Coreceptor Function of Mutant Human CD4 Molecules without Affinity to gp120 of Human Immunodeficiency Virus (HIV)

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Running title: Coreceptor function of gp120-non-binding CD4
Abstract

In spite of extensive mutational studies on the human CD4 molecule concerning the affinity to HIV envelope glycoprotein gp120, coreceptor functions of such mutant molecules have not been examined except by indirectly measuring the affinity to class II major histocompatibility (MHC) molecules. In this report, coreceptor functions of mutant human CD4 molecules which have no or reduced affinity to an HIV envelope protein, gp120, were assessed in a murine T cell receptor (TCR)/class II MHC recognition system. The substitution of human C" β strand with the murine homologous segment resulted in the loss of the coreceptor function as well as in the complete loss of gp120 binding capacity corroborating the consensus that Phe43 in C" β strand plays crucial roles in both situations. However, simultaneous replacement of C'-C" loop along with C" β strand by homologous murine segments rescued the coreceptor function whereas gp120-binding capacity remained negative. Further analysis indicated that insertion of lysine between Gly41 and Ser42 can partially compensate the coreceptor function lost by the Phe43->Val mutation. Although, coreceptor function of these mutant CD4 molecules in a human T cell recognition system are yet to be determined, these observations necessitate re-evaluation of the role played by Phe43 in the coreceptor function.

Examination of the sensitivities of the mutant CD4 molecules expressed on HeLa cells to infection by a T cell tropic HIV-1 strain indicated that only those mutants which had completely lost gp120-binding capacity were resistant to the infection. All mutants having whole C" substitution, irrespective of additional substitutions nor their coreceptor functions, were resistant to the infection.


Introduction

The most serious consequence of the human immunodeficiency virus type 1 (HIV-1) infection is the acquired immunodeficiency syndrome characterized by severe deficiency of CD4+ T cell functions. Such deficiency of CD4+ T cells stems from the affinity of an HIV envelope protein, gp120, to the human CD4 molecule (1). Several etiological mechanisms causing the functional deficiency of CD4+ T cells are involved; 1) targeted infection of CD4+ cells initiated by the binding of the virus to CD4, 2) cell fusion induced by binding of HIV viruses resulting in apoptosis of CD4+ T cells, and 3) inhibition of the specific recognition of antigenic peptide/class II MHC complexes by soluble gp120. Extensive mutational analyses on the human CD4 molecule have identified C'-C'" ridge of D1 domain as the gp120-binding area (2-4). Murine CD4 molecule has no affinity to gp120 whereas it shares 55% sequence homology of the extracellular portion with its human counterpart. Segmental as well as single amino acid substitutions of human CD4 molecule with murine sequences confirmed the crucial role played by an aromatic ring of Phe43 in gp120-binding (5).

Several reports have claimed that the loss of gp120-binding ability of the human CD4 molecule is unavoidably accompanied by the loss of its affinity to class II MHC molecules which is essential for its coreceptor function (6,7). Nevertheless, these studies employed adhesion assays which does not involve specific recognition of MHC molecules by T cell receptor (TCR). The CD4 molecule plays an important role as a coreceptor upon specific recognition of a class II major histocompatibitily complex (MHC) molecule complexed with an antigenic peptide by the T cell receptor (TCR). The CD4 molecule has been shown to perform its coreceptor function by forming a TCR/MHC/CD4 ternary complex contacting with a highly conserved portion of β2 domain of the target class II MHC molecule (8-10) and probably with TCR/CD3 complex laterally (11). The formation of the ternary complex is believed to raise the total avidity of the TCR/MHC interaction and to trigger cytoplasmic signal transduction by bringing a tyrosine kinase p56lck associated with the cytoplasmic portion of the CD4
into the vicinity of the TCR/CD3 complex for phosphorylation of CD3 chains (12-14).

