observed $-3/2$ power dependence of probability on gap length.

The final outcome of the exhaustive matching is a reorganized database that can be rapidly searched using the DARWIN (Data Analysis and Retrieval With Indexed Nucleotide/Peptide Sequences) system (*19*). Each of the 1.7×10^6 aligned pairs of subsequences that result from the exhaustive matching is characterized by an evolutionary distance measured in PAM units. DARWIN, taking a PAM distance from the user, rapidly reconstructs the entire database in the form of sets of "connected components," entries joined by a match with every other entry in the component at or below the user-designated PAM. Because the PAM distances are accompanied by a statistical variance, evolutionary trees (*20, 21*) constructed from these distances by DARWIN are rigorous; they are accompanied by a probability score for the most probable connectivity, probabilistic sequences for the ancestral proteins at the nodes of the tree, and a multiple alignment.

At very low PAM distances, the connected components include very similar sequences, multiple entries in the database, and entries that differ only because of sequencing or entry error. At increasing PAM distances, however, connected components grow to include families and superfamilies of proteins. Repetitive sequences are the only feature that significantly joins apparently nonhomologous entries into connected components. From the total number of connected components plotted as a function of PAM distance (Fig. 4), the number of different protein types in the database can be estimated. Even conservative estimates indicate the existence of several thousand separate families of proteins (*8*). Finally, from these connected components, proteins and metabolisms can be reconstructed for various ancestors of modern organisms (*10*). Several of these reconstructed ancient proteins have now been prepared and studied in these laboratories (*22*).

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$$10\log(P) = -39.21 + 7.75\log(\text{PAM distance}) - 1.65(k - 1)$$

For users of alignment programs that do not permit variation with different PAM distances, the following scoring is recommended:

$$10\log(P) = -20.63 - 1.65(k - 1)$$

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Total Chemical Synthesis of a D-Enzyme: The Enantiomers of HIV-1 Protease Show Demonstration of Reciprocal Chiral Substrate Specificity

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The D and L forms of the enzyme HIV-1 protease have been prepared by total chemical synthesis. The two proteins had identical covalent structures. However, the folded protein-enzyme enantiomers showed reciprocal chiral specificity on peptide substrates. That is, each enzyme enantiomer cut only the corresponding substrate enantiomer. Reciprocal chiral specificity was also evident in the effect of enantiomeric inhibitors. These data imply that the folded forms of the chemically synthesized D- and L-enzyme molecules are mirror images of one another in all elements of the three-dimensional structure. Enantiomeric proteins are expected to display reciprocal chiral specificity in all aspects of their biochemical interactions.

The inherent chirality of "natural" organic compounds as products of physiological processes was first described by Pasteur (*1, 2*). The studies of Emil Fischer in the latter part of last century on the action of enzymes on chiral sugars led him to formulate his "lock and key" hypothesis as an explanation for the ability of the "asymmetrically constructed agent from yeast cells" (that is, an enzyme) to discriminate enantiomeric forms of a sugar substrate (*3*). On the basis of such observations of stereochemical specificity, Fischer believed that biological macromolecules (carbohydrates and proteins) were composed exclusively of the D-sugars and the L-amino acids. It is now well established that the biosphere is inherently chiral, that each class of biological macromolecules is made up of monomer molecules of uniform chirality (*4*), and that the biochemical interactions of biological macromolecules are inherently chiral.

Enzymes, for example, invariably act only on one enantiomer of a chiral substrate, or generate only one diastereomer from a prochiral substrate (*5*). This specificity can be related to the chiral structure of the enzyme molecule, including the three-dimensional folding of the polypeptide backbone and the orientation of the amino acid (aa) side chains in the folded protein molecule (*3, 5, 6*). To date only

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L-enzymes have been described; this leaves the presumed properties of D-enzymes, which include folded structure, enzymatic activity, and chiral specificity, as unexplored questions.

Advances in the total chemical synthesis of proteins (*7–9*) have made possible the reproducible production of homogeneous crystalline L-[Aba^{67,95,167,195}]HIV-1 protease (L-HIV PR) (*10–12*). We undertook the total chemical synthesis of D-[Aba^{67,95,167,195}]HIV-1 protease (D–HIV PR), and compared the properties [covalent structure, physical properties, circular dichroism (CD) spectra, and enzymatic activity] of the D- and L-enantiomeric forms of this enzyme.

