The Roles of Processing, Presentation and T Cell Receptor Recognition in the T Lymphocyte Response to a Protein Antigen

by

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Abbreviations

cI: Lambda phage repressor protein, 12-26: cI residues 12 to 26, 12-24: cI residues 12 to 24, etc., P12-26: the peptide corresponding to residues 12-26 of cI, etc., WT: wild-type, IPTG: Isopropyl-thio-galactoside, LB: Luria Broth, Ig: Immunoglobulin, CDR: Complementarity Determining Region, TCR: T Cell Receptor, LMP: Low Molecular weight Polypeptide, ER: Endoplasmic Reticulum, I_i: Invariant Chain,

Introduction

The capacity of the immune system to respond to foreign pathogens depends upon its ability to produce cells bearing an enormous diversity of receptors which can bind these pathogens at high affinity. At the same time, in order to avoid autoimmunity, the immune system must eliminate or render non-responsive all cells bearing receptors capable of reacting with self proteins. In order to do this an elaborate system of regulation has evolved, with separate, but related mechanisms for dealing with each of the two principal classes of pathogens: extracellular, such as bacteria or toxins, and intracellular, such as viruses or tumor-inducing gene products.

For intracellular pathogens, the goal is to eliminate cells harboring the pathogen. Proteins expressed by infecting viruses or involved in tumor production are "processed" intracellularly into peptide fragments which are bound specifically by MHC Class I proteins, and are displayed on the cell surface. These complexes are recognized and the cells are eliminated by a subset of T lymphocytes expressing the co-receptor CD8 (Townsend and Bodmer, 1989). This permits them to specifically recognize Class I molecules.

In order to be able to respond to the full diversity of antigens which the immune system will face, the receptor by which T cells recognize these peptide-MHC complexes (the T cell receptor or TCR) has a binding site for antigen which is assembled randomnly from several genes, as is the case for immunoglobulins (Davis and

Bjorkman, 1988). Several similar models have been proposed for the structure of the TCR based on the structure of immunoglobulins (Chothia, et al., 1988; Claverie, et al., 1989; Davis & Bjorkman, 1988). This parallel is based on the observation that a great many elements previously found to be unique to the structure of antibodies also appear in the TCR. Crystal structures of antibodies show close intra- and inter-chain contacts at many positions (Amit, et al., 1986; Amzel, et al., 1974; Amzel and Poljak, 1979; Colman, et al., 1987). The residues participating in these contacts are conserved between different antibody sequences, (Chothia, et al., 1985; Novotny and Haber, 1985) and many of these residues are also conserved in TCR sequences (Becker, et al., 1985; Chothia et al., 1988; Novotny, et al., 1986).

As for immunoglobulins, the predicted combining site of the TCR is formed by three separate Complementarity Determining Regions (CDRs), two of which are formed by the products of the V_α and V_β genes, with a third being encoded by sequences lying at the junctions of the V_α and J_α, and V_β, D_β, and J_β genes. TCR genes, unlike immunoglobulin genes (Tonegawa, 1983), do not undergo somatic hypermutation in the periphery (Chien, et al., 1984; Hayday, et al., 1985). This has the important consequence that T cells emerging from the thymus after maturation (described below) do not change their specificity.

In terms of its implications for antigen recognition, the most important structural difference between immunoglobulins and the TCR is that a much greater part of the diversity between TCRs lies in the CDR3 compared to what is the case in immunoglobulins. While

there are estimated to be 62,500-250,000 possible combinations of V_L and V_H for immunoglobulins, there are only about 1/100 this many possible combinations of V_α and V_β for the TCR (Davis & Bjorkman, 1988). By contrast, because of a greater number of J elements, and insertion of non-germline-encoded N-region additions in the junctions, the TCR has an estimated 10^{15} possible junctional combinations, while immunoglobulins have only 10^{11} (Davis & Bjorkman, 1988). The TCR is specialized for recognition of the complex of a relatively non-diverse MHC molecule binding peptides which can be quite different from one another. Current models all hold that these junctional regions (CDR3) are primarily responsible for interaction with peptide antigen, and the V gene-encoded residues are responsible for recognition of the MHC molecule. This model is supported experimentally by the observation that correlations can be found between the fine-specificity of T cells for peptide antigens, and sequences in their junctional regions (Danska, et al., 1990; Engel and Hedrick, 1988; Jorgenson, et al., 1992; Lai, et al., 1990). An MHC interaction site has been mapped to CDRs 1 and 2 (Hong, et al., 1992).

One consequence of this mechanism for generation of diversity is that some TCRs will recognize self-antigen. In order to avoid responding to self, cells bearing these receptors undergo a maturation process in the thymus. In a first step, termed negative selection, cells expressing receptors which can recognize complexes of peptides derived from endogenous antigen bound by Class I are eliminated (Kisielow, et al., 1988; Teh, et al., 1988). It is not clear against what percentage of proteins from the entire organism

selection occurs at this stage. Theories predict that part of the role of the thymus is to express all proteins at low levels to permit tolerance (Lo, et al., 1989; Matzinger and Guerder, 1989; Moraham, et al., 1989). However, it is known that albeit more poorly characterized mechanisms also exist in the periphery to tolerize cells, so this may not be necessary (Arnold, et al., 1992; Jones, et al., 1990; Rocha and Von Boehmer, 1991).

The second step is positive selection where T cells are permitted to proliferate if they have a certain minimum reactivity with the self MHC molecule itself (Kisielow et al., 1988). In this case, various theories predict either that the T cell receptor is recognizing an "empty" MHC class I molecule, or an MHC molecule containing specialized peptides dedicated to positive selection (Marrack and Kappler, 1988; Marrack, et al., 1989; Nikolic-Zugic and Bevan, 1990). By these means, T cells leaving the thymus are expected to have a certain minimum specificity for the MHC molecules expressed in the organism, but not one which is sufficient for activation. Additional interactions between the TCR and peptides derived from pathogens presented by these molecules would then provide the additional affinity necessary for activation of the T cell.

In order to produce the peptides which can bind MHC class I and introduce them to the compartment where they can interact with these proteins, both in the thymus and in periphery, an additional elaborate system exists. It appears that proteins must first reach the cytoplasm (Monaco, 1992). Prevailing opinion is that they are then digested by a protease or proteases which have not been

identified, and the sequence specificity of which has not been characterized. A likely candidate for this protease is the so-called Low Molecular Weight Polypeptide complex (LMP). Its role is suggested by the fact that it possesses proteolytic activity, and at least two of its subunits map to a region of the MHC known to be important for antigen processing (Brown, et al., 1991; Kelly, et al., 1991; Martinez and Monaco, 1991; Monaco and McDevitt, 1982; Monaco and McDevitt, 1984; Monaco and McDevitt, 1986). However, there is no direct evidence implicating it in antigen processing.

Subsequently, according to current models, these peptides are transported across the membrane of the ER. This function is most likely carried out by a heterodimer of the products of two genes, known variously as mtp 1, mtp2, RING1, RING4, PSF1, PSF2, HAM1, HAM2, and in a new unified nomenclature as TAP1 and TAP2 (Deverson, et al., 1990; Monaco, et al., 1990; Spies, et al., 1990; Trowsdale, et al., 1990). The piece of good fortune that permitted their identification by these means is that most Class I molecules are unstable and become rapidly degraded if they reach the cell surface without bound peptide (Ljunggren, et al., 1990; Townsend, et al., 1989). Thus, screening for lack of class I expression identified cells that could not transport peptides across the ER membrane. Cells with a deletion of only one of these genes are still able to present some peptides, which indicates that a heterodimer constitutes the normal functional protein, and that a homodimer may retain some function (Hosken and Bevan, 1992). Once peptides have gained entry to the ER by these transporters they can presumably bind MHC class I and be brought to the cell surface by the normal

surface membrane protein pathway. It is important to note that there is no direct evidence to support the theory that proteolysis occurs prior to translocation. It remains possible that translocation occurs first and proteolysis occurs within the ER. Indeed, one report has demonstrated that cells transfected with a protease for expression *within* the secretory pathway are more efficient at presentation of some peptides expressed in the cytoplasm (Eisenlohr, et al., 1992). Futhermore, in these transfected cells, peptides as long as 32 amino acids were transported from the cytoplasm and trimmed within the ER. The most important piece of evidence suggesting that proteolysis does occur before transport is that peptides derived from proteins in the secretory pathway can be eluted from Class I molecules in TAP mutants, but have lengths that vary widely from those eluted from wild-type cells (Henderson, et al., 1992). This suggests that these peptides are not produced by the same proteases responsible for the digestion of the peptides bound to Class I molecules in normal cells. Peptides which enter the ER by other pathways can also bind MHC. For example, peptides preceded by a leader that allows them to enter by the normal SRP pathway can sensitize TAP mutant cells for lysis by T cells (Anderson, et al., 1991).