In this report, we have examined coreceptor functions of the mutant human CD4 molecules. The coreceptor function was evaluated as the ability of the CD4 molecule to assist the recognition of murine class II MHC antigen (I-A\textsuperscript{k}) by murine allo-specific T cell hybridoma. The study confirmed that the mutations causing the loss of gp120-binding, including the substitution of Phe43 to Val, simultaneously resulted in the loss of the coreceptor function. Further studies, however, revealed that the basis for the roles played by Phe43 in gp120-binding and in the coreceptor function assessed in the murine system are different.
Experimental Procedures

**Mutant CD4 cDNA and plasmids.**

Mutagenesis of human CD4 using single-stranded DNA and synthetic oligonucleotides with mutant sequences was performed according to Kunkel's method as described previously (5). Table 1 enlists mutant human CD4 molecules studied in this report with their amino acid sequences. The wild and mutant human CD4 cDNAs were cloned into expression vectors pEneoAf- and pMkitneo. The plasmids were purified by equilibrium centrifugation in CsCl-ethidium bromide gradients and used for transfection.

**Transfection.**

Purified plasmid (40µg) was transfected into parental 4QBW cells (5x10⁶) by electroporation. Stable transfectants were selected by culturing in medium containing 1mg/ml G418 and transfectants were cloned by limiting dilution at 0.3 cells/well and screened by staining with fluorescein isothiocyanate (FITC)-labeled OKT4 antibody. All mutants with a Phe43->Val substitution lost the reactivity to Leu3a and M#18 (Arg59->Glu) did not react with OKT4A (Table 2). None of the mutants gained reactivity to GK1.5 (data not shown).

**Cells and culture.**

The murine T cell hybridoma 4QBW was generated by the fusion between a murine CD4⁺ T cell clone 4Q11 and murine thymoma BW5147. 4Q11 was derived from a TCR-transgenic mouse expressing TCRαβ chains of an allogeneic I-Ak specific T cell clone QM11 (15). TA3 was an I-Ak positive B cell hybridoma TA3 (H-2d/a) originally produced by Glimcher (16). The cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS), nonessential amino acids, sodium pyruvate, 2-mercaptoethanol, and 10mM HEPES buffer.

**Antibodies.**

Antibody inhibition studies were performed using mAbs to I-Ak β chain (10-2-6, mouse IgG2b), to the mouse CD4 (GK1.5, rat IgG2b) (17), and to human CD4 (OKT4 and
OKT4A; mouse IgG2a, and Leu3a; mouse IgG1) (18,19). TCR expression of mutant CD4 transfectant 4QBW cell lines were determined using biotinylated mAb specific for the idiotype of QM11 (ID11, mouse IgG1) (15) followed by addition of phycoerythrin (PE)-conjugated avidin. PE-labeled anti-mouse CD4 (RM4-5, rat IgG2a) and unconjugated Leu3a were purchased (Pharmingen, San Diego, CA and Becton Dickinson Immunocytometry Systems, San Jose, CA). FITC-labeled as well as non-labeled OKT4 and OKT4A were purchased from Ortho Diagnostic Systems (Raritan, NJ). FITC-labeled goat anti-mouse IgG Fc portion (Organon Teknika, PA) was used for secondary staining. Biotin-conjugated gp120 was prepared in our laboratory and used with PE-avidin as a secondary reagent. For negative controls, cells were stained with the FITC-labeled goat antibody to mouse IgG Fc portion alone or PE-avidin alone. The gp120-binding capacity of mutant CD4 molecules was determined as relative binding index (RBI) as described previously (5). Briefly, BW5147 cells expressing transfected mutant CD4 were stained with FITC-OKT4 and biotin-gp120+PE-avidin separately. RBI was calculated as:

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RBI(\%) = \frac{(Me_{FL1}-Mc_{FL1})_{\text{mutant}}}{(Me_{FL2}-Mc_{FL2})_{\text{mutant}}} \times 100
\]

where Me and Mc stand for mean fluorescence values of experimental and control stainings respectively, FL1 for staining with OKT4, FL2 for staining with gp120. The staining of wild type CD4 was included in every set of experiments for the calculation.

