methyl alcohol and dimethyl sulfoxide; melting point, 201° to 203°C; infrared: 3200, 3000, 1720 to 1680 band range (br), 1620 to 1550 br cm^{-1}; high-resolution mass spectrometry: requires 147.010 for $C_8H_5NO_2$, found 147.030; ^1H nuclear magnetic resonance, 360 MHz ($CDCL_3$): δ 8.2 (s, 1 H), 7.6 (dd, 1 H, J = 7.7 Hz), 7.5 (d, 1 H, J = 7.8 Hz), 7.1 (dd, 1 H, J = 7.7 Hz), 7.0 (d, 1 H, J = 7.8 Hz). The metabolite isolated was identical in all regards to a sample of 2,3-indolinedione (isatin) obtained from commercial sources.

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24. Each group of embryos was suspended by a thread in a Nalgene centrifuge tube containing 50 ml of autoclaved 50% seawater/pondwater with individual aeration through a Pasteur pipette.
25. Embryos were dipped in a solution (100 mg/liter) of penicillin-G (1600 units per milligram) for 6 hours on the first and second days of the experiment.
26. On day 3 of the experiment embryos were dipped for 2 hours in a liquid culture (medium used was 2216 marine broth) of the bacterium strain I-2.
27. On days 3, 5, and 7 of the experiment embryos were dipped in a solution of 200 μg of 2,3-indolinedione per milliliter for 1 hour.
28. Scanning electron micrographs showed that embryos from this group had become heavily infected with *L. callinectes*.
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A Single Amino Acid Interchange Yields Reciprocal CTL Specificities for HIV-1 gp160

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For the IIIB isolate of human immunodeficiency virus type–1 (HIV-1), the immunodominant determinant of the envelope protein gp160 for cytotoxic T lymphocytes (CTLs) of H-2^d mice is in a region of high sequence variability among HIV-1 isolates. The general requirements for CTL recognition of peptide antigens and the relation of recognition requirements to the natural variation in sequence of the HIV were investigated. For this purpose, a CTL line specific for the homologous segment of the envelope from the MN isolate of HIV-1 and restricted by the same class I major histocompatibility (MHC) molecule (D^d) as the IIIB-specific CTLs was raised from mice immunized with MN-*env*–recombinant vaccinia virus. The IIIB-specific and MN-specific CTLs were completely non–cross-reactive. Reciprocal exchange of a single amino acid between the two peptide sequences, which differed in 6 of 15 residues, led to a complete reversal of the specificity of the peptides in sensitizing targets, such that the IIIB-specific CTLs lysed targets exposed to the singly substituted MN peptide and vice versa. These data indicate the importance of single residues in defining peptide epitopic specificity and have implications for both the effect of immune pressure on selection of viral mutants and the design of effective vaccines.

CYTOTOXIC T LYMPHOCYTES ($CD8^+$) recognize a complex of peptide and class I MHC molecules on the surface of target cells (*1*). With the availability of the crystal structure of a human class I (HLA) molecule (*2*) and the results of structure-function studies of mutant class I molecules and variant peptide antigens (*3, 4*), the structural basis of CTL recognition of these surface molecules is being elucidated. However, the general requirements for an effective peptide antigen and the relation between peptide sequence, MHC binding capacity, and the specificity of recognition by the T cell receptor are still poorly understood. Although the minimal length of antigenic peptides has been well characterized in several studies, the role of individual residues in controlling class I MHC binding and dictating T cell specificity has been carefully examined in only a few model systems (*4*), with variable results in terms of the number of residues critical for each function. Our definition of a minimal peptide determinant of 15 amino acids (RIQRGPGRAFVTIGK, residues 315 to 329) in the HIV-1 gp160 molecule, recognized in the context of H-$2D^d$ (*5*), allowed us to study both the general requirements for peptide antigenicity and the relation between viral sequence variation and immunogenicity of this defined determinant. This was possible because the sequence lies in a segment of the gp160 molecule that shows a high degree of variability among HIV-1 isolates.