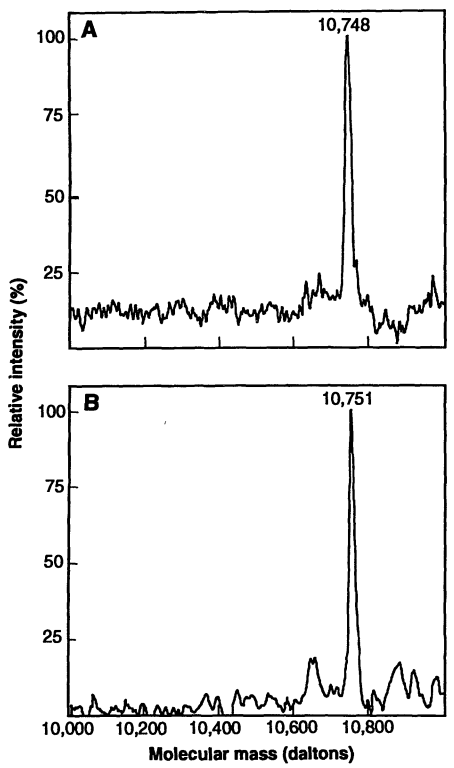


Fig. 1. Covalent characterization of the D- and L-enzyme enantiomers. Deconvoluted ion-spray mass spectrum of (**A**) the L-enzyme; observed monomer molecular mass 10,748 ± 4 daltons; and (**B**) the D-enzyme; observed monomer molecular mass 10,751 ± 3 daltons. Calculated mass: 10,748.0 daltons (monoisotopic), 10,754.7 daltons (average). Purified, folded, chemically synthesized [Aba^{67,95}]HIV PR samples in pH 6.5 MES buffer–10% glycerol were subjected to desalting by reversed-phase high-performance liquid chromatography. The collected protein peak was analyzed by ion-spray mass spectrometry (*29*). Under the conditions used (50% acetonitrile, 50% water, 0.1% trifluoroacetic acid) the enzyme is denatured. In the deconvoluted mass spectra shown, the raw mass-to-charge data have been subjected to a high-pass digital filter and then sorted to yield all parent molecular species between 10 and 11 kD. This deconvolution procedure mathematically reduces the multiple charge states observed for a given molecular species to a single molecular mass.

In separate experiments, the protected polypeptide chains corresponding to the L- and the D-sequences of the [Aba^{67,95}]HIV PR 99-aa monomer (*12*) were prepared by total chemical synthesis (*13*). The products were deprotected and worked up individually, and the synthetic enzymes prepared by folding from denaturant as previously described (*14*).

Analytical reversed-phase high-performance liquid chromatography (HPLC) gave identical retention times for the two synthetic polypeptide chains, and the two products had the same molecular weight, within experimental uncertainty, by ion-spray mass spectrometry (Fig. 1). The complete amino acid sequence of the D-enzyme 99-aa monomer was determined (*15*) and was shown to be the same as that of the L-enzyme. Thus, the two synthetic enzyme molecules had identical covalent structure.

Differences between the two molecules were revealed in chiral interactions. The CD spectra of the individual D- and L-HIV PR enantiomers revealed equal and opposite optical rotations (*16*), as expected for enantiomeric protein molecules. The enzymatic properties of the enantiomeric proteins were evaluated with a fluorogenic assay in which a hexapeptide analog of a natural GAG cleavage site was used as substrate (*17*). The two synthetic enzyme molecules were equally active, but showed reciprocal chiral specificity in that the L-enzyme cleaved only the L-substrate whereas the D-enzyme cleaved only the corresponding D-substrate (Fig. 2). Similarly, the enantiomers of the pseudopeptide inhibitor, MVT101 (Ac-Thr-Ile-Nle-ψ-[CH_2NH]-Nle-Gln-Arg·amide) (Ac, acetyl, and Nle, norleucine) (*11*), were evaluated for their effect on D- and L-HIV PR (Table 1). As expected, the chiral inhibitors were effective only against the corresponding enantiomer of the enzyme, that is, L-MVT101 inhibited L-HIV PR but not the D-HIV PR–catalyzed reaction, and D-MVT101 inhibited D-HIV PR but had no effect on the L-enzyme–catalyzed reaction.

