzee CD4 with a hCDR3 loop (19).

Rat (r)CD4 shares only 50% homology with hCD4 and does not function as a receptor for HIV-1. We have taken advantage of this to determine the regions of CD4 involved in HIV-1 infection by replacing rCD4 sequence with the equivalent hCD4 residues. Using this approach initial studies established that the gp120 binding site of CD4 is encompassed by residues 33–62 of domain 1 (20). In the present study we have taken the analysis a stage further and determined whether regions outside the gp120 binding site of CD4 are involved in the postbinding events of viral entry and syncytium formation. The results show that a mutant form of rCD4 containing residues 33–62 of hCD4 is an efficient mediator of HIV-1 infection and syncytium formation. This suggests that regions outside residues 33–62 are unlikely to be involved in these postbinding events.

Materials and Methods

Cell Lines. The human carcinoma cell line, HeLa (21), the mouse fibroblast cell line, L929 (22), and the baby hamster kidney cell line, BHK-21 (23), were obtained from Flow Laboratories, Inc. (McLean, VA). H32 cells, HeLa cells expressing HIV-1 Env, were obtained from Dr. Lee Bacheler (DuPont Co., Wilmington, DE).

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[1] *Abbreviations used in this paper:* h, human; m, mutant; m.o.i., multiplicity of infection; r, rat.

These cell lines were cultured in DME containing 10% (vol/vol) FCS (Gibco-BRL, Gaithersburg, MD), 2 mM L-glutamine (Sigma Chemical Co., St. Louis, MO), and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml neomycin). In addition, H32 cells were maintained in 0.6 mg/ml G418 (Sigma Chemical Co.). The human T lymphotropic virus type 1–transformed T cell line, C8166 (24), and the T lymphoblastoid cell line, H9 (25), were obtained from the National Institutes of Health AIDS Research Reagent Program. These cell lines were maintained in RPMI 1640 with 10% FCS, L-glutamine, and antibiotics.

Generation of HeLa Cell Lines Expressing CD4. The cDNAs of CD4, rCD4, and mutant (m)2 and m6 were subcloned into the pKG5 vector (26). HeLa cells were stably transfected using the calcium phosphate precipitation method, as described (27). Cells expressing the different constructs were selected and maintained in DME complete medium containing 0.6 mg/ml G418 (Sigma Chemical Co.). The transfected cells were analyzed and sorted by FACS® (Becton Dickinson & Co., Mountain View, CA). RF-T4 mAb (28) (MRC AIDS-Directed Programme) and MRC-OX-71 mAb (directed against domains 3 and 4 of rCD4; A. N. Barclay, personal communication) were used to determine the levels of h- and rCD4, respectively. MRC-OX-21 mAb (anti–human C3b inactivator, c3bINA) was used as a negative control, and MRC-W6/32 mAb (anti–human MHC-I) as a positive control. FITC-conjugated rabbit anti–mouse Ig (RAM-FITC) (Serotec Ltd., Oxford, UK) was used as the second antibody.

Viruses. HIV-1$_{IIIB}$ (29) was obtained from the NIH AIDS Research and Reference Reagent Program and propagated by acute infection of C8166 cells. The culture supernatants were clarified, filtered through 0.45-μm filters, treated with 40 U/ml DNase I (Sigma Chemical Co.) for 30 min at room temperature to remove detectable proviral DNA, and stored in 1-ml aliquots at −80°C. Cocal Virus (COC) (30) was obtained from Robert Shope (Yale Arbovirus Unit, New Haven, CT). BHK-21 cells were infected with COC at a multiplicity of infection (m.o.i.) of 10^{-5} and, after 24 h, cell supernatants were harvested, clarified, and stored at −80°C in 1-ml aliquots.

Titration of Viruses. HIV-1$_{IIIB}$ infectivity was determined as follows. $10^{0.5}$-fold dilutions of virus were incubated with 0.1 ml of C8166 cells (5 × 10^5 cells/ml; eight replicates per dilution) for 4 d at 37°C or with 5 × 10^3 HeLa CD4 cells for 8 d. The cells were lysed and proviral DNA amplified by PCR using LTR-specific primers (31). The mean number of infectious units per sample was determined from the frequency of null samples at a given dilution using the Poisson distribution. COC was titrated by plaque assay on BHK-21 cells (32).