We found that we could induce CTLs specific for the immunodominant region when BALB/c mice were immunized with recombinant vaccinia virus carrying the IIIB isolate of human immunodeficiency virus type 1 (HIV-IIIB) gp160 envelope gene and their spleen cells were restimulated with the target peptide (*6*). Therefore, we immunized mice of several haplotypes, such as BALB/c (H-2^d), C3H (H-2^k), and C57BL/6 (H-2^b), with recombinant vaccinia virus expressing gp160 of the MN isolate of HIV-1 (HIV-MN) and restimulated them with interleukin-2 (IL-2) plus a peptide corresponding to the sequence in the HIV-MN gp160 envelope protein homologous to the previously identified CTL epitope of HIV-IIIB (*5*). We found that we could elicit CTLs specific for the homologous region of HIV-MN gp160 (RIHIGPGRAFYTTKN) (18MN) (Table 1) that can also kill in a highly specific manner targets infected with recombinant vaccinia virus expressing whole gp160 MN (Table 2). CTLs were not induced by stimulation in vitro of cells from unimmunized mice with peptide and IL-2, indicating that in vivo immunization was necessary. These MN-specific CTLs are conventional $CD4^-CD8^+$ CTLs, because they are killed by treatment with monoclonal antibodies to CD8 plus complement but not by antibody to CD4 plus complement (*6*). The CTLs directed against MN do not kill IIIB or RF recombinant vaccinia virus–infected targets, in agreement with our previous results that a $CD4^-CD8^+$ CTL line specific for gp160 IIIB did not cross-reactively kill targets infected with recombinant vaccinia viruses expressing the envelope gene from the RF and MN isolates (*7*). We could induce such variant MN-specific CTLs in BALB/c mice, but could not detect any specific CTLs from C3H and C57BL/6 mice, consistent with our findings using the HIV-IIIB envelope (*5*). Therefore, it was of interest to determine the class I MHC restriction of the newly identified CTL determinant in the MN variant. By using a panel of concanavalin A blast targets, we found that these MN-

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Table 1. Cross-reactive CTL activity for the homologous portion of gp160 from different HIV-1 isolates. Portions of the sequences underlined are conserved among these isolates.

HIV-1 isolates	Sequence	Specific lysis (%)* at various peptide concentrations					
		IIIB-specific CTLs			MN-specific CTLs		
		10 μ*M*	3 μ*M*	1 μ*M*	10 μ*M*	3 μ*M*	1 μ*M*
	315 329						
HIV-IIIB	R I Q R G P G R A F V T I G K	56.6	60.7	66.6	1.4	1.0	1.0
HIV-MN	R I H I G P G R A F Y T T K N	7.6	4.8	4.0	72.2	73.5	73.1
HIV-RF	S I T K G P G R V I Y A T G Q	0.7	−0.1	0.3	1.1	0.7	0.1
HIV-SC	S I H I G P G R A F Y A T G D	5.2	5.1	4.6	70.0	70.5	64.6
HIV–WMJ-2	S L S I G P G R A F R T R E I	−0.7	−1.9	−1.5	−1.6	−1.5	−1.3

*Effector to target ratio is 10:1.

specific CTLs recognized peptide presented by the D^d class I MHC molecule, exactly the same restriction element as seen by IIIB-specific CTLs (*8*). The fact that CTL responses to this determinant were detected with only one of seven class I MHC molecules tested (K^d, D^d, L^d, K^k, D^k, K^b, and D^b) suggests that the potential number of effective gp160 class I MHC molecule complexes that can be formed may be very limited, as has been observed with other viral proteins (*5, 9*).