Table 1. Chiral inhibitors show reciprocal chiral specificity against D- and L-HIV PR. The D- and L-enzymes were separately assayed by the fluorogenic assay method (*17*) with the corresponding chiral substrate, in the presence of 5 × IC_{50} concentration of inhibitor. The inhibitor Evans Blue is a nonpeptide, achiral mixed competitive-uncompetitive inhibitor of HIV PR (*28*).

Enzyme	L-MVT101	D-MVT101	Evans Blue
L-HIV PR	+	−	+
D-HIV PR	−	+	+

Interestingly, the achiral inhibitor Evans Blue, which shows mixed inhibition kinetics, was a potent inhibitor of both enantiomers of the enzyme (Table 1).

The HIV PR exists as a homodimer; that is, a single enzyme molecule is made up of two identical 99-aa folded polypeptide chains (*10, 11*). HIV PR is highly active, showing rate enhancement of about 10^{10}-fold over uncatalyzed peptide-bond hydrolysis (*18, 19*). It is a highly specific enzyme that cleaves peptides as well as proteins (*18, 20*) and its specificity is determined by the interactions of the three dimensionally folded enzyme molecule forming a complex with six consecutive amino acid residues in

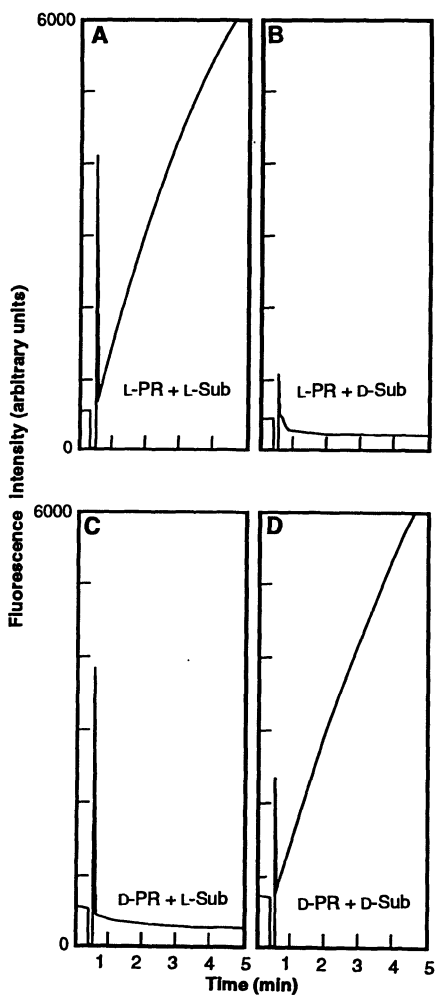
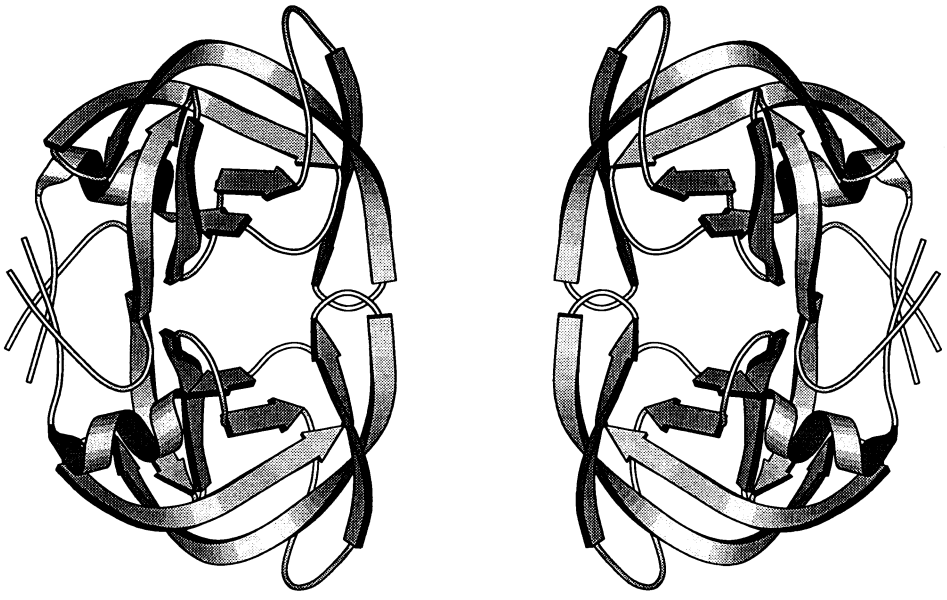