That the ER is the meeting point for antigen and class I explains the observation that presentation of Class I-restricted, but not Class II-restricted antigen can be blocked by the drug Brefeldin A (BFA) (Nuchtern, et al., 1989). BFA inhibits transport into the *cis*-Golgi, and collapses the Golgi (Pelham, 1991). Thus, peptide

antigens and MHC that meet in the ER cannot be transported to the surface and be presented.

Binding to MHC molecules is a specific interaction, of a classical ligand-receptor type. The crystal structure of the MHC class I molecule has been solved (Bjorkman, et al., 1987a). It contains a deep antigen binding groove formed by α-helices with a β-sheet floor. The molecule is highly polymorphic, and the polymorphic residues are concentrated around this groove thus giving different molecules different peptide-binding specificities (Bjorkman, et al., 1987b; Parham, 1988).

Several studies have shown that class I molecules preferentially bind peptides of length 9±1aa (Cerundolo, et al., 1991; Schumacher, et al., 1991; Tsomides, et al., 1991). In general class I molecules show very high specificity for binding peptides containing specific amino acids at particular positions (Falk, et al., 1991; Rotzschke and Falk, 1991). The preferred amino acids and their relative position are different for each Class I protein, and constitute motifs that appear to be shared among among all peptides that bind the same Class I.

The peptide-Class I complex, once it has reached the surface, can be recognized by CD8 positive T cells. In this case the cell is then killed (Taylor and Cohen, 1992).

For the class I antigen presentation pathway, several important questions remain to be anwered, among them: How is it that proteins which are not expressed in the cytoplasm, such as nuclear proteins can be presented? Do there exist dedicated pathways to bring peptides derived from these proteins to the ER, or

does presentation depend upon a "leakiness" of the expression of these proteins into the cytoplasm? Is the proteosome indeed involved in antigen processing? What is the peptide specificity of enzyme involved in processing? Is processing completed in the cytoplasm, or is some sort of trimming of peptides required in the ER to produce the mature peptides which bind to MHC? How does one account for the tight limitation in sizes of peptides which are found bound to Class I? Does this indicate that processing enzymes tend to produce peptides of this length, or are a variety of peptides produced, and selection performed at the level of binding to Class I? Besides the TAP proteins, Class I proteins and proteases, what additional proteins are involved in processing? Two genes coding for members of the hsp70 family have been found in the MHC suggesting that a chaperone function may be necessary (Sargent, et al., 1989). Finally, it is important to determine the nature of peptides bound in the groove of Class I proteins during negative and positive selection, whether these are all peptides from the organism, none, or special peptides dedicated to these functions.

T cells specific for exogenous pathogens express CD4 and recognize antigen bound by class II molecules (Parnes, 1989). They use the same genes to form their T cell receptors and undergo a similar process of maturation involving positive and negative selection in the thymus. The principle differences between the two classes of cells are their effector mechanisms, and the pathways by which antigen is processed and presented to them. Recognition of the class-II/peptide complex by CD4-positive T cell leads to stimulation of T cell help in the form of interleukin secretion

necessary for the maturation of immature B cells into antibody-producing plasma cells (Vitetta, et al., 1989), and in other cases, probably to forms of help necessary for stimulation of CD8-positive T cells. (Fayolle, et al., 1991; Guerne, et al., 1984; Mizuochi, et al., 1986).

The peptides presented by class II molecules can come from a greater diversity of sources than is the cases for class I-restricted antigens (Brodsky and Guagliardi, 1991). The most classical is the endocytic route. The necessity of internalization for processing and presentation of protein antigens is demonstrated by the fact that fixation of cells by formaldehyde blocks presentation of intact proteins, but not peptides (Unanue, 1984). Entry into the endocytic pathway can occur either by internalization of antigen bound to surface immunoglobulin on B cells (Lanzavecchia, 1990), or by fluid phase endocytosis (Unanue and Allen, 1987). In either case, internalized antigen is directed to early endosomes. This compartment contains the proteolytic enzymes Cathepsin B and D which have been implicated in processing (Diment, 1990; Shaw and Chain, 1989; Takahashi, et al., 1989). Processing of protein antigen is therefore believed to occur at this stage, although whether it can be completed may depend upon the antigen. Recently, a third enzyme, Cathepsin E, has been implicated in processing (Bennett, et al., 1992), and localized to a non-lysosomal compartment of the endosomal system, i.e. perhaps the early endosome.

Although a great deal of evidence indicates that the specific sites of cleavage of a protein play an important role in which sequences within a protein are antigenic, the sequence specificity of

these enzymes has not yet been well characterized (Bond and Butler, 1987). This evidence includes the observation that presentation of some epitopes, but not others within a protein, is reduced by inhibitors of various proteases, again, particularly members of the Cathepsin family (Bennett et al., 1992; Diment, 1990; Takahashi et al., 1989; Vidard, et al., 1991).

The optimum pH for these enzymes is approximately 5.5, and proteolysis may be facilitated by denaturation of proteins caused by a gradual acidification that occurs during transport toward lysosomes (Mellman, et al., 1986). Class II presentation is abrogated by ammonium chloride or chloroquine (Ziegler and Unanue, 1982), drugs that raise the pH of intracellular compartments. Cells that are mutant for endosome acidification also have diminished presentation of Class II-restricted antigens (McCoy, et al., 1989).

The intracellular location where peptide antigens bind Class II molecules remains controversial. Three studies using techniques of subcellular fractionation indicate that the endocytic pathway intersects the transport pathway of Class II molecules in the early endosome (Guagliardi, et al., 1990; Lamb, et al., 1991; Lotteau, et al., 1990). However, a more recent one using electron microscopy indicates that it occurs in a post-Golgi compartment related to lysosomes (Peters, et al., 1991). Another study used specially composed liposomes to target protein antigens to lysomes bypassing endosomes, and found that they were processed efficiently, recycled and presented to T cells (Harding, et al., 1991). Furthermore, the efficiency of presentation was higher than for protein antigens delivered directly to endosomes. This indicates that protein

antigens can be degraded in a compartment late in the endocytic pathway and their processing products can come in contact with MHC Class II molecules. It also suggests that for some antigens, such a step may be necessary for complete processing. The reasons for the different conclusions obtained in the references cited above are not clear, but all studies are in agreement with the conclusion that peptides derived from protein antigens come in contact with Class II molecules via the endocytic pathway.

As the initial pathways followed by class I and class II molecules are identical, it is important that a means exist to prevent the saturation of class II molecules by peptides in the ER and Golgi. Otherwise, Class II molecules would not be able to present exogenous antigens. This role appears to be played by the Invariant Chain (I_i). The Invariant chain has been shown to bind Class II dimers in the rough ER following their synthesis, probably forming a nine protein complex (Marks, et al., 1990; Roche, et al., 1991). Class II-I_i complexes are unable to bind peptides *in vitro* (Roche and Cresswell, 1991a), and cells expressing a secreted version of I_i have a diminished capacity to present peptides *in vitro* (Teyton, et al., 1990).

The Invariant chain has also been implicated in the targeting of Class II to the endosomal pathway. Transfected cells expressing only Class II α- and β-chains transport the assembled molecules to the cell surface (Peterson and Miller, 1990; Sekaly, et al., 1986). However, this is probably due to the normal transport of all proteins in the exocytotic pathway via the Golgi that occurs by default (Rothman, 1987). This conclusion is based on the observation that

cells transfected only with Class II stain for expression in the ER, Golgi and on the cell surface, whereas cells co-transfected with I_i also stain for Class II in endosomes (Lotteau et al., 1990). The pathway by which Class II reaches endosomes in the co-transfectants has been examined by pulse-chase experiments. It does not appear to occur by transport to the surface and re-internalization (Lotteau et al., 1990). In the absence of Class II expression, I_i is retained in the ER (Bakke and Dobberstein, 1990; Lotteau et al., 1990). Experiments involving transfection of cells with truncated variants of I_i lacking the N-terminus indicate that this is due to an ER retention signal lying in the cytoplasmic extension (Bakke & Dobberstein, 1990; Lotteau et al., 1990). Binding to $\alpha\beta$ dimers apparently suppresses this signal and allows a second one targeting it to the endosome to dominate. Truncated variants of I_i lacking the ER retention signal also seem to be transported directly to the endosome. The signal for I_i to dissociate from the Class II molecule to permit binding of peptides appears to be induced by its cleavage by proteases in the endosome (Blum and Cresswell, 1988; Machamer and Cresswell, 1984; Nguyen, et al., 1989; Nowell and Quaranta, 1985) It has been demonstrated that intact Class II molecules capable of binding peptide are released from I_i by *in vitro* digestion by Cathepsin B (Roche and Cresswell, 1991b). Thus I_i can protect Class II molecules from becoming saturated with intracellular peptides before coming in contact with exogenous peptides in this compartment. At this time it is not clear by what pathway peptide-Class II complexes are transported to the cell surface.