From studies of competition by overlapping or substituted inactive peptides, we found that in the IIIB determinant, the critical region for binding to D^d is the COOH-terminal half of the peptide, with residues 322 (R) and 324 (F) being predominant (*10*). There are six amino acid differences between 18IIIB and 18MN (Table 1). Consistent with the binding of both peptides to D^d, positions 322 and 324 are shared. As a result of preliminary data with substituted peptides, we focused on the hypervariable amino acid 325, which was Val in 18IIIB and Tyr in 18MN, as a candidate for one of the key residues controlling the difference in specificity observed for the MN and IIIB CTL responses. Peptides with the complete sequence of either MN or IIIB in positions 315 to 329, except for a reciprocal exchange of the residue at position 325, were synthesized and used in CTL lysis experiments. This single amino acid substitution was sufficient to interchange the specificities of MN and IIIB (Fig. 1). Thus, if the Tyr at position 325 of the 18MN peptide was changed to Val, the MN-specific CTLs no longer recognized the substituted peptide except very weakly and only at very high concentration (more than 10 μ*M* compared to 0.01 μ*M* for 18MN), but surprisingly, despite five other remaining differences in sequence, the substituted peptide was recognized by the IIIB-specific CTL line almost as well as the peptide 18IIIB, although this IIIB-specific CTL line could not recognize the wild-type peptide 18MN at all. Conversely, when the Val at position 325 of the 18IIIB peptide was changed to Tyr, potency for recognition by the IIIB-specific CTL line was diminished more than 1000-fold, but now the MN-specific CTL line, which could not recognize the wild-type peptide 18IIIB, killed targets sensitized with this substituted peptide as effectively as it killed targets sensitized with the MN peptide containing all six differences. Therefore, a single amino acid substitution was sufficient to reciprocally alter the CTL epitope specificity of this

Table 2. Specificity of IIIB- and MN-derived CTL lines. As targets, BALB/c 3T3 fibroblasts were incubated with 10^7 plaque-forming units per milliliter of these recombinant vaccinia viruses expressing HIV-1 envelope gp160 genes (vMN and vIIIB), or with control vaccinia virus (vCon), or pulsed with 1 μ*M* (18IIIB) or 5 μ*M* (18MN) of peptide for 60 min.

CTL line	Specific lysis (%) of various targets*					
	vMN	vIIIB	vCon	18MN	18IIIB	None
IIIB	0.4	31.2	−4.7	2.6	67.4	3.2
MN	29.7	−1.0	−5.8	47.5	1.2	2.8

*Effector to target ratio is 10:1.