**IL-2 assay and stimulation of transfectant T hybridoma.**

In the coreceptor function assay, responder cells (2x10⁴) were co-cultured with 5x10⁴ TA3 cells in a total volume of 200µl on 96 well U-bottomed microtiter plates. After 14 hours of incubation, culture supernatants were harvested. The harvested supernatants were serially diluted with fresh culture medium and the concentration of murine IL-2 was determined by enzyme-linked immunosorbent assay (ELISA). For murine IL-2 ELISA, capturing monoclonal antibody (mAb) (JES6-1A12, rat IgG2a), biotinylated detecting mAb (JES6-5H4, rat IgG2b) and peroxidase-labeled streptavidin (SIGMA, ST.Lois, MO) were used. As a coloring substrate 3,3′,5,5′-tetramethylbenzidine (TMB)
HIV-infection analysis

Sensitivity of mutant CD4 molecules to HIV infection was assessed on CXCR4-positive HeLa cells expressing mutant CD4 generated by calcium phosphate transfection and G418 selection. The expression of the transfected CD4 was determined by staining with FITC-OKT4. A T cell-tropic HIV-1SF2 had been molecularly cloned as previously reported (20). HeLa transfectants were treated with 2 μg/ml of polybrene (Sigma Chemical Co., St. Louis, Mo) for 30 min. at 37°C and exposed to 1ml of culture supernatant containing viruses (p24 antigen at 5ng/ml) for 2 hours at 37°C. Cells were washed and cultured. The levels of p24 core antigen in the culture fluids were determined by ELISA (Abbott, Wiesbaden-Delkenheim, Germany) at 4 day intervals.
Results.

Developing an assay system for evaluating the coreceptor function of mutant human CD4 molecules.

Several reports have indicated that the human CD4 molecule could substitute the murine CD4 in restoring the class II MHC-restricted specific recognition of murine T cells. (21-23). Thus attempts were made to develop a murine recognition system in which the coreceptor function of the human CD4 could be assessed. Human wild type CD4 cDNA in an expression vector pMkit was transfected into a murine CD4+ T cell hybridoma, 4QBW, specific for an allogeneic class II MHC antigen. 4QBW recognized I-A^k in a CD4-dependent manner and secreted IL-2 in response to the recognition. Several human CD4-expressing clones simultaneously expressing murine CD4 and TCR in comparable amounts with those of the mother line 4QBW were isolated. Fig. 1 shows the expression of TCR, murine and human CD4 by 4Q/wild, one of such transfectant clones. 4Q/wild as well as 4QBW produced IL-2 when co-cultured with a B cell hybridoma TA3 which expressed I-A^k. As shown in Fig.2A, these responses were inhibited by a monoclonal antibody to I-A^k, 10-2-16, confirming the specificity of the response. The efficiencies of the recognition by 4Q/wild and that by 4QBW appeared comparable as judged from the inhibition curves of the responses by graded concentrations of the antibody. Fig.2B shows the inhibitory effect of GK1.5, an anti-murine CD4 monoclonal antibody, on the recognition of I-A^k by 4QBW or by 4Q/wild. The complete inhibition of the IL-2 production response of 4QBW by GK1.5 confirmed the coreceptor-dependency of the recognition by TCR of this hybridoma. On the contrary, the recognition of I- A^k by 4Q/wild was only partially inhibitable by this antibody. Therefore, in the presence of an excessive amount of GK1.5 (1:10^4), graded amounts of monoclonal antibody to D1 domain of human CD4, OKT4A or Leu3a, were added to the mixed cultures of 4Q/wild and TA3. As shown in Fig.2C, the GK1.5-resistant component of the response of 4Q/wild was inhibitable by these anti-human
CD4 antibodies, indicating that wild type human CD4 molecules compensated the coreceptor function of murine CD4 molecules when they were blocked. These anti-human CD4 antibodies did not have detectable inhibitory effect on the response in the absence of GK1.5. Similar experiments were performed on three other independently cloned human CD4 transfectant hybridomas with essentially the same results. These results confirmed the earlier reports that the human CD4 could function as a coreceptor in the recognition of murine class II MHC molecules by murine TCR (21-23). Therefore, this assay system was employed for evaluating the coreceptor function of mutant human CD4 molecules.

Coreceptor function of mutant CD4 molecules.

Mutants #1-#5 and #18 were examined for their coreceptor function. Mutants #1-#5 have segmental replacement of C' β strand, C'-C" loop, C" β strand, C"-D loop and D β strand, respectively, by murine homologous segments. Mutant CD4 cDNA cloned in an expression vector pMkit was transfected into 4QBW and at least three independent clones with reasonable expression levels of the transgenic CD4, endogenous CD4 and TCR were established for each mutant (data not shown). Antigen-induced IL-2 production responses of these clones were examined and representative results are summarized in Fig.3. In these graphs, the relative amount of IL-2 produced by a given transfectant is expressed as % of the amount produced by the same clone in response to antigenic stimulation by TA3 in the absence of inhibitory antibodies (cross-hatched dark bar).