The purification and sequencing of peptides bound to Class II molecules has been achieved more recently than for Class I-bound peptides (Rudensky, et al., 1991). There are several characteristics that distinguish the two types. First, the Class I molecule has been shown to preferentially bind peptides of a defined length (Cerundolo et al., 1991; Schumacher et al., 1991; Tsomides et al., 1991), while Class II molecules are apparently capable of binding peptides varying greatly in size. This is reflected by the observation that typical peptides eluted from Class I molecules are approximately 9 amino acids (Falk et al., 1991; Rotzschke & Falk, 1991), while those eluted from Class II molecules range from 13-17 amino acids (Hunt, et al., 1992; Rudensky, et al., 1992; Rudensky et al., 1991). Second, several different truncated variants of the same peptide are found bound to Class II molecules (Hunt et al., 1992; Rudensky et al., 1992; Rudensky et al., 1991), whereas a given peptide bound to Class I molecules in normal cells has always been found to have the same N- and C-termini (Falk et al., 1991; Rotzschke & Falk, 1991). Once again, in the case of Class I molecules, this appears to reflect a preference for peptides of a defined length, as well as a particular positioning of the key amino acids constituting the Class I-binding motif within the groove. It may also partly reflect the role of antigen processing and selectivity of transport of certain peptides into the ER by TAP proteins. Peptides eluted from Class II molecules have different N- and C-termini suggesting that the proteins they are derived from are first digested into relatively long peptides, and that these peptides are subsequently trimmed to the mature peptides found bound to the Class II molecule. Although motifs have

been identified for binding to Class II molecules by sequence comparison of epitopes having the same restriction, and by *in vitro* binding experiments, they are generally less precisely defined and less stringent than those identified for Class I binding. For example, motifs for Class I binding are generally constituted of two or three particular amino acids with a fixed separation within the sequence. By contrast, the motif for binding the Class II A^d molecule, which has now been verified by elution and sequencing of peptides (Hunt et al., 1992), has relatively low stringency with respect to substitutions at each position, but is six amino acids long (Sette, et al., 1989; Sette, et al., 1988). Thus although the Class II protein is considered to be structurally similar to Class I (Brown, et al., 1988), there appear to be fundamental differences in the way the two bind peptides, and in the nature of the peptides bound by each.

As is the case for the Class I antigen presentation pathway, a number of important questions remain for the Class II antigen presentation pathway. The most elusive of these have to do with the mechanism of peptide generation from exogenous protein. Although various proteases have been implicated, no conclusive evidence has been found for the involvement of any one in particular. If members of the Cathepsin family are involved in processing, it is not known if the peptides produced from their action are the mature peptides found bound to Class II molecules, or whether trimming is required. There are several examples of sequences within proteins which are capable of binding MHC and stimulating T cells if immunization is with peptide, but to which there is no response if the intact protein is used. One would therefore like to identify definitively the sites

of proteolysis necessary for presentation of a given epitope as means of determining the role processing plays in the hierarchy of epitopes within a protein. It remains unclear in which compartment processing occurs, the endosome, or the lysosome. It may be that processing and transport to compartments containing Class II molecules is a continuous and progressive process. Some epitopes may be produced by the relatively mild proteolytic conditions in the early endosome, whereas others may require more extensive proteolysis in the late endosome or lysosome. The possible role of other proteins, such as chaperonins, remains to be investigated. More extensive analysis of the binding of peptides to Class II molecules is required. Beyond this, one would like to know the reasons for differences in the peptides bound by Class II and Class I antigens. Finally, the molecular means by which the peptide-MHC complex is recognized by the T cell receptor requires further investigation. The degree to which the functions of MHC- and peptide-recognition are divided between different domains of the TCR requires further clarification. Such knowledge is ultimately essential for a complete understanding of the molecular basis for positive and negative selection of T cells in the thymus, and will be useful in determining how to improve the immune response to foreign pathogens.

The work described here was performed in order to understand the respective roles of antigen processing, presentation and T cell recognition in response to a single Class II-restricted epitope. The epitope chosen was that which was previously identified as the immunodominant epitope from the protein λ–repressor (cI) in d-and

k-haplotype mice (Lai, et al., 1987). In the first chapter we analyze the binding of the peptide to the Class II E^k molecule, and determine the critical residues in the peptide sequence involved. In the second chapter, we describe the response of a panel of T cells to the epitope, identify the key residues for their response, and identify correlations between responsiveness of the cells and conserved elements in the sequences of their TCR genes. Finally, in the third chapter we examine the role of the residues outside the epitope in the processing of the epitope in attempt to determine the structural cues in the protein which are important for proper proteolysis, and identify proteins which are processed less efficiently than the wild-type.

Materials and Methods

Cell lines

T cell hybridoma A128, is A^d-restricted and specific for residues 46-62 of cI. It was a gift from Dr. Francesco Ria (Universita Cattolica, Rome, Italy). Hybridoma IG6.4, specific for Hen Egg Lysozyme residues 1-14, and 2H6.41, specific for HEL residues 105-120, both E^k-restricted, were from Dr. Serge Fuchs, Sandoz Ltd., Basel, Switzerland. E^k-restricted hybridomas 3I, 4I, 8I, 10I (Lai et al., 1987; Lai, et al., 1988) were from Dr. Ming-Zong Lai, Institute of Molecular Biology, Taipei, Taiwan. All other 12-26-specific T hybridomas described here were produced by A.S. using the technique previously described by Lai et al. (1987). The fusion partner was the TCR-deleted thymoma line BW5147$\alpha^-\beta^-$ (White, et al., 1989). E^k-restricted hybridomas described in Chapter 2 were produced from CBA mice. The A^d-restricted cells 24.4 and 26.2 were produced from Balb/c mice. Cell 26.2 was produced by immunisation with peptide 12-26 and cell 24.4 by immunisation with peptide 12-24. The antigen presenting cell line TA3 (I-$A^{d/k}$, $E^{d/k}$) was from Dr. L. Glimcher, Harvard University. The IL-II/IV-dependent cell line CTL.L2 was from Dr. D. Raulet, University of California, Berkeley. All cell lines were grown and assays carried out using complete RPMI 1640 medium (RPMI 1640 (Seromed, France) with 10% fetal

calf serum, 250μg/ml glutamine, 100 μg/ml penicillin and 62μg/ml streptomycin).

Measurement of T Hybridoma Response to Peptides and Proteins

Assays of this type were conducted in 96-well plates using serial dilutions of antigens. Each well contained $2.5X10^4$ T cells and 10^4 TA3 as APC in a volume of 100μl. Cultures were incubated for 18-24 hours before assay of interleukin secretion. To assay hybridoma supernatants for interleukin content, 50μl of the supernatant was transfered to a second plate which was frozen for two hours at -80°C and thawed at 37°C. 10^4 CTL.L.2/well were added in 50μl of complete RPMI. 18 hours later, 0.5μCi/well of tritiated thymidine (Amersham) was added. After further incubation for 6 hours, the cells were harvested onto glass fiber filters and thymidine incorporation measured using a Skatron 96-well harvester and an LKB Beta-Plate scintillation counter.

Competition Binding Experiments

Serial two-fold dillutions of competitor peptides (in general, analogs of P12-26) were prepared in complete RPMI along rows of 96-well plates. Each well contained 25μl. Subsequently, 25μl of glutaraldehyde-fixed (see below) TA3 cells were added at a concentration of $4X10^5$ cells/ml, and the plates were incubated at 37° for six hours to allow peptides to bind E^k. At this time, a fixed concentration of an un-related E^k-restricted stimulator peptide was

added to all wells in 50µl along with $2.5X10^4$ T cells specific for this peptide. The concentration of the stimulator peptide to be used was in each case determined two days previous to the competition experiment as that which induced 75% maximal stimulation in the absence of competitors. The stimulator peptide used for the experiments described in Chapter 1 was HEL 1-14, or HEL 105-120, and the T hybridomas used were IG6.4, or 2HG.41, respectively. The plates were incubated for a further 18 hours, and frozen for 2 hours below -80°C. After thawing at 37°C, supernatants were assayed for interleukin content by measuring thymidine incorporation in the CTL.L assay described above. The relative effectiveness of peptides as competitors was determined as the concentration of P12-26 necessary to reduce stimulation of the appropriate T cell by 50% divided by the concentration of a given analog necessary to achieve the same effect. To confirm that a reduction in stimulation of was not due to toxicity of the peptide, experiments were conducted where the competitor peptide was added *subsequently* to the stimulator peptide. Under these conditions, no significant inhibitory effect was observed for any peptide.

Glutaraldehyde Fixation of Antigen Presenting Cells

TA3 cells were washed three times by centrifugation and resuspension in PBS and resuspended PBS with 0.05% glutaraldehyde (Sigma). The cells were incubated 60 seconds after which a five-fold volume excess of 0.25 M HCl-lysine in PBS was added to quench glutaraldehyde. After washing twice in PBS, the cells were

resuspended at $2X10^5$/ml in complete RPMI. Cells fixed by this method are as effective for presentation of P12-26 as un-fixed TA3 cells. Intact cI protein is not processed by fixed APC, and was used as a positive control for fixation.