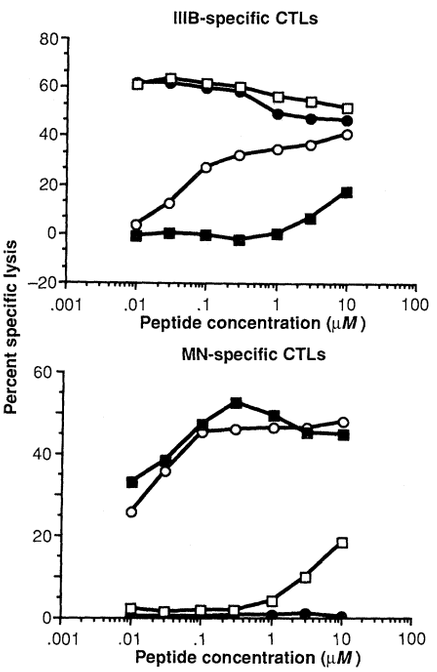

Fig. 1. Reciprocal interchange of specificity by substitution of a single amino acid. Mice were immunized intravenously with 10^7 plaque-forming units of recombinant vaccinia virus. Three to 14 weeks later, immune spleen cells [5×10^6 per milliliter in 24-well culture plates in complete T cell medium (CTM): a 1:1 mixture of RPMI 1640 and Eagle-Hanks amino acid medium containing 10% fetal bovine serum, 2 m*M* L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 5×10^{-5} *M* 2-mercaptoethanol] were restimulated in vitro repetitively with either 2×10^5 of mitomycin-treated per milliliter (100 μg/ml for 30 min at 37°C) gp160 gene–transfected, MHC-identical fibroblasts (*5*), or 1 μ*M* of peptide 18IIIB or 5 μ*M* of peptide 18MN together with 4×10^6 of 3300-rad irradiated syngeneic spleen cells in the presence of 10% concanavalin A supernatant-containing medium (Collaborative Research). Cytolytic activity of CTL lines was measured by a 6-hour assay with various ^{51}Cr-labeled targets. For testing the peptide specificity of CTL, effectors and ^{51}Cr-labeled BALB/c 3T3 fibroblasts targets were mixed with various concentrations of peptide [●, 18IIIB; ○, 18IIIB(V→Y); ■, 18MN; or □, 18MN(Y→V)] at the beginning of the assay. The peptide 18IIIB(V→Y) consists of the 18IIIB sequence (Table 1) with residue 325 Val replaced by Tyr (RIQRGPGRAF**Y**TIGK). The peptide 18MN(Y→V) consists of the 18MN sequence (Table 1) with 325 Tyr replaced by Val (RIHIGPGRAF**V**TTKN). The percent specific ^{51}Cr release was calculated as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton-X 100. Spontaneous release was determined from target cells incubated without added effector cells. Standard errors of the mean of triplicate cultures were all <5% of the mean.

immunodominant peptide from the HIV gp160 protein, despite five other differences in sequence.

Only a few cases have been reported in which amino acid substitutions in defined peptides have allowed interchange of specificity among sets of T cells reactive with the parent peptides (*11, 12*) and these deal with class II MHC presentation, not class I. In all of these cases, several amino acids were altered and, in all but one case, the responses to the recombinant peptides were substantially weaker than the responses to the parental forms. In the one case in which a comparable response was obtained (*12*), five amino acid substitutions were required, and the interchange was not reciprocal. Other studies have demonstrated, however, that a single amino acid change at a critical position in a short peptide immunogen can elicit T cells uniquely specific for that form of the peptide, and T cells elicited under such conditions show the reciprocal recognition properties described here for related peptides varying only in the critical epitopic residue (*13*). In these cases, though, the related peptides differed only in that single position, so that the single change converted one peptide into the other. The finding here is that five other amino acid mismatches between the MN and IIIB peptides are permitted, as long as the key residue at 325 matches the immunogen eliciting the CTL. These findings suggest that position 325 in the gp160 envelope protein may be one of the critical amino acids for CTL epitope recognition. Furthermore, the MN-specific CTL line cross-reactively killed targets sensitized with the corresponding HIV-SC–derived peptide, but did not kill the targets sensitized with the corresponding peptide from HIV-RF or HIV–WMJ-2 (Table 1). The SC sequence shares the sequence 316 to 325 with MN, including the Tyr at position 325, whereas the WMJ-2 sequence shares the sequence 318 to 326 with MN, except for the nonconservative substitution of Arg for Tyr at position 325. In contrast, the RF sequence shares the Tyr at position 325 with MN but differs in the three residues surrounding this one. Therefore, these other residues at positions 323, 324, and 326 may also be specificity-determining residues, or may influence the configuration of the Tyr at position 325, or may affect D^d binding. Failure of 18RF to compete with 18IIIB in target sensitization experiments suggests that one or more of these residues may affect binding to D^d, in agreement with the assignment of this function to residue 324. However, the IIIB-specific CTL line did not cross-reactively kill targets sensitized with any of the above HIV isolates (Table 1). Also, the MN-specific line recognized the HIV-SC epitope in association with the same D^d class I MHC molecule (Table 1) (*8*). These results suggest that residue 325 may be a critical epitopic amino acid for other HIV-1 variants. The current results may be relevant in view of recent findings that more than 80% of patients tested in the United States have sera that react with the MN sequence in this region, and that about three-fourths of 51 of these isolates have the critical Tyr at position 325, defined here (*14*).