The transfectants expressing mutant CD4 molecules showed variable sensitivities to the blocking effect of an excessive amount of GK1.5, whereas the response of the mother hybridoma line 4QBW was almost completely inhibitable. The responses of Mutants #1 and #5 (4Q/M1 and 4Q/M5) showed GK1.5-resistant components comparable to that of 4Q/wild, which were inhibitable by additional anti-human CD4 antibodies. Therefore it was concluded that the coreceptor function of these mutant molecules were not impaired by these mutations. Mutants #2 and #4 showed
somewhat reduced coreceptor activities. Mutants #3 and #18 did not show significant GK1.5- resistant responses. The same experiments were repeated with 10 (M#3) and 3 (M#18) independent clones with essentially the same results. Based on these observations, it was concluded that M#3 and M#18 were non-functional as a coreceptor. A salient correlation between the coreceptor function and gp120-binding ability (ref. 5, Table 2) of these mutant CD4 molecules was observed.

Since M#3 had three amino acid substitutions within C" strand, contributions of the individual substitutions were studied. As shown in Fig.4, Phe43 was the crucial amino acid for the coreceptor function (M#6) and other two amino acid substitutions had no obvious effects (M#7 and 8). Phe43 had also been shown to be the key amino acid in gp120-binding (Table 2, ref. 5). The results of the two experiments apparently confirmed the general correlation between gp120-binding and affinity to class II MHC observed with mutant CD4 molecules (6,7).

Functional mutants with no gp120-binding.

As the wild type human CD4 molecule functioned as a coreceptor in the murine recognition system, it was rather puzzling that the substitution of C" strand of a functional human CD4 by the sequence of a fully functional murine CD4 resulted in a function-less molecule. Thus, we speculated that this substitution might have generated a certain structural incompatibility between the murine C" strand and surrounding structures of the human CD4 molecule causing certain local distortion of the ternary structure. Therefore three additional mutants were produced to test this possibility. In M#19, C'-C" loop along with C" strand was replaced by the murine counterpart. M#20 has murine sequences in C" strand and C"-D loop. In M#21, C" and the two flanking loops were replaced by murine counterparts altogether.

The gp120-binding capacities of the three mutants were examined by cell surface staining assay on murine thymoma cell BW5147 tansfected by the respective mutant CD4 cDNA (see Material and Methods and reference 5). All three mutant molecules had
completely lost gp120-binding ability as indicated by relative binding index in Table 2. The IL-2 production assays were carried out on 3 to 10 transfectant clones for each mutant and representative results are shown in Fig.5. Mutants #19 and #21 had coreceptor activities comparable to that of the wild type human CD4 despite their loss of the affinity to gp120. Since these three mutants had lost Leu3a epitope as M#3 did, their GK1.5-resistant IL-2 production was not inhibitable by this antibody. Mutant #20 did not function as a coreceptor. These results suggest a requirement of compatibility between C'-C" loop and C" β strand for the coreceptor function.

In order to investigate possible contributions of the length of murine C'-C" loop and the charged amino acid within the loop in compensating the function, Gly41 was replaced to Lys (M#22) or Lys was inserted between Gly41 and Ser42 (M#23) along with the Phe43->Val substitution (Fig. 6). The insertion of Lys between 41 and 42 (M#23) partially restored the coreceptor function while M#22 (Gly41->Lys, Phe43->Val) had marginal activity. The results indicated that both the length of the loop and lysin did contribute, but were not sufficient for, the restoration of the coreceptor function.