Peptides and Proteins

P12-26 and truncated analogs were synthesized in the laboratory of Dr. A.D. Strosberg, Institut Cochin de Génétique Moléculaire, Paris, France using an Applied Biosystems apparatus. Analogs of P12-26 containing single substitutions were a gift of Dr. F. Borras-Cuesta, Pamplona, Spain. cI protein, and variants containing substitutions were purified from E. coli according the previously described procedure (Johnson, et al., 1980).

Strains and plasmids

Propagation of plasmids was conducted in the F'lacI^Q E. coli strain X90 (Hanahan, 1983). Expression of cI and cI mutants was carried out in X90 carrying the plasmid plys-s (Studier, et al., 1990). This plasmid contains the gene encoding lysozyme, and cells carrying it are thus susceptible to lysis by freeze-thaw. The plasmids pAS101 and pAS102 carry the cassette gene for the entire coding sequence of cI. They were made by ligating the small *Pvu* I-*Eco*RI fragment of plasmids pWL103 and pWL105, respectively (Reidhaar-Olson and Sauer, 1990) into the backbone of plasmid pRB200 (Breyer and Sauer, 1989). The pWL plasmids were a gift of

Dr. Robert Sauer (MIT), and pRB200 was a gift of Dr. Richard Breyer, (Vanderbilt University). pAS101 differs from pAS102 in that it contains an *Nco*I site overlapping codon 1 in order to permit mutagenesis at the N-terminus and thus codes for glycine at residue 1 instead of the wild-type serine. The wild-type amino acid is reintroduced during mutagenesis.

Cassette mutagenesis

The procedure of Oliphant (Oliphant, et al., 1986) was used to generate mutations at one to four codons at a time in the cI gene. For this purpose pAS101 or pAS102 was digested with two restriction enzymes, one on either side of the desired site of modification. The short fragment produced was replaced by a synthetic oligonucleotide cassette. One oligonucleotide of each pair was synthesized using an equimolar mixture of all four bases at each mutagenized position. The complementary oligonucleotide contained inosine residues at the corresponding positions. Synthesis for mutagenesis at position 20 and 24-27 was performed using an Applied Biosystems 391 apparatus. Other oligonucleotides were obtained from the laboratory of Dr. Robert Sauer (MIT). For mutagenesis at positions 8-11 an *Nco*I site in pAS101 beginning 4 bases upstream of the the first codon and a *BssH*II site beginning at codon 15 were used. For mutagenesis C-terminal to position 17 the *BssH*II and *Sac*I sites in pAS102 were used.

Induction of Protein Expression and Preparation of Lysates

Individual colonies carrying cI variants were picked and grown overnight in LB with 100μM ampicillin and 25μM chloramphenicol. 50 μl of the saturated cultures were diluted into 10 ml of LB and the cultures were grown with rotary agitation until one being monitored reached an OD_{600} of cI. IPTG (Sigma) was then added to a final concentration of 500μM. Three hours later the bacteria were spun down at 4000 RPM for 10 minutes in a Jouan GR412 centrifuge resuspended in 1ml of RPMI tissue culture media and transferred to microcentrifuge tubes. Lysis of the bacteria was performed by three cycles of freezing in liquid nitrogen and thawing at 23°C. In some early experiments lysis was performed by sonication using a Sonics and Materials sonicator (Danbury, Conn.) Debris was spun down at 10,000 RPM and supernatants were kept.

Screening of Lysates Containing cI Variants

10μl of each lysate was added per well in a microtiter plate containing $5X10^4$ TA3 cells (Walker, et al., 1982) and $1X10^5$ of the appropriate T cell in 100μl of complete RPMI. 100μg/ml Gentamycin was included to reduce the risk of contammination from any remaining live bacteria in the lysate. 18-24 hours later 50μl of each supernatant was transferred to a second microtiter plate and assayed for interleukin content by thymidine incorporation in CTL.L cells.

Chapter 1

Abstract

The epitopes recognized by T cells are peptides specifically bound by molecules of the Major Histocompatibility Complex. In a number of cases sequence motifs have been proposed which are necessary for the binding of different peptides which can be presented by the same MHC molecule. In order to determine the critical residues for binding of 12-26 to the E^k-molecule, we conducted competition binding experiments using a set of 63 analogs differing from 12-26 by single substitutions. The results indicate that binding of 12-26 to E^k is primarily due to interactions between the MHC molecule and residues 18 and 26 of the peptide. These residues correspond to those most conserved among other E^k-restricted peptides. We propose a simplified E^k-binding motif that consists of a basic residue (preferably a lysine) preceeded variably 7-8 residues before by a hydrophobic amino acid. Binding of peptides to E^k may therefore be similar to binding to class I molecules which have recently been shown to recognize two principal anchor residues at the extremities, but to accomodate a variable number of intervening residues. Substitutions at nearly

every other position do generally affect binding to some extent. The effect tends to be negative, as no example was found where a substitution appeared to significantly increase binding to MHC.

Introduction

Recognition of peptide antigens by T cells can essentially be subdivided into two independent problems, binding of the peptide to MHC, and recognition of the peptide-MHC complex by the TCR.

The first of these two essential steps can be thought of as a classical ligand-receptor binding interaction. Class II molecules have been demonstrated to have a unique binding site (Guillet, et al., 1986), and peptides restricted by the same class II molecule have been shown to compete for binding against one another both in functional assays (induction of interleukin secretion by T cells) (Guillet et al., 1986) and in direct binding experiments (Babbitt, et al., 1986). Similar observations have been made for class I molecules (Maryanski, et al., 1988; Pala, et al., 1988). The K_d for the binding of various peptides to class II molecules has been measured and is typically on the order of 1μM (Babbit, et al., 1985).

Several attempts have been made to identify sequence patterns or secondary structures common to all T cell epitopes that might be required for binding to MHC. Different groups have found that sequences consistent with β-sheet (Spouge, et al., 1987), α-helical (Allen, et al., 1987; Rothbard, et al., 1988) and in particular amphipathic α-helical structures (De Lisi and Berzofsky, 1985; Margalit, et al., 1987) exist in many epitopes and would correlate with the distribution of Ia-contact and TCR residues in a peptide. One group identified a short sequence pattern that was common to T

cell epitopes of several different MHC restrictions (Rothbard and Taylor, 1988).

A variety of evidence now suggests that no such general structures are necessary for a sequence to become a T cell epitope, and that specific sequences which vary depending upon the MHC restriction of a given peptide play a more important role. The first of these showed that proline or glycine residues could be introduced into an epitope from ovalbumin without affecting its ability to bind MHC (Sette, et al., 1989), thus making it unlikely that strict α-helical or β-sheet conformations are necessary. Other groups have shown that peptides incorporating a few crucial residues into either polyalanine (Jardetzky, et al., 1990), or polyproline or polyglycine (Maryanski, et al., 1990) backbones can bind efficiently to MHC.

The crystal structure of the human class I molecule HLA-A2 has been solved (Bjorkman et al., 1987a). It contains a long groove 25Å in length by about 10Å in width bounded by α-helices containing the majority of the residues which are polymorphic between class I molecules. This groove, which is the nominal antigen binding site, would not be wide enough to accept a peptide in α-helical conformation, but could do so in extended conformation (Claverie et al., 1989). Although the structure of the class II molecule has not been solved, a hypothetical model based on sequence homologies suggests that it has a similar antigen binding site.

More recently, the structure of other class I molecules has been determined, and in one case has been shown to contain a nonamer peptide in extended conformation in its binding site (Madden, et al., 1991). These studies have permitted the

identification of unique pockets in the structure of individual class I molecules that are probably responsible for their specificity of binding (Garrett, et al., 1989). Peptides eluted from a given class I molecule have been shown to contain conserved residues at two or three positions in their sequences (Falk et al., 1991; Jardetzky, et al., 1991). Modeling studies indicate that the conserved residues would fit well into the specificity pockets of the class I molecule. The other residues have a high degree of variability, indicating that it is primarily the interaction of the MHC molecule with the primary structure of peptides that determines binding. It is thus likely that the conformation of the bound peptide is largely imposed on it by the MHC molecule.

Peptides bound by class II molecules are much more heterogeneous in sequence and length than those binding class I. No sequence motifs or apparent critical binding residues could be identified in the first peptides eluted from class II molecules (Rudensky et al., 1991). More extensive analysis has revealed such motifs, but in general their stringency is significantly less than for class I-binding peptides (Hunt et al., 1992; Kropshofer, et al., 1992; Rudensky et al., 1992).

The most successful means of identifying motifs for class II binding have been the comparison of sequences from various minimal peptides binding the same class II molecule (Sette et al., 1989) and the identification of critical residues in epitopes using substituted peptides. This was originally performed in either direct binding (Sette, et al., 1987), or competition binding experiments (Allen et al., 1987) using alanine-substituted peptides.