The results of the present studies, combined with our previous data (*10*) on the key residues involved in controlling binding of the IIIB determinant to D^d, suggest that only a small number (two to four) of the amino acids in a minimal peptide sequence are crucial for the specificity of the two essential features of MHC molecule association and T cell receptor recognition. This conclusion is in agreement with the results of two very recent studies, one involving a class II–associated peptide (*12*) and the other a CTL target determinant (*15*), although in the former study a greater number of residues needed to be exchanged for transfer of specificity, which was not reciprocal, and in the latter study, an exchange experiment was not performed. The overall length (usually seven to ten residues) and spacing requirements observed for peptide antigens may thus reflect the importance of sequence nonspecific factors, perhaps binding stabilization via the peptide backbone, and orientation of the epitopic residues in the proper spatial relation to the site of T cell receptor–MHC interaction. The similarity in overall charge, although too gross a property to contribute in a specific way to T cell recognition, may also have a nonspecific role in interaction with the MHC molecule (*16*). Nevertheless, the fact that for peptides 18IIIB and 18MN the interchange in specificity was reciprocal and resulted in full reversal of activity suggests that, as long as appropriate structures are available for interaction with the class I MHC molecule and orientation of the peptide, a single amino acid may contribute most of the binding energy defining the specificity of both CTL receptors.

CTLs may contribute to immune control of HIV-1 infection (*17*). Changes in epitopic residues (that is, those that affect CTL specificity rather than MHC binding) might affect susceptibility of the virus to immune defense mechanisms if the CTL repertoire varied significantly among individuals. However, T cell receptor repertoires are thought to be broad enough to accommodate recognition of almost all mutated virus proteins as long as the viral proteins are still processed and presented in association with the available MHC molecules of the individual (*13*). The primary means of escape from immune effector targeting after such epitopic changes would be due to mutations that lead to self-mimicry (*18*). A second mode of escape from immune destruction after epitopic alteration would be somewhat unique to HIV. Because there is a progressive loss of $CD4^+$ T cells after infection, as each new mutant virus arises, there will be a diminished capacity to generate a new wave of specific CTLs because of the decrease in available T helper cell function. At some point, the new virus will escape the cytotoxic T cell system entirely as immune function reaches a nadir. This may in fact presage the more severe or overt stages of acquired immunodeficiency syndrome (AIDS) and argues that the observed latent state in asymptomatic seropositive individuals reflects the effective functioning of the immune system before its collapse. Changes in agretopic residues (those that affect MHC binding) would also be expected to affect CTL recognition in that, without binding of peptide to a suitable class I MHC molecule, CTLs can no longer recognize the mutated immunodominant site of the virus at all.

Recent preliminary data indicate that five of the humans who were immunized with recombinant vaccinia virus expressing HIV-IIIB envelope gp160 protein (vSC25) (*19*) showed specific CTL activity against cells pulsed with the 18IIIB antigenic site (*20*), although no single MHC molecule was shared by all the responders. Also, a number of HIV-infected humans have CTLs that kill autologous targets pulsed with peptide 18IIIB (*21*). This suggests that more than one of the human polymorphic class I MHC molecules may have the ability to bind this immunodominant site of HIV-1. Thus, a common way for the virus to escape from human immune pressure, especially from specific CTL recognition, may be to alter one or more of the epitopic amino acids. Our results are compatible with the human data cited above in that HIV-1 mutate their epitopic residue 325. Furthermore, it has been shown that the HIV-1 virus can mutate within the course of disease in a single individual (*22*). This emphasizes the importance of investigating the relation among the human HLA type, the infecting HIV-1 isolate, and the mutated HIV-1 isolate during the progress of the disease.

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Fibroblasts Transformed with v-*src* Show Enhanced Formation of an Inositol Tetrakisphosphate

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The tyrosine kinase pp60$^{v\text{-}src}$, encoded by the v-*src* oncogene, seems to regulate phosphatidylinositol metabolism. The effect of pp60$^{v\text{-}src}$ on control points in inositol phosphate production was examined by measuring the amounts of inositol polyphosphates in Rat-1 cells expressing wild-type or mutant forms of the protein. Expression of v-*src* resulted in a five- to sevenfold elevation in the steady-state amount of an isomer of inositol tetrakisphosphate, whereas the concentrations of inositol trisphosphates or other inositol tetrakisphosphates were not affected. The activity of a key enzyme in the formation of inositol tetrakisphosphates, inositol (1,4,5)-trisphosphate 3-kinase, was increased six- to eightfold in cytosolic extracts prepared from the v-*src*–transformed cells, suggesting that this enzyme may be one target for the pp60$^{v\text{-}src}$ kinase and that it may participate in the synthesis of novel, higher order inositol phosphates.

