that they may act as transcriptional regulatory factors to modulate changes in gene expression. The high level of specific transcription of these proto-oncogenes in type B spermatogonia therefore suggests their potential involvement in mediating changes in gene expression at this stage of germ cell differentiation. Because the type B spermatogonia represents the last mitotic stem cell division before entry into the prophase of meiosis, it is possible that the nuclear proto-oncogenes are involved in altering programs of gene expression at this developmental transition.

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The MHC-Binding and gp120-Binding Functions of CD4 Are Separable

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CD4 is a cell surface glycoprotein that is thought to interact with nonpolymorphic determinants of class II major histocompatibility (MHC) molecules. CD4 is also the receptor for the human immunodeficiency virus (HIV), binding with high affinity to the HIV-1 envelope glycoprotein, gp120. Homolog-scanning mutagenesis was used to identify CD4 regions that are important in class II MHC binding and to determine whether the gp120 and class II MHC binding sites of CD4 are related. Class II MHC binding was abolished by mutations in each of the first three immunoglobulin-like domains of CD4. The gp120 binding could be abolished without affecting class II MHC binding and vice versa, although at least one mutation examined reduced both functions significantly. These findings indicate that, while there may be overlap between the gp120 and class II MHC binding sites of CD4, these sites are distinct and can be separated. Thus it should be possible to design CD4 analogs that can block HIV infectivity but intrinsically lack the ability to affect the normal immune response by binding to class II MHC molecules.

CD4 IS A CELL SURFACE GLYCOPROTEIN found on those T cells that recognize antigen presented by class II MHC molecules (*1*). It is thought that the extracellular region of CD4, which comprises four immunoglobulin-like domains (V_1 to V_4), interacts with nonpolymorphic determinants of class II MHC and that this interaction is important in the efficient recognition of antigen by these cells (*2*). Gene transfer experiments have indicated that CD4 can function as an adhesion molecule that binds to class II MHC molecules on antigen-presenting cells and improves the interaction of the T cell receptor (TcR) with its ligand (*3, 4*). In addition, it has been reported that CD4 may associate with the TcR as part of the antigen recognition process (*5*) and may be directly involved in the resultant signal transduction (*6*). The CD4 molecule serves as the receptor for HIV-1 (*7*), binding with high affinity to the viral envelope glycoprotein, gp120 (*8, 9*). The relationship between the regions of CD4 that are important in its normal function and those involved in HIV-1 binding has been unknown; while several residues within the V_1 domain of CD4 are known to be important in gp120 binding (*10–13*), the residues involved in class II MHC binding have not been mapped.

As soluble forms of CD4 are currently under investigation as AIDS therapeutics (*9, 14–16*), we thought it important to examine whether the regions of CD4 responsible for its different functions are distinct. Although monovalent soluble CD4 analogs have not been found to affect the normal function of CD4-bearing cells (*15, 17*), it is possible that multivalent derivatives [such as CD4 immunoadhesins (*16*)] might do so. We have used homolog-scanning mutagenesis (*18*) to begin to define the MHC-binding regions of CD4 and their relationship to those involved in gp120 binding. We find that the sequences involved in these two functions are not identical, and, although possibly overlapping, can be separated.

Our strategy for generating mutants was based on the observation that, although human and mouse CD4 share a high degree of amino acid sequence identity (*19*), the murine homolog does not bind gp120 (*11*) and does not interact with the human class