Previous studies have been conducted to determine the shortest derivitive of P12-26 capable of binding E^k (Sette, et al., 1989). Direct binding experiments using truncated variants of P12-26 indicate that the C-terminal lysine is essential for E^k-binding. The effect of truncations at the N-terminus is less clearcut. Removal of the amino acids at positions 16-18 leads to a progressive loss of binding. Here, in order to produce a more detailed description of binding of P12-26 to E^k with specific identification of the requirements and tolerance for charge, size and hydrophobicity of amino acids at each position, we have employed a set of 63 analogs of P12-26 containing single substitutions. By examination of the effectiveness of each of these peptides in competition binding experiments, and comparison to sequences of other E^k-binding peptides we propose a refined model, involving only two principle contacts between the peptide and MHC, for the binding of peptides to E^k in general.

Results

In order to identify MHC contact residues within P12-26, competition binding experiments were performed using a large panel of substituted analogs of P12-26. In general 5 different substitutions were made at each position from residue 14-26 in order to test not only whether a given residue was essential for binding, but also to probe the chemical environment in its interaction with the MHC molecule. Substitutions at positions 12 and 13 were not made because previous work has shown that removal of these residues has no effect on the capacity of the peptide to bind E^k. The substitutions made were to aspartate, leucine, lysine and tyrosine. In addition, each position was also substituted with alanine which is generally considered the substitution causing the most minor perturbation of protein structure. If the normal amino acid at a given position was one of the above, another amino acid from the same class was used. At positions 18 and 20 only four substitutions were made.

In these experiments, dilutions of the various substituted analogs were incubated with "fixed" (see materials and methods) APC for 6 hours. Subsequently, a set concentration of an unrelated E^k-restricted peptide derived from hen egg lysozyme (HEL residues 1-14) was added along with T cells specific for this peptide. The concentration of the second peptide was chosen so as to produce 75% maximal stimulation of the HEL 1-14-specific hybridoma in the absence of competitor peptides. If in the first step a given peptide

was able to bind to E^k, this would be reflected by a decreased amount of free E^k available to bind HEL 1-14, and therefore a decreased stimulation of the HEL 1-14-specific T cell. The results are expressed in Table 1.1 in terms of the relative capacity of a given analog to achieve the same inhibitory effect as P12-26. Smaller decimal fractions therefore correspond to peptides that bind less well to E^k. As can be seen, these experiments implicate primarily two residues, numbers 18 and 26, in MHC binding. Peptides containing substitutions at position 18, for example, are .02 - .006 times as effective as P12-26 as competitors for binding of HEL 1-14. Similarly, substitutions at position 26 reduced the effectiveness of the peptides from .15 times that of P12-26 for a conservative arginine substitution to .004 times that of P12-26 for alanine and tyrosine substitutions.

At least one other substitution at nearly every other position also affected the effectivenes of the P12-26 analogs as inhibitors of binding to E^k. In particular, some substitutions at positions 19, 21, and 23 had effects on the order of 10- to 20-fold. These effects however were at least 10-fold less severe than the effects of substitutions at positions 18 and 26. Furthermore, while even quite conservative substitutions (leu to ala at position 18, lys to arg at position 26) decreased the effectiveness of the analogs as inhibitors by roughly 10-fold or more, conservative changes at positions 19, 21, and 23 had little effect. These results suggest that while several residues within 12-26 can affect binding to E^k, positions 18 and 26 play the most important roles.

T cells have occasionally been seen to cross-react with other peptides of the same MHC restriction. To control for the possibility that the lack of competition seen for some peptides was not due to a cross-reactivity of IG6.4 for certain P12-26 analogs, some of the experiments were repeated using a T hybridoma specific for a different E^k-restricted hybridoma. In this case the cell 2H6.41, specific for HEL residues 105-120 was used. The results of these experiments are also included in Table 1.1. As can be seen, there is a close correlation with the results obtained with cell IG6.4. The experiments also implicate primarily residues 18 and 26 in binding to E^k. In fact, in this case, none of the peptides containing substitutions at these positions except that containing a conservative argine substitution at position 26 are capable of achieving half-maximal inhibition of stimulation of 2H6.41.

The results of these experiments are summarized in schematic form in Figure 1.1. In this figure the principle MHC contacts are indicated by filled triangles. Residues which do not play a critical role, but still appear to affect binding are indicated by open triangles.

Discussion

An alignment of several other E^k-binding peptides can be made to 12-26 based on sequence homologies (Figure 1.2). In this alignment, all except HEL 1-14, a weakly binding peptide (Guéry, et al., 1992), contain lysine at the position corresponding to 26. Furthermore, all except HEL 1-14 and HEL 102-116, contain either ala, leu or ile at the residue corresponding to position 18 in 12-26. Thus our competition binding data and sequence homologies to other good E^k-binders agree well with a simple E^k-binding motif encompassing a basic residue preceded 8 residues before by a hydrophobic amino acid.

The most significant exception to this rule would be HEL 102-116. Adorini and co-workers have investigated the binding behavior of this peptide, and proposed the following motif for E^k-binding: Two hydrophobic (A, V, I, L) short chain, (such as S or T), or aromatic (Y, W, F) residues six to eight positions before a basic residue, (K, R, H). This model was based on the observation that binding was affected to a certain extent by substitutions of the trp at the position correponding to leu-18 in 12-26. Nevertheless, the binding capacity of this peptide was more strongly dependent on the valine C-terminal to it (Leighton, et al., 1991). Thus in a more refined model, based on our data and the sequence homologies shown in Figure 1.2, the aromatic residue may not play an important role. The most important residues in the motif are simply the basic residue, which by sequence comparison and effectiveness in our competition

experiments is almost always a lysine, and the hydrophobic residue which may variably be located either 7 or 8 residues away from the basic residue.

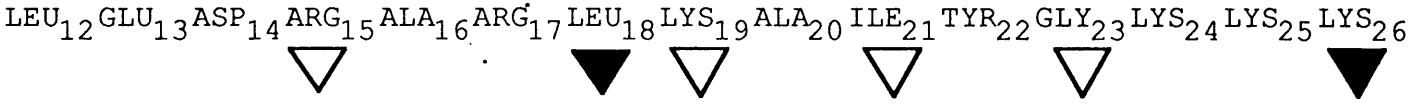
Such a model would permit several unifying and simplifying principles. First, this refined motif would explain the E^k-binding of two peptides studied by Adorini and co-workers which did not contain their proposed motif (Leighton et al., 1991). These two peptides were entirely non-natural sequences containing alternating hydrophobic and charged residues. They would fit a simple motif of a basic residue preceded 8 residues before by a hydrophobic residue, and they were found to be among the best E^k-binders of the peptides studied.

Second, it would imply that binding of a peptide to a specific class II molecule is primarily dependent upon two critical residues in the sequence. Thus, binding to E^k would be more similar than is presently proposed to binding to class I, where identification of motifs based on sequence analysis of peptides eluted from class I molecules has generally identified only two key residues as being necessary (Falk et al., 1991).

For such a model to be valid, it would imply that the two critical residues for MHC binding could be situated a variable distance apart. A similar conclusion has recently been reached for class I MHC proteins as the result of crystallographic studies (Guo, et al., 1992). This work has shown that different peptides binding the same MHC molecule are tightly bound at the anchoring residues, but that different numbers of intervening residues can be accommodated.

As a final point, it is remarkable that we identified few substitutions which seem to *increase* binding, considering the fact that substitutions at many positions can significantly decrease binding to E^k. This may simply be a reflection of the fact that 12-26 has essentially already been selected as having a relatively high affinity for E^k, because it has been identified as the dominant epitope of cl. This means it has a certain minimal affinity as well as a relative affinity that is greater than that of other epitopes in the protein. This and the fact that it matches quite closely the other known E^k epitopes may indicate that its affinity for E^k is already near a maximum.

Figure 1.1



The figure illustrates the important MHC contacts of 12-26 as determined by competition binding experiment. Critical contacts constituting the E^k-binding motif are indicated by filled triangles. Non-essential contacts which affect binding to a lesser degree are indicated by empty triangles.

Table 1.2

PEPTIDE			12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Reference
cl 12-26			LEU	GLU	ASP	ARG	ALA	ARG	**LEU**	LYS	ALA	ILE	TYR	GLU	LYS	LYS	**LYS**	Lai, et al, 1987
MCC 88-103		ALA	ASN	GLU	ARG	ALA	ASP	LEU	**ILE**	ALA	TYR	LEU	LYS	GLN	ALA	THR	**LYS**	Buus, et al, 1987
PCC 88-104	LYS	ALA	GLU	ARG	ALA	ASP	LEU	ILE	**ALA**	TYR	LEU	LYS	GLN	ALA	THR	ALA	**LYS**	Buus, et al, 1987
HEL 1-14 (1-18)				LYS	VAL	PHE	GLY	ARG	**CYS**	GLU	LEU	ALA	ALA	ALA	MET	LYS	**ARG**	Guéry, et al, 1992
HEL 81-96		SER	ALA	LEU	LEU	SER	SER	ASP	**ILE**	THR	ALA	SER	VAL	ASN	CYS	ALA	**LYS**	Buus, et al, 1987
HEL 102-116 (107-116)			GLY	ASP	GLY	MET	ASN	ALA	TRP	**VAL**	ALA	TRP	ARG	ASN	ARG	CYS	**LYS**	Leighton, et al, 1991
Nase 83-97 (81-100)			ASP	LYS	TYR	GLY	ARG	GLY	**LEU**	ALA	TYR	THR	TYR	ALA	ASP	GLY	**LYS**	Schaeffer, et al 1989
Nase 96-110 (101-120)			GLY	LYS	MET	VAL	ASN	GLU	**ALA**	LEU	VAL	ARG	ASN	GLY	CYS	ALA	**LYS**	Schaeffer, et al 1989

Sequence alignment of reported E^k-binding peptides. Boxed residues represent the putative MHC-contact residues.