ONCOGENES ARE ALTERED OR overexpressed forms of genes whose products participate in normal growth control, and oncogene products are presumed to induce malignancy by performing in an unregulated fashion activities that the proto-oncogene products control in normal cells (*1*). Thus, certain oncogene products are closely related to growth factors, mitogen receptors, and nuclear regulatory proteins (*1*). The protein encoded by the v-*src* oncogene, pp60$^{v\text{-}src}$ (*2*), may interact with inositol lipid signaling pathways because it regulates a membrane-bound phosphatidylinositol kinase (*3*) and stimulates phospholipid breakdown (*4*). However, control of the inositol signaling pathways is complex, with regulatory sites in both the membrane and cytoplasm (*5*).

To investigate the effect of expression of the v-*src* oncogene on inositol polyphosphate concentrations, we incubated growing, nearly confluent cultures of normal and *src*-transformed Rat-1 fibroblasts for 24 hours with [^{3}H]*myo*-inositol and examined the steady-state amounts of those isomers that are thought to serve as intracellular messengers, the inositol tris- and tetrakisphosphates (*5*). We added serum to the incubation medium so that the normal cells would continue to divide, thereby allowing us to compare normal growing cells with the constitutively proliferating v-*src*–transformed cells. The inositol polyphosphates present in extracts of normal and v-*src*–transformed cells were resolved on a strong anion-exchange column by high-performance liquid chromatography (HPLC) (*6*). When the results from five such experiments were averaged and corrected for sample protein content, the steady-state amount of inositol tetrakisphosphate (IP_4) was 6.2 (±1.9) times as great in v-*src*–transformed cells as in normal fibroblasts, whereas the amounts of inositol (1,4,5)-trisphosphate [$I(1,4,5)P_3$] and inositol (1,3,4)-trisphosphate [$I(1,3,4)P_3$] were similar in the two cell types (Fig. 1). The amount of IP_4 was not increased in Rat-1 cells containing the psV-*neo* vector alone. Our studies suggest that the high concentration of IP_4 in v-*src*–transformed cells is not due to an agonist

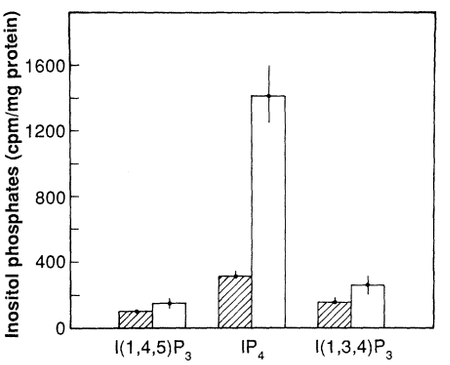

Fig. 1. Effect of expression of v-*src* on the steady-state amounts of inositol polyphosphates in Rat-1 cells. Normal (hatched bars) and v-*src*–transformed (open bars) Rat-1 fibroblasts were labeled with [^{3}H]*myo*-inositol for 24 hours, and ^{3}H-labeled inositol polyphosphates were extracted from the cells (*6*). The $I(1,3,4)P_3$, $I(1,4,5)P_3$, and IP_4 in the sample were resolved by HPLC on a strong anion-exchange column, and the counts in each compound were corrected for the protein corresponding to the sample applied to the column. Data represent the mean ± SEM from five experiments performed on two to three dishes of cells per experiment. The effect of v-*src* on IP_4 concentrations was significant at the $P < 0.05$ confidence interval using a one-tailed, paired *t* test.

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