**Sensitivity of mutant CD4 molecules to HIV infection.**

In order to examine the sensitivity of the mutant CD4 molecules to HIV infection, the mutant genes were transfected into a chemokine receptor (CXCR4)-positive human cell line, HeLa. The transfected cells were exposed to a T cell-tropic HIV-1 strain, SF2, and cultured. Fig.7 shows the relative amounts of HIV core antigen p24 in the culture supernatants determined on indicated culture days. The transfectant expressing wild type human CD4 was sensitive to the infection by SF2. The cells expressing Mutants #2 and #4 were also infectable despite their significant loss of gp120-binding ability, indicating that the residual weak binding activities were sufficient for HIV infection. This conclusion was enforced by another mutant with residual gp120-binding activity, i.e. M#6 (Phe43 -> Val), which also showed sensitivity to the infection (data not shown). On the contrary, M#3 which had completely lost the
binding activity did not confer the sensitivity to HIV infection to the transfectant. 
Mutants #19 and #21, as expected from the total loss of gp120-binding capacity, did not 
succumb to the infection either.
Discussion

The consensus constructed through extensive mutational analyses of the CD4 molecule has been that all mutations resulting in the loss of gp120 binding capacity were unavoidably accompanied by the loss of the affinity to the class II MHC molecule and consequently the loss of the coreceptor function (6,7). The present report largely confirmed the observations by showing that mutations on human CD4 molecule causing the loss of the affinity to gp120 simultaneously resulted in the loss of the coreceptor function and that Phe43 played crucial roles in both situations. However, more careful analysis revealed that the bases for the roles played by Phe43 in the two functions of the CD4 molecule might be different.

Chen et al. reported that synthetic mimetic of the β-turn formed by amino acid residues 40-43 of the human CD4 was capable of inhibiting the binding of gp120 to human CD4 (25), indicating the direct involvement of this portion of the molecule in binding to gp120. In our previous reports, it was shown that the presence of an aromatic side chain at position 43 was essential for gp120 binding and therefore it was speculated that π electrons might be involved in the binding (5). It is known that the murine CD4 molecule does not function as a coreceptor in human TCR/class II MHC recognition system. On the other hand, the fact that the wild type human CD4 molecule functioned as a coreceptor in the murine recognition system indicates that differences in amino acid sequences between human and murine CD4 molecules are not critical in the other direction. Therefore the loss of the coreceptor function in the murine system resulting from the substitution of C" strand of a functional human CD4 by the sequence of a fully functional murine CD4 suggests that this substitution might have generated a certain structural incompatibility between the murine C" strand and surrounding structures of the human CD4 molecule generating a local distortion of the ternary structure. C'-C" loop of the human CD4 consists of only 2 amino acids forming a hairpin loop (β-turn) (Fig.8). It was speculated that the maintenance of such a structure might somehow be dependent on surrounding structures (26). The homologous loop of the murine CD4
molecule consists of three amino acids with two glycines at both ends making the loop more flexible than the human counterpart. Thus the structure of the murine C'-C" loop is likely to be much less dependent on surrounding structures. If this is the case, the combination of murine C" and human C'-C" loop might not reconstitute the right ternary structure. The results obtained in this report are consistent with this model.

Another possibility, nevertheless, can also be envisioned. There might be certain differences in the basis of CD4/class II MHC interaction between human and murine molecular pairs, in the human pair Phe43 playing a decisive role while in the murine pair the interaction being largely determined by the charged side chains of C'-C" loop. In fact, the insertion of lysine between Gly41 and Ser42 (M#23) partially restored the coreceptor function lost in Phe43->Val mutation. However, the restoration by this insertion was merely partial. The difference between mutants #19 and #23 indicates the involvement of not only the lysine residue but also glycine at position 42 suggesting a requirement for more flexibility at this position. Thus it appears that three factors, i.e. lysine in the middle of C'-C" loop, the length of the loop, and serine at position 42, contributed the restoration of the coreceptor function. Structural analyses are necessary to elucidate the actual structural changes introduced by these mutations. More importantly, coreceptor functions of the mutant molecules have to be assessed in a human TCR/class II MHC recognition system to see whether or not the observations are reproducible in the human system. It may be argued that they wouldn't since the murine CD4 molecule fails to function in the human system mainly because of the substitution of Phe43 to Val. However, it is also possible that the failure observed in such experiments was due to the putative structural change resulting from the incompatibility between the amino acid in the 43rd position and surrounding structures rather than the direct effect.