Table 1.1 Relative binding of 12-26 analogs to E^k

	Position																	
	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26			
Peptide	LEU	GLU	ASP	ARG	ALA	ARG	LEU	LYS	ALA	ILE	TYR	GLU	LYS	LYS	LYS		Hel 1-14	HEL 105-120
D14-A	•	•	ALA	•	•	•	•	•	•	•	•	•	•	•	•		.75	NT
E	•	•	GLU	•	•	•	•	•	•	•	•	•	•	•	•		.60	NT
L	•	•	LEU	•	•	•	•	•	•	•	•	•	•	•	•		1.3	NT
K	•	•	LYS	•	•	•	•	•	•	•	•	•	•	•	•		.005	NT
Y	•	•	TYR	•	•	•	•	•	•	•	•	•	•	•	•		1.0	NT
A15-S	•	•	•	SER	•	•	•	•	•	•	•	•	•	•	•		.01	NT
D	•	•	•	ASP	•	•	•	•	•	•	•	•	•	•	•		.004	NT
L	•	•	•	LEU	•	•	•	•	•	•	•	•	•	•	•		.13	NT
K	•	•	•	LYS	•	•	•	•	•	•	•	•	•	•	•		.15	NT
Y	•	•	•	TYR	•	•	•	•	•	•	•	•	•	•	•		.17	NT
R16-A	•	•	•	•	ALA	•	•	•	•	•	•	•	•	•	•		.13	NT
D	•	•	•	•	ASP	•	•	•	•	•	•	•	•	•	•		.08	NT
L	•	•	•	•	LEU	•	•	•	•	•	•	•	•	•	•		4.0	NT
K	•	•	•	•	LYS	•	•	•	•	•	•	•	•	•	•		7.5	NT
Y	•	•	•	•	TYR	•	•	•	•	•	•	•	•	•	•		1.0	NT
R17-A	•	•	•	•	•	ALA	•	•	•	•	•	•	•	•	•		.60	NT
D	•	•	•	•	•	ASP	•	•	•	•	•	•	•	•	•		.24	NT
L	•	•	•	•	•	LEU	•	•	•	•	•	•	•	•	•		.75	NT
K	•	•	•	•	•	LYS	•	•	•	•	•	•	•	•	•		.60	NT
Y	•	•	•	•	•	TYR	•	•	•	•	•	•	•	•	•		.60	NT
L18-A	•	•	•	•	•	•	ALA	•	•	•	•	•	•	•	•		.02	-
D	•	•	•	•	•	•	ASP	•	•	•	•	•	•	•	•		.004	-
K	•	•	•	•	•	•	LYS	•	•	•	•	•	•	•	•		.006	-
F	•	•	•	•	•	•	PHE	•	•	•	•	•	•	•	•		.01	-
K19-A	•	•	•	•	•	•	•	ALA	•	•	•	•	•	•	•		.13	.22
D	•	•	•	•	•	•	•	ASP	•	•	•	•	•	•	•		.06	-
L	•	•	•	•	•	•	•	LEU	•	•	•	•	•	•	•		.05	.14
R	•	•	•	•	•	•	•	ARG	•	•	•	•	•	•	•		.55	2.2
Y	•	•	•	•	•	•	•	TYR	•	•	•	•	•	•	•		.04	.65
A20-S	•	•	•	•	•	•	•	•	SER	•	•	•	•	•	•		.12	.40
V	•	•	•	•	•	•	•	•	VAL	•	•	•	•	•	•		.30	.88
K	•	•	•	•	•	•	•	•	LYS	•	•	•	•	•	•		.11	.47
F	•	•	•	•	•	•	•	•	PHE	•	•	•	•	•	•		.46	1.9
I21-A	•	•	•	•	•	•	•	•	•	ALA	•	•	•	•	•		1.5	2.5
D	•	•	•	•	•	•	•	•	•	ASP	•	•	•	•	•		.05	-
L	•	•	•	•	•	•	•	•	•	LEU	•	•	•	•	•		.29	.75
K	•	•	•	•	•	•	•	•	•	LYS	•	•	•	•	•		.08	.08
F	•	•	•	•	•	•	•	•	•	TYR	•	•	•	•	•		.05	.09
Y22-A	•	•	•	•	•	•	•	•	•	•	ALA	•	•	•	•		1.2	1.7
D	•	•	•	•	•	•	•	•	•	•	ASP	•	•	•	•		.38	1.1
L	•	•	•	•	•	•	•	•	•	•	LEU	•	•	•	•		2.0	1.5
K	•	•	•	•	•	•	•	•	•	•	LYS	•	•	•	•		1.1	.66
F	•	•	•	•	•	•	•	•	•	•	PHE	•	•	•	•		1.7	1.4
E23-A	•	•	•	•	•	•	•	•	•	•	•	ALA	•	•	•		.15	.05
D	•	•	•	•	•	•	•	•	•	•	•	ASP	•	•	•		.30	.15
L	•	•	•	•	•	•	•	•	•	•	•	LEU	•	•	•		.03	.04
K	•	•	•	•	•	•	•	•	•	•	•	LYS	•	•	•		.02	.08
Y	•	•	•	•	•	•	•	•	•	•	•	TYR	•	•	•		.08	.02
K24-A	•	•	•	•	•	•	•	•	•	•	•	•	ALA	•	•		.60	.9
D	•	•	•	•	•	•	•	•	•	•	•	•	ASP	•	•		.05	-
L	•	•	•	•	•	•	•	•	•	•	•	•	LEU	•	•		.34	.34
R	•	•	•	•	•	•	•	•	•	•	•	•	ARG	•	•		.23	.25
Y	•	•	•	•	•	•	•	•	•	•	•	•	TYR	•	•		.21	.85
K25-A	•	•	•	•	•	•	•	•	•	•	•	•	•	ALA	•		.75	.9
D	•	•	•	•	•	•	•	•	•	•	•	•	•	ASP	•		.05	.05
L	•	•	•	•	•	•	•	•	•	•	•	•	•	LEU	•		4.0	3.0
R	•	•	•	•	•	•	•	•	•	•	•	•	•	ARG	•		2.4	2.3
Y	•	•	•	•	•	•	•	•	•	•	•	•	•	TYR	•		.43	1.1
K26-A	•	•	•	•	•	•	•	•	•	•	•	•	•	•	ALA		.004	-
D	•	•	•	•	•	•	•	•	•	•	•	•	•	•	ASP		.007	-
L	•	•	•	•	•	•	•	•	•	•	•	•	•	•	LEU		.02	-
R	•	•	•	•	•	•	•	•	•	•	•	•	•	•	ARG		.15	.09
Y	•	•	•	•	•	•	•	•	•	•	•	•	•	•	TYR		.004	-

Relative effectiveness of 12-26 analogs as inhibitors for binding to E^k. Values shown are the inverse of the relative concentrations required to to achieve half-maximal inhibition of presentation of two other E^k-restricted peptides, HEL 1-14, and HEL 105-120. The relative efficiency of unsubstituted 12-26 was defined as 1. Results shown are averages of duplicates. Dashes indicate that half-maximal inhibition of presentation could not be achieved at the highest concentrations tested.