Fig. 2. Comparative activity of the HIV PR enantiomers on enantiomers of a chiral fluorogenic substrate. (**A**) L-Enzyme with L-substrate. (**B**) L-Enzyme with D-substrate. (**C**) D-Enzyme with L-substrate. (**D**) D-Enzyme with D-substrate. The L-enzyme acts only on the L-substrate, and the D-enzyme acts only on the D-substrate. Aliquots containing equal amounts (as determined by amino acid analysis) of the purified, folded enzyme preparations were used in a fluorogenic assay (*17*). The increase in fluorescence was recorded on a continuous chart recorder.



L–HIV protease D–HIV protease

Fig. 3. Ribbon representations (*30*) of the polypeptide backbone of the homodimeric HIV PR. (**Left**) L-[Aba^{67,95,167,195}]HIV PR, based on the x-ray crystallographic coordinates (*11*) of the chemically synthesized enzyme complexed with a substrate-derived peptide inhibitor (inhibitor is not shown). (**Right**) The folding of the D-[Aba^{67,95,167,195}]HIV PR polypeptide backbone suggested by the data in this report. This model was generated by performing a mirror-image transformation of the L-enzyme data. The folded backbone "ribbon" structures are nonsuperimposable mirror images and contain numerous chiral elements of secondary, tertiary, and quaternary structure. Note, for example, the relatedness of the flaps to one another, the relatedness of the helix segments to the neighboring β strands, the characteristic twist (*31*) (right-handed, in the L-protease) of the antiparallel β strands in each flap, and the handedness of the helical segments.

the substrate polypeptide chain (*11, 18*). As with all enzymes, HIV PR owes its specificity and catalytic activity to the precise three-dimensional (3-D) structure formed by specific folding of the polypeptide chain (6), and to precise geometric interactions in the specific complexes formed with substrates (*3, 5*). The observed reciprocal chiral specificities, therefore, show that the folded forms of the D- and L-enzyme molecules are mirror images of one another in all elements of the 3-D structure responsible for the enzymatic activity. The extensive nature of these interactions (*11*) implies that the two enzyme molecules are mirror images in every respect (*21*), consistent with the observed equal and opposite CD spectra. Most notably, the folded form of the polypeptide backbone (that is, ignoring the side chains) is itself a chiral entity that must exist in mirror image form in the two protein enantiomers (Fig. 3).

The folded 3-D structure of an enzyme molecule contains numerous chiral elements in secondary and supersecondary structure, in the tertiary structure, and in the quaternary structure (Fig. 3). Since the only chiral element introduced in the chemically synthesized polypeptide chains is the stereochemistry at the amino acid Cα atoms (and the Cβ atoms of Thr and Ile), our results demand that all stereochemical aspects of the folded enzyme molecule, from secondary to quaternary structure, are determined simply by the stereochemistry of the polypeptide backbone. Thus, the reciprocal chiral properties of the chemically synthesized enzyme enantiomers are a fundamental demonstration that the final folded 3-D structure and consequent biological activities of this 21.5-kD homodimeric enzyme molecule are completely determined by the amino acid sequence (*22*).

The observed reciprocal chiral properties of the mirror-image enzyme molecules described in this report serve to reinforce and generalize the chiral nature of biochemical interactions of proteins. The chiral properties of the protein molecules themselves, which give rise to this behavior, are given only cursory attention in biochemical texts. We can now state, based on experimental evidence, that protein enantiomers should display reciprocal chiral specificity in their biochemical interactions.

The observation that both enantiomers of HIV PR were equally affected by the achiral inhibitor Evans Blue suggests a number of potentially significant implications. First, the unnatural enantiomer of an enzyme that operates on an achiral substrate and yields an achiral product (such as carbonic anhydrase) should be fully functional in vivo. This may have important potential therapeutic applications. D-Enzymes are expected to be long-lived in vivo (in an L-protein biosphere) because they would be resistant to naturally occurring proteases that would in general attack only proteins made up of L-amino acids. D-Proteins may also be nonimmunogenic (*23*).