There has been only a limited number of studies examining the coreceptor function of mutant CD4 molecules. The majority of the studies employed adhesion assays in which binding between CD4-expressing transfectants and class II MHC
antigen-expressing cells was measured. We have not been able to develop reliable adhesion assay systems mainly because of the subjectiveness of the assay and highly variable background adhesions. Although the assay system employed in the present report does not allow us to extract pure information on the affinity between MHC class II molecules and mutant CD4 molecules, it provides more comprehensive information concerning the coreceptor function of the CD4 molecule. The CD4 molecule has been shown to perform its coreceptor function by forming a TCR/MHC/CD4 ternary complex in contact with a highly conserved portion of β2 domain of the target class II MHC molecule (8-10) and probably laterally with TCR/CD3 complex (11). The formation of the ternary complex is believed to raise the total avidity of the TCR/MHC interaction and to trigger cytoplasmic signal transduction by bringing a tyrosine kinase p56lck associated with the cytoplasmic portion of the CD4 into the vicinity of the TCR/CD3 complex for phosphorylation of the ζ chain (12-14). In one series of studies, ability of mutant CD4 molecules to assist the recognition of class I MHC molecules by class I restricted TCR was studied (27,28). In this situation, TCR/MHC/coreceptor ternary complexes should not have been formed. The significance and actual existence of TCR-independent interactions between coreceptor molecules and MHC molecules in physiological cellular interactions is not clear (Eshima et al. manuscript submitted for publication). Therefore, it is essential to evaluate the function of coreceptor molecules in systems involving specific recognition of MHC molecules by TCR. We had to compromise and utilize a murine recognition system for evaluating coreceptor functions of the mutant human CD4 molecules since our repeated long-lasting efforts to develop human systems have not been successful so far.

The focused insults on CD4+ T cells by HIV would be avoided if CD4 molecules did not have affinity to gp120. Attempts to block the binding of gp120 to CD4 molecules in vivo by soluble CD4 molecules or peptides were hampered by the development of CD4-independent variant of HIV-1 in patients and by tactical difficulties in maintaining high levels of soluble inhibitors in vivo. The advancement of the gene targeting technology has opened a path to a novel strategy for the
reconstitution of the HIV resistant immune system. If CD4 structural genes of innocent hematopoietic stem cells of an HIV infected patients were replaced (knock-in) by a CD4 gene encoding a gp120-non-binding yet functional mutant molecule, the progeny mature CD4+ T cells arising from the engineered stem cells would be resistant to T cell tropic HIV-1 infection. Although this strategy may not eradicate viruses in the patient's body, particularly those of the CD4-independent variants, it is expected to reconstitute a functional CD4+ T cell subpopulation and to correct the most serious consequence, i.e., immunodeficiency. Needless to say, this approach requires the developments of a technology for massive propagation of hematopoietic stem cells in vitro and an efficient method for replacing a gene in somatic cells. Recent development of human embryonic stem cell (ES cell) lines (29) might provide another less difficult approach. Replacement of genes in ES cells is a routine technology now. In addition, the manipulated ES cells can differentiate in vitro into cells of hematopoietic lineage including lymphoid precursors but are devoid of mature immunocompetent cells. Such cells may well be taken by patients with severe immunodeficiency without causing graft versus host reactions. Efforts are being made along these lines.

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Footnote

Abbreviations used in this paper.

HIV: human immunodeficiency virus

TCR: T cell receptor

MHC molecule: major histocompatibility complex molecule

FITC: fluorescein isothiocyanate

DMEM: Dulbecco's modified essential medium

FCS: fetal calf serum

PE: phycoerythrin

RBI: relative binding index (This value reflects the affinity of CD4 molecules to gp120)

ELISA: enzyme-linked immunosorbent assay

mAb: monoclonal antibody

TMB: 3,3',5,5'-tetramethylbenzidine

ES cell: embryonic stem cell
FIGURE LEGENDS

Figure 1. Staining profiles of 4QBW and its human CD4 transfectant 4Q/wild.
Human and murine CD4 were stained with FITC-labeled OKT4 and RM4-5, respectively. I-A^k specific TCR was stained with idiotypic Ab, ID11 (15). See legends for Table 2 for description of OKT4, OKT4A and Leu3a.