Measurement of Virus Production. Mutant and wild-type CD4-expressing HeLa cells were challenged with HIV-1$_{IIIB}$ at an m.o.i. of 2 × 10^{-4} (virus titrated on HeLa cells expressing hCD4) and grown in culture for 1 mo. The cells were split and counted every 3 d and samples of culture supernatant were taken for analysis by p24 ELISA and reverse transcriptase assay (33). Standard methods were used for p24 ELISA (34). Capture antibody was affinity-purified sheep anti-HIV-1-p24 Gag polyclonal antibody (1 μg/well; Aalto Bioreagents, Dublin, Ireland). Detection antibody was EH12-E1 (35) biotinylated using D-biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Detection of Viral Entry Into Cells by the PCR. HeLa cells expressing rCD4, m2, m6 or hCD4 were challenged with HIV-1$_{IIIB}$ (m.o.i. of 10^{-2}; virus titrated in HeLa hCD4) for 2 h, washed twice in PBS, and incubated at 37°C for a further 18 h. Lysates were prepared and PCR amplifications performed as described else-

where (31). The lysates were serially diluted, using uninfected HeLa cell lysates as the diluent, and HIV-1 provirus was amplified by PCR to produce a semiquantitative measure of viral entry. The primers used to detect HIV-1 proviral DNA amplified a 540-bp fragment of the LTR. To check that equal numbers of cells from each cell line were used, the lysates were serially diluted using mouse L929 cell lysate as the diluent, and analyzed by PCR using primers to a 238-bp region of human β-globin gene (for primer sequences see reference 31).

Pseudotype Assays. H9 cells were infected with HIV-1$_{IIIB}$ at an m.o.i. of 10^{-2} and incubated for 10 d. The cells were then superinfected with COC at an m.o.i. of 10^{-4} and incubated for a further 24 h. Infected cell supernatants were then harvested, clarified by centrifugation, and stored at −80°C. Virus was preincubated for 30 min at room temperature with either rabbit anti-COC serum (1:100 dilution, having the potency to neutralize >10^6 COC PFU/ml) to detect COC(HIV) pseudotypes; rabbit anti-COC serum and sheep anti-HIV-1 gp120 (MRC AIDS-Directed Programme reference ADP401 used at 1:80 dilution) to detect background PFUs; or with growth medium to titrate total COC PFUs. The preincubated samples were then used in plaque assays with the cells of interest (32).

Syncytium Assays. Trypsinized H32 cells were mixed with an equal number of CD4-expressing HeLa cells, and placed in sterile petri dishes containing a cover slip. After overnight incubation at 37°C, the cells were fixed for 5 min in methanol and stained for 10 min in phosphate buffer, pH 6.8, containing 10% Giemsa's stain and 1% May and Grunwald's stain (BDH Chemicals Ltd., Poole, Dorset, UK). The coverslips were mounted on slides and the number and size of syncytia counted.

Results

m2 and m6 consist of rCD4 in which residues 36–62 and 33–62, respectively, of domain 1 have been replaced with the corresponding sequence of hCD4 (20). The affinities of m2 and m6 for gp120 are 70- and 2-fold lower, respectively, than that of hCD4 (20). HeLa cells were transfected with constructs encoding these two mutants and those encoding h- and rCD4. After up to two rounds of sorting of HeLa rCD4, HeLa m2, and HeLa m6, the level of surface expression of CD4 was essentially the same (Fig. 1) for each cell line.

HIV-1 Replication. The cells were challenged with HIV-1 and samples were collected every 3 d for 1 mo postinfection for analysis by p24 ELISA and reverse transcriptase assay. Similar levels of HIV-1 replication were detected in HeLa hCD4 and HeLa m6, although the kinetics were delayed by 2–3 d in the latter (Fig. 2). In contrast, no p24 or reverse transcriptase were detected in the cell culture supernatants of either HeLa rCD4 or HeLa m2 even 1 mo after challenge. The growth rate of each cell line was found to be the same (results not shown). During the course of infection cytopathic effects and syncytia were observed first in the culture of HeLa hCD4 cells and a few days later for HeLa m6 cells, but were not seen in either HeLa rCD4 or HeLa m2 cultures. The results show that m6 is an efficient receptor for HIV-1 whereas m2 is not.