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II MHC molecule HLA-DP as assessed by functional and adhesion assays (*20*). Mutants were made by substituting clusters of variant mouse CD4 residues for the corresponding residues in human CD4; mutants M1 to M3 are within the V_1 domain, M4 and M5 are within V_2, and M6 to M8 within V_3 (see Table 1). Although this approach does not address the possible importance of invariant residues, it allows construction of mutants containing relatively large sequence differences while minimizing the contribution of conformational changes. Each mutant was inserted into a retroviral expression vector and transduced into the murine $CD4^-$ $CD8^-$ T cell hybridoma 3DT52.5.8, which expresses a T cell receptor that recognizes an allotypic mouse class I determinant H-$2D^d$ (*21*) (Fig. 1A). Our assay for functional interaction of CD4 with class II MHC molecules uses a target cell expressing both H-$2D^d$ and the human class II molecule, HLA-DP. The 3DT52.5.8 cells are stimulated to produce interleukin-2 (IL-2) when cocultured with these target cells only when they express human CD4; both the interaction of the T cell receptor with H-$2D^d$ and that of CD4 with HLA-DP are required for the IL-2 response (*17, 22*). Similar levels of the wild-type and mutant CD4 molecules were expressed on the cell surface, as demonstrated by staining with two monoclonal antibodies against CD4 (anti-CD4): OKT4, which maps outside the first two V domains (*23*), and OKT4A, which maps in V_1 (*10, 11*) (Fig. 1, Table 1).

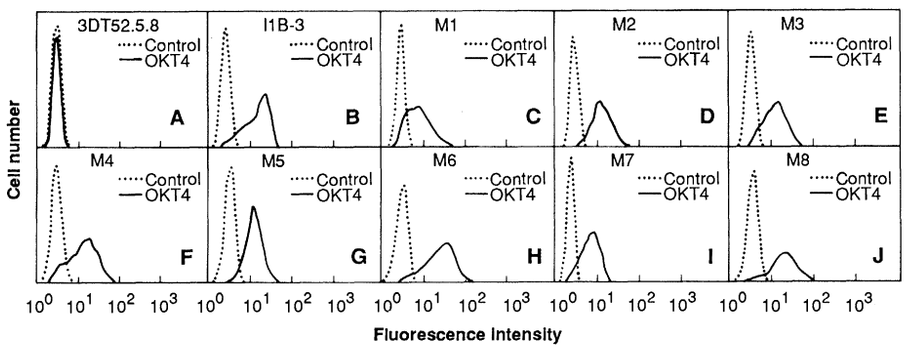

Fig. 1. Cytofluorimetric analysis of effector cells expressing wild-type and mutant CD4 molecules. CD4 mutants (see Table 1) were constructed by oligonucleotide-directed mutagenesis (*32*) with an oligonucleotide primer containing the L3T4 nucleotide sequence replacement flanked on each side with an 18-nucleotide stretch corresponding to the human CD4 sequences surrounding the site of substitution. Mutants were identified by hybridization with an oligonucleotide probe for the substituting L3T4 sequence, and their sequences were confirmed by DNA sequencing. The CD4 mutant coding region was inserted into the retroviral vector MNC and transfected into the amphotropic packaging cell DAMP (*10, 17*). Supernatant from the mutant $CD4^+$ DAMP cells was used to transduce the murine T cell hybridoma 3DT52.5.8. Nontransduced cells (3DT52.5.8) (**A**), cells expressing wild-type CD4 (clone IIB-3) (**B**) and cells expressing the CD4 mutants M1 to M8 (**C** to **J**), were stained with OKT4 and incubated with a fluoresceinated goat antimouse immunoglobulin (solid line). Control cells were stained only with the fluoresceinated antibody (dotted line). Flow cytometric analysis was carried out on a FACSCAN (Becton Dickinson). Live cells were gated by propidium iodide exclusion. A minimum of 10,000 cells were analyzed for each histogram. Fluorescence is plotted on a four-decade logarithmic scale against number of cells.