Figure 2. Compatibility of human CD4 as a coreceptor in murine recognition system.
4QBW and 4Q/wild were stimulated with an I-A^k positive B cell line, TA3. The amount of secreted IL-2 in 14 hour culture supernatant was determined by ELSA. The amount of IL-2 is expressed as % of the amount produced by the respective cell line stimulated in the absence of inhibitory antibodies.
(A) IL-2 production responses of 4QBW (square) and 4Q/wild (triangle). The cell lines were co-cultured with TA3 in the presence or absence of serially diluted ascites of anti-I-A^k mAb (10-2-6). Average amounts of IL-2 produced in the absence of the antibody were 14 and 9.5 ng/ml for 4QBW and 4Q/wild, respectively.
(B) Failure of anti-mouse CD4 antibody to completely inhibit the specific recognition of 4Q/wild. Serial dilutions of an ascites of GK1.5 (anti-murine CD4 mAb) were added to mixed cultures of 4QBW (square) or 4Q/wild (triangle) and TA3.
(C) Sensitivity of GK1.5-resistant component of response of 4Q/wild to antibodies to human CD4. 4Q/wild was cultured with TA3 in the presence of an excessive amount of GK1.5 and serially diluted Leu3a or OKT4A. These antibodies completely blocked the GK1.5-resistant component of the response of 4Q/wild.

Figure 3. Specific antigen-recognition of T cell hybridomas expressing mutant CD4.
The assay conditions are as described in the legend for Fig.2 and in the materials and methods section. M numbers after slash in the names of transfectant lines correspond to the serial numbers of mutants shown in Table 1. The concentrations of added GK1.5 (dotted light bar), OKT4A (dotted dark bar), and Leu3a (checker board bar) were approximately 2.5 μg/ml and 10.0 μg/ml, respectively. The amounts of IL-2 are
expressed in % where 100% values are amounts of IL-2 produced by the respective cell lines stimulated in the absence of blocking antibodies (cross-hatched dark bar). M#3 lost the reactivity to Leu3a and M#18 did to OKT4a (data not shown). The size of the cross-hatched bar approximately represents the contribution of the transfected CD4 molecule.

**Figure 4.** Phe43 is a key amino acid residue of C" strand for coreceptor function.
The assay conditions are as described in the legend for Fig.2 and in the materials and methods section. M numbers after slash in the names of transfectant lines correspond to the serial numbers of mutants shown in Table 1. M#6 lost the reactivity to Leu3a.

**Figure 5.** Coreceptor function of the Mutants #19-21.
The assay conditions are as described in the legend for Fig.2 and in the materials and methods section. M numbers after slash in the names of transfectant lines correspond to the serial numbers of mutants shown in Table 1. M#19 and #21 lost the reactivity to Leu3a as M#3 did.

**Figure 6.** Examination of lysine in C"-C" loop.
The assay conditions are as described in the legend for Fig.2 and in the materials and methods section. M numbers after slash in the names of transfectant lines correspond to the serial numbers of mutants shown in Table 1. Coreceptor function was treated with 4 and 10 independent transfectant clones of #22 and #23, respectively, and the figure shows representative results.

**Figure 7.** HIV-1 infection to HeLa cells expressing transfected mutant CD4.
Sensitivity of mutant CD4 molecules to HIV infection was assessed on CXCR4-positive HeLa cells expressing transfected mutant CD4. HeLa transfectants were exposed to HIV-1SF2 and cultured. Levels of p24 core antigen in the culture fluids were determined by ELISA. In experiment 1, values are expressed as direct readings of
optical density at a single point of dilution. In experiment 2, values are expressed as concentrations of p24 calculated from a standard plotting curve.

**Figure 8.** C'-C" ridge of murine and human CD4.

Human structure is drawn according to the x-ray crystallography (3,4). Murine structure was drawn by arbitrary alignment to the human structure. Shaded ribbons indicate β strands.
| Amino acid sequences (36-59) of CD4 molecules covering C'-C''-D segments are |  |
|---|---|---|---|---|---|---|
| Human | ILGNQGSFLTKG---PSKLNDRADSRR |  |
| Murine | QSFLTKGR |  |
| Mutant |  |
| #1 | QH |  |
| #2 | KG |  |
| #3 | V | IR |  |
| #4 | G | S | QF- |  |
| #5 | | | | F |  |
| #6 | | | V |  |
| #7 | | | I |  |
| #8 | | | R |  |
| #18 | | |  |
| #19 | KG | V | IR |  |
| #20 | V | IR | GS | QF- |  |
| #21 | KG | V | IR | GS | QF- |  |
| #22 | K | V |  |
| #23 | K | V |  |