Syncytium Assay. To determine whether the differences in ability of each mutant to support HIV-1 propagation in culture were mainly determined by the ability of virus to spread by cell-cell fusion, we tested the ability of each molecule to
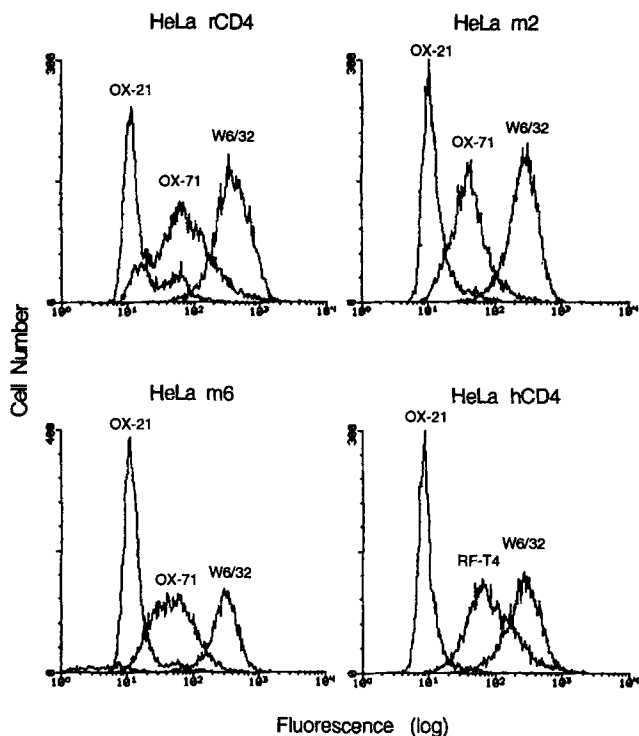
HeLa rCD4   HeLa m2

HeLa m6   HeLa hCD4

Fluorescence (log)

Cell Number

Figure 1. Measurement of mutant and wild-type CD4 expression in transfected HeLa cells by indirect flow cytometry. The levels of CD4 expression were measured by FACS® analysis. Cells were labeled first with mouse mAbs and then with RAM-FITC. The first mAbs were: OX-21 (C3bINA), negative control; W6/32 (anti-human MHC-I), positive control; OX-71 (anti-rat CD4) or RF-T4 (anti-human CD4).

Figure 2. Production of HIV-1 by mutant and wild-type CD4-expressing HeLa cells. HeLa rCD4 (O), HeLa m2 (▽), HeLa m6 (●), and HeLa hCD4 (▼) were challenged with HIV-1, and samples of cell culture supernatant were taken every 3 d postinfection and analyzed for the presence of HIV-1 by (A) ELISA and (B) reverse transcriptase assay. The lower detection limit for each assay was ∼0.1 ng/ml and ∼10³ cpm/ml, respectively.
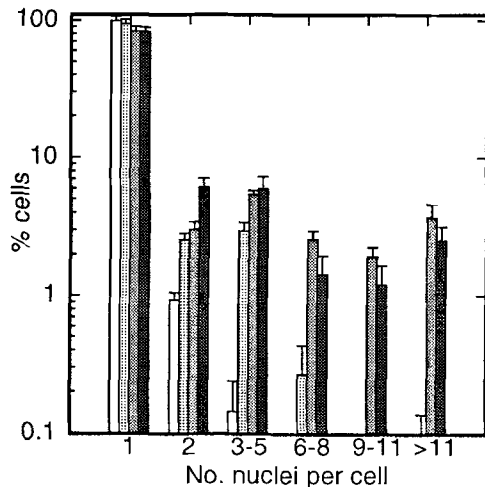
Figure 3. Syncytium-forming ability of HeLa cells expressing mutant and wild-type CD4. HeLa rCD4 (*open columns*), HeLa m2 (*light-shaded columns*), HeLa m6 (*medium-shaded columns*), and HeLa hCD4 (*dark-shaded columns*) were mixed with HeLa cells expressing HIV-1 envelope glycoproteins and the cultures were scored for the frequency and size of syncytia after overnight cocultivation. For each cell line and coculture, 10 fields of view containing at least 200 cells were scored. The columns show the average (± SD) percentage of cells in the population containing one or a specified number of nuclei. No cells containing more than five nuclei were detected in any unmixed cultures.

support HIV-1 Env-dependent syncytium formation in a virus-free system. HeLa cells expressing the HIV-1 Env were mixed with each CD4-expressing cell line and these were scored for frequency and size of syncytia. Fig. 3 shows that rCD4 is unable to support the formation of syncytia. Both HeLa hCD4 and HeLa m6 produced a high level of syncytia, the size and frequency of which could not be distinguished, and m2 supports a low but significant level of syncytium formation.

Viral Entry. Since m6 and hCD4 have indistinguishable syncytium-inducing ability, inefficient cell-cell spread of HIV-1 cannot account for the slight delay in replication kinetics observed in HeLa m6 cells. One possibility is that it may be due to a lower efficiency of infection by cell-free virions since the affinities of m2 and m6 for gp120 are ∼70- and 2-fold, respectively, lower than that of wild-type CD4 (20). To test this, we used three independent measures of viral entry.