D-Protein molecules have other potentially practical applications (*24, 25*). At the present time D-enzymes, and D-proteins in general, are accessible only by total chemical synthesis (*26*). Recent innovations (*27*) in the total chemical synthesis of proteins should considerably increase the utility of this approach to the preparation of protein enantiomers.

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12. Aba is L- or D-α-amino-*n*-butyric acid and is used as an isosteric replacement for Cys residues at positions 67 and 95 in the HIV PR monomer polypeptide chain. This same isosteric replacement was used in the work leading to the original correct structures of HIV PR (*10, 11*).
13. The 99-aa polypeptide chains were assembled from protected L-amino acids and protected D-amino acids, respectively. The tBoc (*tert*-butyloxycarbonyl) D– and L–amino acid derivatives were obtained from the Peptide Institute (Osaka, Japan) and Peptides International (Louisville, KY) except: Boc-L-Aba, Boc-L-Asn(Xan) (xanthyl), Boc-D-Ile and Boc-D-His(Bom) (benzyloxymethyl), obtained from Bachem Bioscience (Philadelphia, PA); Boc-D-Asn(Xan), Boc-D-Asp(OcHex) (*O*-cyclohexyl) and Boc-D-Glu(OcHex), obtained from Bachem California, (Torrance, CA); Boc-D-Lys (ClZ) (chlorobenzyloxycarbonyl), crystallized from the *tert*-butyl amine (TBA) salt obtained from the Peptide Institute; and, D-Aba (Sigma, St. Louis, MO) which was converted to Boc-D-Aba and isolated as the dicyclohexylammonium salt. Other side-chain protecting groups that were used were: Arg(Tos) (tosyl); Tyr(BrZ) (bromobenzyloxycarbonyl), L-His(Tos), D-His(Bom) and Thr(Bzl) (benzyl). The L-enantiomer content of the Boc-D-

amino acid preparations was between 0.01 and 0.08% (manufacturers specifications). Stepwise chain assembly was carried out in a machine-assisted fashion on a highly modified Applied Biosystems 430A synthesizer (0.2 mmol scale with D- or L-Boc-Phe-OCH_2-Pam-resin). Each cycle of amino acid addition (*9*) involved: Nα-deprotection, neat (100%) trifluoroacetic acid (2 × 30-s flow washes, + 1-min batchwise treatment); *N,N'*-dimethyl formamide (DMF) flow wash (1 × 22 s followed by 1 × 38 s); and coupling (1 × 10 min) with simultaneous in situ neutralization [Boc amino acid (2.25 mmol) preactivated by reaction with benzotriazolyltetramethyluronium-hexafluorophosphate (HBTU) (2.22 mmol) and diisopropylethylamine (DIEA) (6.4 mmol) in DMF for 2 min]. The in situ neutralization method has been shown to result in negligible levels of racemization (*9*) [P. Henklein, M. Beyermann, B. Costisella, R. Sohr, M. Haußner, in *Innovation & Perspectives in Solid Phase Synthesis*, R. Epton, Ed. (SPPC Ltd., Birmingham, U.K., in press)]. The assembled peptides were deprotected and cleaved from the resin in 9:1 HF–*p*-cresol [resorcinol and thiocresol were present when His(Bom) was included in the sequence] after removal of the Boc group and the formyl group from Trp (with ethanolamine) (*8*).