Table 1. Constructed Human CD4 Mutants and gp120 Binding Affinity
Amino acid sequences (36-59) of CD4 molecules covering C'-C''-D segments are
shown. Only amino acid residues differing from the wild type human sequence are indicated for murine and mutant CD4 molecules. Positions are numbered according to the human CD4 molecule.
| mutant mutations | gp120-binding | +HIV | #mAb epitopes | resistance | OKT4A | Leu3a |
|------------------|---------------|------|--------------|------------|-------|-------|
| coreceptor       | *(RBI)        |      |              |            |       |       |
| CD4 function     |               |      |              |            |       |       |
| wild             | 100           | -    | +            | +          | ++    |       |
| #1 murine C'     | 93.4          | -    | +            | +          | ++    |       |
| #2 murine C'-C'' | 28.5          | -    | +            | +          | +     |       |
| #3 murine C''    | -2.0          | +    | +            | -          | -     |       |
| #4 murine C''-D  | 12.7          | -    | +            | +          | +     |       |
| #5 murine D      | 95.7          | -    | +            | +          | ++    |       |
| #6 F43V          | 19.0          | -    | +            | -          | -     |       |
| #7 T45I          | 94.8          | nd   | +            | +          | ++    |       |
| #8 K46R          | 98.7          | nd   | +            | +          | ++    |       |
| #18 R59E         | 24.4          | nd   | -            | +          | -     |       |
| #19 murine C'-C'', C'' | 0.3 | +   | +            | -          | ++    |       |
| #20 murine C'', C''-D | -0.1 | nd | + | - | - |       |
| #21 murine C'-C2, C'', C''-D | 0.9 | +  | + | - | ++ |       |
| #22 G41K, F43V   | 5.0           | nd   | nd           | -          | -     |       |
| #23 K between G41 and S42, F43V | 4.0 | nd | nd | - | + |       |

Table 2. Summary of the experiments.

*Calculation of relative binding index (RBI) is as described in the materials and methods section. +Only those mutants which showed no signs of infection during whole period (30-40 days) are scored resistant. #Results of flow cytometry with direct staining. The specificities of OKT4A and Leu3a are directed to the N-terminal D1 domain of CD4 in contrast to OKT4 which is directed to D3 domain, and therefore can be influenced by mutations within D1 domain.
Fluorescence intensity

Cell count

Fluorescence intensity

4QBW

4Q/wild

--- negative staining
--- anti-TCR QM11
--- anti-mo. CD4

--- negative staining
--- OKT4
--- OKT4A
--- Leu3a
**A**

- Ab 10^6 3\times 10^5 10^5 3\times 10^4 10^4 3\times 10^3 10^3
- Dilution of added anti-I-A\(\beta\) soln.

**B**

- Ab 10^6 3\times 10^5 10^5 3\times 10^4 10^4 3\times 10^3 10^3
- Dilution of added GK1.5 soln.

**C**

- Added anti-human CD4 Ab (\(\mu\)g/ml)

---

Added anti-human CD4 Ab (\(\mu\)g/ml)
IL-2 production
(% of positive control)

- 4Q/M6
- 4Q/M7
- 4Q/M8

Legend:
- no stimulation
- TA3 (I-A^k positive)
- + GK1.5
- + GK1.5 + OKT4A
- + GK1.5 + Leu3a
IL-2 production (% of positive control)
IL-2 production (% of positive control)

- no stimulation
- TA3 (I-A^k positive)
- + GK1.5
- + GK1.5 + OKT4A
EXP. 1

p24 in culture sup. units/ml

days after exposure

EXP. 2

p24 in culture sup. units/ml

days after exposure

M#21

M#19

M#2

M#3

M#4 wild

EXP. 1

M#2

M#4 wild

EXP. 2

wild

M#19

M#21
Coreceptor function of mutant human CD4 molecules without affinity to gp120 of human immunodeficiency virus (HIV)

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