To assess the ability of these mutants to bind gp120, we carried out whole-cell saturation binding analysis, using ^{125}I-labeled recombinant soluble gp120 (rgp120) (*8*). This analysis revealed similar numbers of gp120-binding sites in cells expressing wild-type or mutant CD4 (except M1, which had too low a gp120-binding affinity to determine accurately) (Table 1), confirming the

Table 1. Analysis of cell surface expression, gp120 binding, and interaction with class II MHC of CD4 mutants. M1 to M8 are transduced murine T cell hybridomas that express CD4 homolog-scanning mutants in which clusters of four to nine amino acids of the human CD4 (T4) were substituted with the corresponding residues of the murine CD4 (L3T4) at a nonconserved region in V_1 to V_3. Numbering of amino acids and alignment with L3T4 are according to Littman and Gettner (*19*). Mutants were analyzed for their ability to interact functionally with target cells expressing the human class II MHC molecule HLA-DP (D^dDP), as measured by IL-2 production in the presence or in the absence of MAbs to CD4 (*24*), their ability to form rosettes (see Fig. 2) and their ability to bind rgp120 (*31*). Mutants were also tested for their capacity to produce IL-2 after stimulation with cross-linked MAbs to the 3DT52.5.8 TcR (*25*). Methods are described in the legend to Figs. 1 and 2.

Mutant	Amino acid position	Substitution sequence	Mean fluorescence (a.u.)*		rgp120 binding		Functional interaction (IL-2 units/ml) anti-CD4		Rosette formation (D^dDP)	Anti-TcR stimulation (IL-2 units/ml)
			OKT4	OKT4A	K_d (n*M*)	Sites/cell	−	+		
WT	—	—	19	19	5.6 ± 1.6	2730 ± 740	9120	2960	+	21,000
M1	39–43	h NQGSF- m QHGKGV	13	12	>120	—	9760	3200	+	20,000
M2	48–52	h PSKLN- m GSPSQF	16	13	27.0 ± 4.8	3080 ± 420	3040	0	+	24,000
M3	99–105	h GLTANSD m KVTFSPG	16	11	6.1 ± 1.7	2940 ± 310	80	0	−	18,000
M4	121–123	h PPG-S m NSKVS	30	21	4.6 ± 0.3	2960 ± 290	160	0	−	8,500
M5	127–134	h SVQCRSPR m LTECKHKK	16	15	4.4 ± 0.3	4160 ± 880	1040	0	+	20,800
M6	181–186	h KASSIV m STAITA	13	13	9.6 ± 0.8	3750 ± 450	320	20	−	25,000
M7	220–226	h ASSKS m DSFQP	8	11	6.9 ± 1.4	3250 ± 350	80	80	−	32,000
M8	240–252	h MGKK m LKET	22	62	9.2 ± 2.1	2640 ± 420	1280	0	+	

*a.u., Arbitrary units on a four-decade logarithmic scale.

results obtained by anti-CD4 staining. The affinity of each mutant for gp120, determined by Scatchard analysis, is summarized in Table 1. Mutant M1 showed a reduction of more than 20-fold in affinity for gp120, compared to wild-type CD4, mutant M2 had an ≈5-fold lower affinity, and mutant M3 was not significantly different from wild-type CD4 (Table 1). Mutants M4 and M5, as well as M6 to M8, residing within V_2 and V_3, respectively, exhibited gp120-binding affinities comparable to that of wild-type CD4 (Table 1). These results are consistent with previous results localizing the gp120 binding site to the V_1 domain (*10–13*).

A different picture emerged when we assessed the ability of these CD4 mutants to interact functionally with class II MHC molecules. Mutant M1, which had essentially lost gp120 binding, supported the production of IL-2 by 3DT52.5.8 cells as well as wild-type CD4 (Table 1). Mutant M2, whose affinity for gp120 was reduced by ≈5-fold, supported IL-2 production ≈3-fold less well than wild-type CD4 (Table 1). Mutants M3, M4, M6, and M7, were virtually inactive in the IL-2 production assay, although the mutations had not affected gp120 binding. M5 and M8 showed considerably reduced, but significant, IL-2 production and retained gp120 binding. The IL-2 production for each mutant was CD4-dependent, as it was largely blocked by antibodies to CD4 (Table 1) (*24*). The inability of some mutants to support IL-2 production did not appear to be due to an intrinsic defect in the cells, as all the cell lines tested produced high levels of IL-2 when activated with antibody to the T cell receptor (Table 1) (*25*).