Semiquantitative PCR was used with LTR-specific primers to measure the amount of provirus formed during the first round of infection. CD4-expressing HeLa cells were challenged with HIV-1 and lysed after 20 h, before a second round of infection could take place. The lysates were serially diluted ($10^{0.5}$-fold dilutions), and proviral DNA was amplified by PCR (see Fig. 4 *A*). The results show that entry and reverse transcription were ∼10-fold less efficient in HeLa m6 than in HeLa hCD4. In comparison, entry and reverse transcription was ∼300-fold less efficient in HeLa m2 than in HeLa
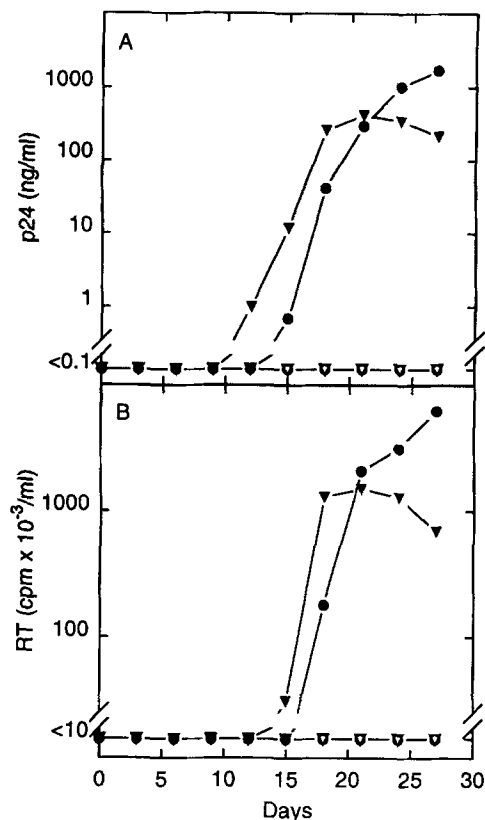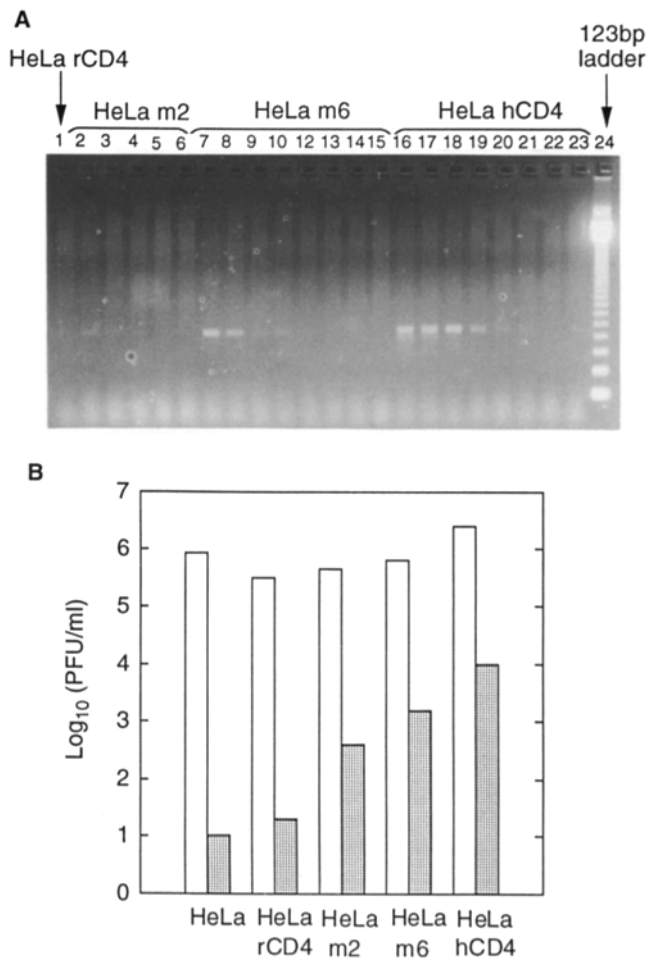
A