Chapter 2

Abstract

The means by which the T cell receptor recognizes peptide-MHC complexes is a subject of intense ongoing study. Models have been proposed which postulate that V gene-encoded domains of the TCR are primarily responsible for recognition of the MHC molecule, whereas features of the peptide ligand are recognized by residues lying at the respective V-J and V-D-J junctions of the α- and β-chains. Support for this model comes in part from previous studies which have demonstrated that the presence of conserved sequences in the junctional regions of TCRs from 12-26-specific T cells corresponds to particular fine-specificities. In order to characterize all residues having important interactions with the TCR and to identify which parts of TCR are responsible for their recognition, we determined the responsiveness of a large panel of 12-26-specific T cells to a set of 63 analogs of P12-26 containing single substitutions. We find that the cells fall into two groups having distinct fine-specificities at each position. For one group, position 22 is the most important, in the sense that cells are most stringent with respect to substitutions at this position. Comparison

of these results with those obtained for T cells specific for the peptide MCC 93-103, indicates that equivalent residues in different E^k-restricted peptides have a similar importance in their respective interactions with the TCR. Thus, the positioning of a given epitopic residue within the MHC groove appears more important than the nature of the amino acid, i.e. polar, hydrophobic, etc. as a determinant of its interaction with the TCR. A second set of cells having dramatically different fine-specificities has also been found. These T cells also differ collectively in requiring a longer minimal peptide. Strikingly, members of each group of cells have been found to use the identical α-chain including V_α and J_α genes and non-germline encoded N region insertions. Thus, in this case, the same α-chain is capable of combining with different β-chains to give recognition of the same peptide-MHC complex, but with almost entirely different fine-specificities. The conserved α-chain may be involved in recognition of position 22 of 12-26, because the cells have similar fine-specificities at this position. Taken as groups, the switch between one type of fine specificity and the other appears attributable to the use of different β-chain junctions and different J_β elements, because both use predominantly $V_\alpha 2$ and $V_\beta 1$. We also present evidence to suggest that the aligment of TCRs specific for different peptide-E^k complexes with these complexes may be largely similar.

Introduction

Knowledge of the means by which the TCR interacts with the peptide-MHC complex is of profound importance for the understanding of the immune system's ability to distinguish self from non-self. Three similar models have been proposed to describe the interaction of the peptide-MHC complex with the TCR (Chothia et al., 1988; Claverie et al., 1989; Davis & Bjorkman, 1988). Based on sequence homologies between the TCR and immunoglobulins, all three postulate that the TCR recognizes antigen similarly to the Fab domain of an antibody. Like the immunoglobulins, TCRs are encoded by interchangeable V, D and J genes which are fused together to create complete α- and β-chains. Again, in both Igs and TCRs the V regions contain two Complementarity Determining Regions (CDRs) that have been shown for Igs to be the principal points of contact for antigen. A third CDR lies at the junctions of the V and J segments (for α) and V, D and J segments (for β). The most striking difference between the TCR and Igs is that while much of the diversity between immunoglobulins comes from the use of a large number of different V genes and thus lies in the first two CDRs, a far greater portion of the diversity in TCRs occurs in CDR3. In the predicted structure of the TCR, this third CDR would lie between the other two. These observations led all three groups to propose that the first two CDRs interact principally with the relatively non-diverse MHC molecules, while the greater diversity of the third CDR could be exploited for recognition of the many different antigens that would bind in the cleft.

Such a structure would explain several additional observations. First, the maturation of MHC-restricted T cells in the thymus requires the specific interaction of the TCR with MHC in the absence of peptide antigen (Kisielow et al., 1988) and leads to selection of T cells bearing certain V_βs with inherent affinity for the endogenous MHC molecule (Blackman, et al., 1989) in combination with a variety of D_β and J_β segments. This shows that significant interactions occur between the first two CDRs of the TCR V regions and the polymorphic domains of the MHC molecules. No particular association has been noted between expression of a given MHC molecule and thymic selection of particular D or J segments. More recently, residues involved in MHC recognition have been mapped to CDR1 and CDR2 in mature T cells (Hong et al., 1992)

Such a structure would also explain the complementary geometries of the MHC and TCR molecules. The width of the binding site in MHC class I molecules is approximately 10Å (Bjorkman et al., 1987a). This would correspond quite closely with the distance between CDRs 1 and 2 in the TCR structure. CDR3 would extend over approximately 15Å permitting it to interact with 5-6 residues of a peptide bound in extended conformation.

Although it remains possible that there is no precisely fixed physical interaction between the T cell receptor and the MHC molecule, evidence is accumulating that the TCR does not have the same sort of flexibility for diverse interactions as antibodies have with protein antigens. For example, the same class I molecules are simultaneously recognized by the TCR and the co-receptor CD8 (Connolly, et al., 1990; Ingold, et al., 1991; Salter, et al., 1990),

which has a precisely defined binding site on the class I molecule. This suggests that the TCR may be largely constrained in its configuration of interaction with MHC.

Recently, Davis and co-workers have produced even stronger evidence for the interaction of CDR3 with bound peptide using mice transgenic for either the α- or β-chains from a TCR specific for moth cytochrome C residues 93-103 (Jorgenson et al., 1992) (MCC). The T cell receptor in these mice is formed from a combination of the protein coded for by the transgene and an endogenous counterpart. If the mice are immunized with MCC, they produce T cells bearing TCRs closely related to the orginal. If the mice are immunized with peptides containing single substitutions at particular positions, responding T cells use TCRs containing compensatory changes in the junctional residues. Specifically, TCR α-chains from β-chain transgenics immunized with wild-type MCC containing a lysine at position 99 all contained either aspartate or glutamate in their α-chain junctions. T cells from the same mice immunized with an aspartate at position 99 had lysine in the α-chain junction. Substitutions in MCC at position 102 led to similar compensatory changes in the β-chain junctions of responding T cells.

It is important to determine whether the correlations one observes between junctional sequences and antigen recognition are the general rule or represent a specific case. One report has shown a strong correlation between the recognition of the same peptide antigen (tetanus toxin) and the use of a particular V_β gene, but no conservation in the junctional regions (Boitel, et al., 1992). In different systems it has been demonstrated that T cells recognizing

a given peptide-MHC complex use a limited set of TCR α/β chain combinations (Acha-Orbea, et al., 1988; Danska et al., 1990; Fink, et al., 1986; Gold, et al., 1991; Lai et al., 1988). It is important to know whether these V regions are involved only in MHC binding, or whether certain ones are needed as frameworks to permit the junctional regions to interact with a given peptide. One would also like to know if the V regions themselves make important positive or negative contacts with bound antigen. If particular domains of the TCR can be shown to interact with the peptide or MHC, one would like to know whether binding is in only one register, or whether it can occur at different ends of the MHC molecule depending upon which TCR genes are used.

Previous studies of I-E^k-restricted, 12-26 specific hybridomas have shown that over 90% use one of two closely related members of the $V_\beta 2$ family, and 70% use $V_\beta 1$ (Lai et al., 1988). Furthermore, close correlations have been identified between sequences in both the α- and β-chain junctions and reactivity to peptides containing substitutions at position 22. In a second study, among eight α-chain sequences determined, six contained identical α-chain junctions (Lai et al., 1990). A seventh differed from the other six only in that its α-chain junction contained a single amino acid deletion. This cell was the only one in the panel incapable of responding to P12-26 containing a tyrosine to phenylalanine substitution at position 22 (P12-26(22F)). This observation, therefore, implicated the α-chain in recognition of position 22. Interestingly, if an alignment is made between 12-26 and MCC 93-103 (see Figure 1.2) position 22 of 12-26 would correspond to

position 99 of MCC 93-103. As noted above, substitutions at this position induce compensatory changes in the α-chain junctions of T cells in β-chain trangenic mice. This parallel suggests that the same region of the T cell receptor might be used for recognition of equivalent residues in different E^k-restricted peptides. Since these T cells use different V_α and V_β genes, this also suggests that different TCRs specific for E^k would align with the MHC molecule in a similar way, and that the most critical residues for recognition might be selected by their positioning in the MHC groove.

However, correlations were also made between fine-specificity at position 22 and sequences in the β-chain. It was observed that among the cells containing the identical α-chain junctions, all but one did *not* accept a tyrosine to histidine substitution at position 22. This T cell's β-chain differed primarily from all the others in that it lacked a conserved non-germline-encoded glutamate in its junction. Thus, there does not appear do be an absolute division whereby fine-specificity at position 22 would be determined entirely by either the α- or β-chains. It is possible, however, that this is merely an indirect consequence of differences in a part of the TCR whose principal function is recognition of another residue of the peptide.

To expand upon these studies we were interested to see how many different residues in the peptide seemed to affect fine-specificity. It was desirable to further examine the observed correlations between fine-specificity at position 22 and sequences in the α- and β-chains using a larger panel of T cells, and search for correlations at other positions. We were also interested in

determining whether only the junctional regions of the TCR were implicated in recognition of these residues, or whether other parts of the TCR were as well. More specifically, we were interested in pursuing possible parallels between the 12-26 system, and the other E^k system, MCC. Davis and colleagues have shown that when charged residues are substituted at positions 99 and 103 in MCC, T cells that recognize these peptides often contain complementary charged residues in their junctions. We were interested to see whether the equivalent residues of 12-26 were of equal importance, and whether a similar means of recognition was employed. If this is indeed the case we would expect to identify position 25 as a critical T cell contact, and, further, might expect to find correlations between the recognition of substitutions at this position and particular sequences in the β-chain junction.

In this chapter we describe the characterization of a large panel of 12-26-specific hybridomas using a set of 63 analogs of P12-26 containing single substitutions. Comparison of the results to those obtained elsewhere indicates that the importance of the interaction of equivalent residues in different E^k-restricted peptides with the TCR is determined by their position within the MHC groove. Additionally, by comparing the sequences of the TCRs of several of the T cells, we are able to ascribe changes in the fine-specificities to particular differences in TCR sequence.