To analyze directly the ability of these CD4 mutants to bind class II MHC molecules, we examined rosette formation between the target and effector cells used in the IL-2 production assay. Rosette formation requires the interaction of both the TcR and CD4 with their respective ligands (D^d and HLA-DP) on the target cells (Fig. 2, a and b), and is specific for CD4 since addition of antibody to CD4 (Fig. 2c) or of rgp120 (Fig. 2d) abolishes rosette formation, and the inhibition by rgp120 can be prevented by soluble recombinant CD4 (rCD4) (*22*). In most cases, the ability of the mutant-expressing cells to form rosettes (Fig. 2, e to l) paralleled their ability to support IL-2 production; however, for two mutants (M5 and M8) rosette formation was observed but IL-2 production was low. These mutants therefore do not appear deficient in MHC binding, but may instead be defective in complex formation with the TcR (*5*) or in signal transduction (*6*). We have obtained similar functional and adhesion results with target cells expressing the class II MHC molecule HLA-DR (*20*), indicating that the interactions between CD4 and different human class II MHC isotypes are conserved.

Clayton *et al.* (*26*) recently reported the existence of mutations in CD4 that inhibit interactions with MHC class II without affecting gp120 binding. We further describe a mutant (M1) which has lost gp120 binding and can still interact with class II MHC molecules in both functional and adhesion assays. These results indicate that the regions of the CD4 molecule that interact with gp120 and with class II MHC molecules are distinct and that these two functions are separable by mutation. The interaction with class II MHC CD4 can be abrogated by mutations in at least the first three immunoglobulin-like domains of CD4 domains (V_1 to V_3), whereas only the V_1 domain is required for significant gp120 binding (*13, 27*). Mutant M2 showed partial reduction in both gp120 binding and class II MHC binding, consistent with the possibility that residues within this region are important for both functions. Indeed, the idea that the two binding sites may partially overlap is supported by our finding that rgp120 can block the interaction of CD4 with class II MHC (Fig. 2d) (*17, 28*) as can antibodies that recognize the gp120-binding site of CD4 (*22, 29*). Of the eight mutants described here, all but one showed a reduction in class II MHC binding, consistent with the suggestion that a relatively large surface area of CD4 interacts with class II MHC (*30*). However, identification of other CD4 sequences important for class II MHC or gp120 binding, including invariant residues, will require more extensive mutational analysis.

Soluble CD4 analogs can inhibit HIV infection of T cells and monocytes (*9, 14–16*), and are currently under investigation as potential AIDS therapeutics. Our finding that the gp120-binding region of CD4 can be retained while deleting the interaction with class II MHC may therefore have practical importance, particularly for multivalent forms of CD4 (*16*).