14. After deprotection and cleavage the crude peptide products were precipitated with ether and dissolved with 6 M guanidine hydrochloride in a pH 8.0 $NaHCO_3$ buffer prior to semi-preparative C_{18} reversed-phase high-performance liquid chromatography enrichment and folding by dialysis in 10% glycerol, 25 mM NaH_2PO_4 buffer, pH 7.0 (*10, 11*). After concentration under high vacuum to a solution in glycerol, the enzymes were quantitated by amino acid analysis and stored at 4°C.
15. Samples taken in the assembly of the D-monomer polypeptide chain were cleaved and deprotected, and the sequence was determined by matrix-assisted laser desorption time-of-flight mass spectrometric readout (B. Chait and S. B. H. Kent, unpublished results).
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21. The empty (uncomplexed) HIV PR molecule is highly symmetric and has a C_2 axis; that is, a 180° rotation about an axis running between the flaps and between the active site Asp residues generates an identical structure (*10*). This rotational symmetry does not affect the chiral asymmetry of the enzyme molecule.
22. The L- and D-enzymes in this study have not been exposed to biosynthetic conditions and have thus not been in contact with biochemical factors of any sort. Interestingly, the simple homodimeric enzyme molecule studied here is formed rapidly (both folding and assembly) and accurately even at the relatively low concentrations used in the assay conditions, as well as in more normal dialysis-from-denaturant folding conditions. The results obtained in this study are conclusive evidence that whatever their proposed role, biosynthetic factors are not required for the formation of the correct, functional folded and assembled form of the protein. This result suggests that caution should be exercised in hypotheses concerning biosynthetic oligomeric protein folding [J. E. Rothman, *Cell* **59**, 591 (1989)]. Our observations are consistent with the original proposal [C. B. Anfinsen, *Science* **181**, 223 (1973)] that the amino acid sequence alone determines the folded 3-D form of the protein molecule.
23. Although D-amino acids and small peptides containing D-amino acids may function as haptens in an immune response, it is not expected that a long polypeptide chain made up entirely of D-amino acids could be processed and presented by the immune system.
24. Enzyme enantiomers may have use as chiral catalysts in the production of both enantiomers of a fine chemical.
25. Protein enantiomers can potentially contribute to the acquisition of phase data in x-ray crystallography [A. L. Mackay, *Nature* **342**, 133 (1989)]. Centro-symmetric crystals of a D-, L-protein pair would have greatly simplified phases, and more reliable structures may be obtained in this way.
26. Ribosomal synthesis of polypeptide chains, even in in vitro translation systems, does not incorporate D-amino acids [J. A. Ellman, D. Mendel, P. G. Schultz, *Science* **255**, 197 (1992)].
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Lymphoid Development in Mice Congenitally Lacking T Cell Receptor αβ–Expressing Cells

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Vertebrate T cells express either an αβ or γδ T cell receptor (TCR). The developmental relatedness of the two cell types is unresolved. $\alpha\beta^+$ T cells respond to specific pathogens by collaborating with immunoglobulin-producing B cells in distinct lymphoid organs such as the spleen and Peyer's patches. The precise influence of $\alpha\beta^+$ T cells on B cell development is poorly understood. To investigate the developmental effects of $\alpha\beta^+$ T cells on B cells and $\gamma\delta^+$ T cells, mice homozygous for a disrupted TCRα gene were generated. The homozygotes showed elimination of $\alpha\beta^+$ T cells and the loss of thymic medullae. Despite this, $\gamma\delta^+$ T cells developed in normal numbers, and there was an increase in splenic B cells.

In all vertebrates examined, T cells express either an αβ TCR or a γδ TCR (*1*). The better understood, αβ TCR, is expressed by most systemic T cells (*1*). It recognizes specific peptides within a polymorphic cleft of major histocompatibility (MHC) antigens (*2*). αβ T cells that populate the peripheral immune system of adult animals are mostly specific for MHC complexed with peptides encoded by foreign agents or pathogens. The effector response to this recognition is variable, depending on largely uncharacterized factors, but the end result is that αβ T cells contribute to the eradication of foreign pathogens by direct cytotoxicity toward infected cells and by the stimulation of B cell production of antigen-specific antibodies (*3*). However, the degree to which B cell development is uniformly dependent on αβ T cells is unclear. Some B cell responses are apparently T cell–independent (*4*), and there may also be a negative influence of T cells on B cell development (*5*). Experiments that examine B cell development in congenitally athymic, nude mice have frequently yielded conflicting results (*6*), presumably because this mutant does not eliminate $\alpha\beta^+$ T cells comprehensively.

In contrast to cells that bear the αβ TCR, the biological function of γδ TCR–bearing cells is unknown. There is strong similarity between the structures of αβ and γδ TCR (*7*), and cell surface expression of both occurs in association with a cluster of proteins termed CD3. γδ T cells that recognize peptides and MHC antigens have

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