HeLa rCD4

HeLa m2　　HeLa m6　　HeLa hCD4

1 2 3 4 5 6 7 8 9 10 12 13 14 15 16 17 18 19 20 21 22 23 24

123bp
ladder

B

Figure 4. Infectability of HeLa cells expressing mutant and wild-type CD4 by HIV-1. (*A*) Semiquantitative PCR assay of first-round provirus formation. Mutant and wild-type CD4-expressing HeLa cells were challenged with HIV-1 at an m.o.i. of 0.01. The cells were lysed 20 h post infection and then serially diluted in uninfected L929 cell lysate ($10^{0.5}$-fold series). PCR was used to amplify a 540-bp fragment of the HIV LTR. (Lane *1*) HeLa rCD4, 10^0 dilution; (lanes *2–6*) HeLa m2, 10^0–10^{-2} dilutions; (lanes *7–15*) HeLa m6, 10^0–$10^{-3.5}$ dilutions; (lanes *16–23*) HeLa hCD4, 10^0–$10^{-3.5}$ dilutions. Semiquantitative PCR of the HeLa CD4 lysates using globin-specific primers confirmed that there were equal numbers of cells in each of the lysates (results not shown). The position of molecular weight markers is shown (lane *24*). (*B*) Pseudotype assay. Untransfected and CD4-expressing cells were challenged with COC(HIV) pseudotypes. Unshaded columns show the titer of COC in each cell line. Shaded columns show the titer of pseudotype (HIV-I-dependent entry).

hCD4. As expected there was no HIV-1 entry into HeLa rCD4 cells (Fig. 4 *a*).

Viral entry was also studied using COC(HIV) pseudotype assays. The pseudotypes have the genome of COC, a plaque-forming virus, and the envelope glycoprotein of HIV-1, thereby enabling HIV-1 gp120-dependent entry of COC genome. Fig. 4 *B* shows entry of COC(HIV) pseudotypes in hCD4-, m6-, and m2-expressing HeLa cells. The results confirm the PCR data, with HeLa hCD4 being more susceptible than HeLa m6, which in turn are more susceptible than HeLa m2. A few

plaques were observed in HeLa rCD4 cells and untransfected HeLa cells, and this may reflect the previously described inefficient CD4-independent entry of HIV-1 into HeLa cells (36).

Finally, a stock of HIV-1$_{IIIB}$ with an infectivity of $\sim 10^7$ C8166-infectious U/ml was titrated on HeLa m6 and HeLa hCD4 cells. The titer of this stock was 2.2 ± 1 and 6.9 ± 3 × 10^3 infectious U/ml in HeLa m6 and HeLa hCD4, respectively. Since an infectivity assay of this sort is only a reliable measure of entry if a single infection event produces sufficient progeny virus to be measured, the infectivity for HeLa rCD4 and HeLa m2 were not investigated by this method.

Discussion

Taken together, these results show that m6 is a functional receptor for HIV-1 although the efficiency with which cell-free virus infects HeLa m6 is between 3- and 10-fold lower than that with which it infects HeLa hCD4 cells. This reduced efficiency at the initial stage of infection is probably sufficient to account for the slight delay in replication kinetics (see Fig. 2). Our results also suggest that syncytium formation depends only on high affinity binding of CD4 to gp120 and not, as has previously been proposed (16, 17, 19), on the sequence of the CDR3-like loop (residues 86–89), which is substantially different between hCD4 (VEDQ) and rCD4 (LENK). In agreement with our results, a recent study in which hCD4 residues 81–92 were replaced by those of chimpanzee, rhesus, or mouse CD4, and residues 79–92 were comprehensively mutated, no mutants were found in which syncytium formation was affected without gross changes in the structure of the molecule (37). Similarly, cells expressing chimpanzee CD4 are able to form syncytia with HIV-1 Env-expressing cells as efficiently as those expressing hCD4 (37a, and Quentin Sattentau, personal communication). Recent work shows that the CDR3-derived peptides that inhibit syncytium formation are not specific since they also interfere with HTLV-I entry and noncompetitively inhibit the binding of both gp120 and a CDR2-directed mAb to CD4 (38).

The reduction in infectability of HeLa m6 compared with HeLa hCD4 (3–10-fold) is proportionally greater than might be expected from the twofold reduction in monomeric affinity. The interaction between CD4 and gp120 on the viral surface may be multimeric (18, 39, 40). If this were the case, the consequent decrease in avidity of m6 for gp120 could account for the greater than expected drop of infectability. To test this possibility, a comparison is needed between the predicted and actual levels of viral entry using other mutants whose affinities and on and off rates of binding to gp120 are known. The present data suggest that the 70-fold reduction in affinity of m2 for gp120 results in a substantial decrease in both infectability and syncytium-forming potential leading to abortive infection of HeLa m2 cultures.

In summary, the results presented here strongly suggest that the presence of an effective gp120 binding site is all that is required for efficient HIV-1 receptor function of CD4 both in the formation of syncytia and the infection of cells by virions.

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