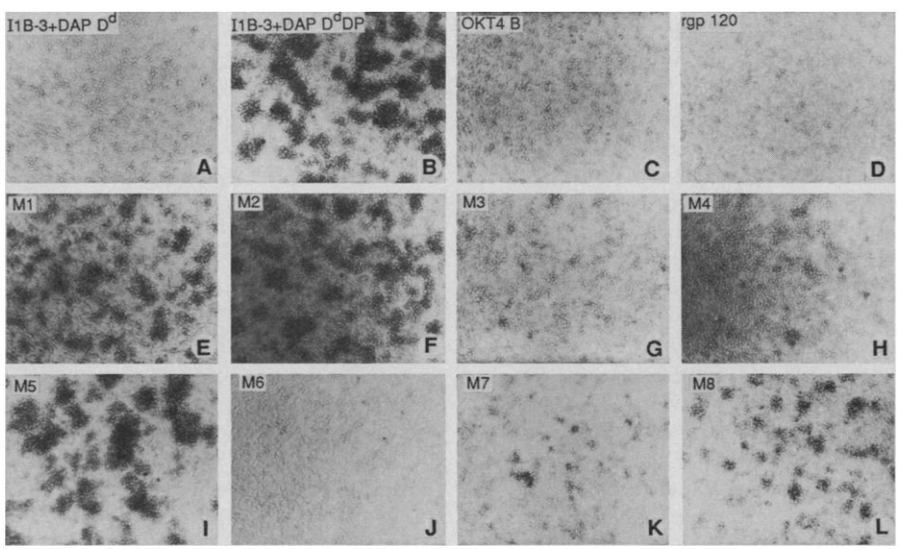

Fig. 2. Binding of the target DAP-3 D^dDP to the murine T cell hybridoma 3DT52.5.8 expressing cell surface wild-type (IIB-3) or mutant CD4 molecules. IIB-3 cells (**B**) and M1 to M8 (**E** to **L**), 10^5 cells, were cocultured with the murine fibroblastic DAP-3 D^dDP cell line (10^4 cells) for 20 hours at 37°C. Negative controls include the coculture of IIB-3 cells with (**A**) DAP-3 expressing only H2-D^d (DAP D^d), (**C**) DAP-3 D^dDP in presence of 20 n*M* of OKT4B antibody, (**D**) DAP-3 D^dDP in presence of 100 n*M* rgp120. Cells were then observed by phase contrast microscopy (Nikon Diaphot) and were photographed with Kodak Tri-X pan 400 ASA film under 100× magnification.

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24. T cell hybridomas expressing the mutant CD4 molecules were preincubated with the appropriate murine antihuman CD4 monoclonal antibody (MAb) at 20 n*M* for 60 min at 37°C prior to addition of 10^5 DAP-3 D^dDP cells. After a 20-hour co-culture, the culture supernatants were assayed for the presence of IL-2 using the IL-2–dependent cell line CTLL.2. The results are expressed as units of IL-2 per milliliter. M1, M2, M4, M7, and M8 expressed levels of the OKT4B epitope identical to the cells expressing wild-type CD4 and were treated with the OKT4B antibody. Monoclonal antibody (MAb) inhibition experiments with M3, M5, and M6 were performed in the presence of Leu3a antibody since these mutants expressed level of Leu3a comparable to those of cells expressing wild-type CD4.
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31. The dissociation constants (K_d) for rgp120–CD4 interaction were measured by saturation binding analysis of ^{125}I-labeled rgp120 to intact 3DT52.5.8–transduced cells. Cells (0.5×10^6 to 1.0×10^6) were incubated with ^{125}I-labeled rgp120 at concentrations ranging from 0.1 to 20 n*M* (1 hour, 37°C). Nonspecific binding was measured in the presence of 200-fold excess of unlabeled rgp120. The values reported are means ± SD of at least two independent experiments done in triplicate.
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Structure and Specificity of a Class II MHC Alloreactive γδ T Cell Receptor Heterodimer

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Two distinct CD3-associated T cell receptors (TCRαβ and TCRγδ) are expressed in a mutually exclusive fashion on separate subsets of T lymphocytes. While the specificity of the TCRαβ repertoire for major histocompatibility complex (MHC) antigens is well established, the diversity of expressed γδ receptors and the ligands they recognize are less well understood. An alloreactive $CD3^+CD4^-CD8^-$ T cell line specific for murine class II MHC (Ia) antigens encoded in the I-E subregion of the H-2 gene complex was identified, and the primary structure of its γδ receptor heterodimer was characterized. In contrast to a TCRαβ-expressing alloreactive T cell line selected for similar specificity, the TCRγδ line displayed broad cross-reactivity for multiple distinct I-E–encoded allogeneic Ia molecules.

TCRγδ T LYMPHOCYTES CONSTITUTE a distinct T cell lineage (1% to 10% of $CD3^+$ T cells) with cytolytic activity and the capacity to produce lymphokines (*1*). The major unresolved question concerning TCRγδ T cells is the nature of the ligands they recognize. The TCRαβ repertoire displays specificity for both class I and class II MHC (Ia) antigens. Several developmental parallels between TCRγδ and TCRαβ suggest that some TCRγδ might also recognize MHC antigens. For example, the TCRδ locus is situated with the TCRα locus and V_α gene elements can rearrange productively to DJδ (*2–4*).

In this light, several murine and human alloreactive TCRγδ T cell lines and clones specific for class I MHC antigens have been identified (*3, 5*). Evidence for different antigen-processing pathways for presentation of peptides by class I versus class II MHC molecules (*6*) suggests that class I and class II MHC–restricted T cells may recognize distinct sets of foreign protein antigens. Therefore, in order to better characterize the potential diversity of the MHC-specific TCRγδ repertoire, it was important to establish whether TCRγδ T cells have the capacity for class II MHC molecule recognition.

Alloreactive T cell lines were derived from the peripheral lymph nodes of athymic B10 ($H\text{-}2^b$) *nu/nu* mice by in vitro stimulation of T cells with H-2–congenic B10.BR ($H\text{-}2^k$) splenic antigen-presenting cells (APCs) (*7*). The structure of the receptor proteins expressed by these lines was examined by performing immunoprecipitations with a monoclonal antibody specific for the CD3ε chain of the TCR complex (*8*). One $CD3^+CD4^+CD8^-$ line, LBK4, expressed a CD3-associated heterodimer with a molecular mass of 41 to 43 kD, which appeared to be an αβ receptor (Fig. 1A and Fig. 1B, lane 1). That LBK4 was an αβ T cell line was confirmed by showing that an antiserum to $C_\beta 1$ (*9*) precipitated an identical 41- to 43-kD heterodimer (Fig. 1B, lane 2).

A second independently derived line, LBK5, with a $CD3^+CD4^-CD8^-$ surface phenotype, expressed a markedly different CD3-associated heterodimer consisting of proteins of 31 and 45 kD (Fig. 1C). A clone derived from this line, G11, expressed an identical heterodimer (Fig. 1D, lane 1). After reduction and alkylation of the immunoprecipitate of the G11 clone obtained with an antibody to CD3, the 31-kD and 45-kD proteins were immunoprecipitated by an antiserum to the $C_\gamma 1/C_\gamma 2$ peptide (*9*) (Fig. 1D, lane 3) and an antiserum to TCRδ (*10*) (Fig. 1D, lane 2), respectively.

The specificities of the TCRαβ-bearing LBK4 line and the TCRγδ-expressing LBK5 line were tested in cell-mediated cytotoxicity assays with target cells from various H-2 recombinant mouse strains (Fig. 2A). Because TCRγδ T cells cultured in the presence of exogenous growth factors may develop nonspecific cytolytic activity (*5*), the assays of cytolytic T lymphocytes (CTLs) were performed with cells that had been cultured in the absence of interleukin-2 (IL-2) for 24 to 48 hours. First, the MHC-linked specificity of both lines was shown by the fact that they lysed H-2 congenic B10.BR ($H\text{-}2^k$) (not shown) and B10.A ($H\text{-}2^a$), but not syngeneic B10 ($H\text{-}2^b$) or allogeneic B10.D2 ($H\text{-}2^d$) target cells (Fig. 2A, Exp. 1). The LBK5 but not the LBK4 T cells also killed B10.A(5R) ($H\text{-}2^{i5}$), B10.S(9R) ($H\text{-}2^{t4}$), and B10.RIII ($H\text{-}2^r$) targets (Fig. 2A, Exp. 2).

More detailed analysis of the specificity of the γδ receptor expressed by LBK5 was

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