Results

T cells specific for 12-26 were made from CBA mice after a primary response induced either by immunization with cI protein or P12-26 peptide. Fusions were performed between the T cell receptor deficient thymoma BW5147$\alpha^-\beta^-$ (White et al., 1989) and lymph node cells from three different mice. In an initial screening, 45 hybridomas from P12-26-immunized mice and 27 hybridomas from cI-immunized mice were tested against P12-26 and the truncated peptide PP15-26 and their MHC restrictions determined. As had previously been observed in a study of 12-26-specific hybridomas (Lai et al., 1987), all those produced in k-haplotype mice by immunization with cI responded equally well to PP15-26 as to P12-26 and were E^k-restricted Table 2.1. Notably, however, in these experiments hybridomas produced by immunization with 12-26 fell into two distinct categories, those which responded equally well to P12-26 and PP15-26, and those which responded to P12-26, but did not respond at all to concentrations of PP15-26 as high as 50μg/ml.

Several of the hybridomas which proliferated stably in culture were selected for further characterization. In the study of 12-26-specific hybridomas mentioned above, A^d-restricted hybridomas from Balb/c mice could be subdivided into two categories based on their minimal peptide requirement, those which respond better to the peptide PP15-26 than to the peptide P12-24, and those with the opposite preference for peptide. We therefore tested all cells

against P12-24. Unlike in A^d, no cell was able to respond to concentrations of 12-24 as high as 100μg/ml (not shown). It was observed, however, that the P15-26-responders had a sensitivity for 12-26 which is significantly higher on average than for the non-responders. The average concentration of 12-26 necessary for half maximal stimulation among the responders shown in Table 2.2 is 0.27μM. P15-26-non-responders, by contrast, require an average concentration of 1.4μM.

Because of the difference in the types of hybridomas produced by immunization with P12-26 or by cI protein, we considered the possibility that processing of the protein is at the origin of the exclusive production of hybridomas which respond to P15-26 in cI-immunized mice. Such a situation might arise, for example, if the exact peptide produced by processing of cI were capable of stimulating only a limited subset of T cells which can respond to the peptide P12-26 which does not require processing in order to be presented. To test this hypothesis, we examined the responsiveness to cI protein of the different sets of hybridomas which responded either only to P12-26, or to both P12-26 and P15-26. Figure 2.1 shows the results of such an experiment. A representative P15-26-responder (1.9) can be stimulated by cI protein, although, as has been previously observed (Lai et al., 1987), about 10-fold more protein than peptide on a molar basis is required. By contrast, two of the P15-26-non-responders, 1.21, and 2.4, are not stimulated by the protein, and the third, 2.19, requires significantly more protein on a molar basis. This is not simply a consequence of a difference in sensitivity, because the hybridomas shown have approximately the

same sensitivities to peptide. A similar observation has been made for an E^d-restricted hybridoma (26.1.E) that differs from other E^d-restricted hybridomas studied to date in that it too does not respond to P15-26 or to cI protein (not shown). This hybridoma was also produced by immunization with P12-26 peptide. These results suggest that processing of cI protein produces a peptide to which only a limited set of E^k-restricted T cells can respond. Furthermore, this effect is probably at least partially responsible for the fact that the 12-26-specific T cells previously described from k-haplotype mice are more homogenous than those in d-haplotype mice (Lai et al., 1987). Because of this additional diversity in fine-specificities, the experiments described below were conducted using T cells derived from mice immunized with P12-26.

We then tested some of these T cells for their responsiveness to the various substituted analogs described in chapter 1. A total of 23 different 12-26-specific E^k-restricted hybridomas, including three which had been previously isolated and partially characterized (Lai et al., 1987; Lai et al., 1988) were tested. Although initial examination of the results revealed no clear pattern of responsiveness when the cells were considered as an ensemble, it was observed that if P15-26-responders and non-responders were grouped separately, patterns did emerge. The results are presented in Figure 2.2 grouped in this way. In order to facilitate the presentation of the data for all of the hybridomas in one figure, the following format has been used. The substitutions made in the peptides are indicated in the left-hand column. The names of the

various T cells tested are in the top row. Each rectangle in the figure corresponds to the response of a given T cell to a peptide, and the sensitivity of a given T cell to a given peptide is indicated by the darkness of the rectangle. The two groups of cells show clear differences in their stringencies with respect to substitutions at each position. For example, T cells on the left-hand side of the panel (cells which respond to P15-26) in general accept a variety of different substitutions at positions 16 and 17. By contrast, with two minor exceptions (cells 1.11 and 8I), they all accept either no substitutions at all, or only phenylalanine in place of the wild-type tyrosine at position 22. T cells on the right-hand side of the panel (P15-26-non-responders), by contrast, in general readily accept substitutions at position 22, but do not at positions 16 and 17.

Within each group there are distinct preferences for certain substitutions at several positions. For example, P15-26-non-responders all accept only serine as a substitution at position 20. P15-26-responders, however, taken together, have no preferred substitution at this position. At position 21, P15-26-responders again show a preference for hydrophobic side chains, in this case alanine and leucine. P15-26-non-responders, in general do not accept substitutions at this position. As mentioned above, P15-26-responders accept only aromatic amino acids at position 22, while P15-26-non-responders accept a variety of substitutions, but show a clear preference for aspartate. At position 25, P15-26-responders show a preference for hydrophobic residues, alanine and leucine, while P15-26-non-responders prefer the basic arginine. Finally, at

position 26, P15-26-responders cells prefer arginine, while in this case P15-26-non-responders prefer leucine.

These results show that different residues in 12-26 play roles of variable importance for the recognition of the epitope by the TCR. For P15-26-responders, position 22 appears to be the most important, in that T cells are the most stringent with respect to substitutions here, although other positions are restrictive with respect to substitutions as well. An key question is whether the importance of a given residue is determined by its localization within the MHC groove, or by the particular amino acid at the position, i.e. polar, hydrophobic, etc. One means of determining this is to compare different peptides having the same MHC restriction to see if the equivalent residues appear to play the same role for recognition by the TCR. Figure 2.3 shows such a comparison between the results obtained here for P15-26-responders and those reported for another E^k-restricted peptide, MCC (Fox, et al., 1987). The peptide alignment is the same as that shown in Figure 1.2. Although not all positions were tested for the MCC peptide, where the comparison can be made, the equivalent positions do appear to play the same role. Position 99 in MCC, the equivalent of position 22 in 12-26 is the most stringent with respect to substitutions. This cannot be the result of the particular class of amino acid at this position because in MCC, position 99 is a lysine, and in 12-26 position 22 it is a tyrosine. Similarly, positions 98 in MCC and 21 in 12-26 are both spacer residues, apparently making no important contacts with the TCR (or MHC). MCC positions 101 and 102 also appear to have similar roles to positions 24 and 25 of 12-26,

despite the difference in the amino acids found at these positions. These results, therefore, suggest that the importance of a given residue for its interaction with the TCR depends upon its localization in the MHC groove.

Possible binding of 12-26 to E^k in alternative conformations

The results obtained for P15-26-non-responders are more difficult to interpret. For these cells, the pattern of fine-specificities appears different at virtually every position from that obtained for P15-26-responders. To explain this contrast, we considered the possibility that 12-26 might bind to E^k in more than one conformation. The justification for such a hypothesis is based on the following reasoning: MHC binding is a necessary but not sufficient condition for stimulation of all T cells. Therefore, if a given substitution prevents the binding of the peptide to MHC, all of the T cells in the panel should no longer respond. Furthermore, at every position that contacts MHC there should be a certain hierarchy of preferred substitutions. For example, if a hydrophobic residue is important for MHC binding at a certain position, leucine and alanine substitutions may not affect binding at all, tyrosine may work less well, and lysine and aspartate may both work poorly. In contrast, the interaction of the TCR with the same peptide-MHC complex may be different for each T cell. Thus, for those positions that do not interact with MHC, some will be TCR contacts for a given T cell and not for others. Furthermore, even for two TCRs that contact the same residue, the nature of their interactions with it will not

necessarily be the same. That is, in one case the interaction may be based on a charge pair, while in another it may be a hydrogen bond. Thus, the hierarchy of acceptable substitutions at a given position is expected to be different between T cells for residues whose predominant interaction is with the TCR.

To see if these differences in the roles of various residues were indeed reflected in the responsiveness of T cells, we compared the results obtained in the competition binding experiments described in the preceding chapter to the fine-specificities of the T cells we have studied. As can be observed in Figure 2.4, the correlation is indeed quite high for P15-26-responders. The two residues identified as being most important for MHC binding, 18 and 26 give fairly uniform patterns of responsiveness for all of the cells. Furthermore, there is a direct correlation between the extent to which a given substitution affects binding to MHC in the competition assay, and the extent to which the T cells in this group respond. At position 18 no cell responds to the substitution, an aspartate, which reduces binding most in the competition assay. At position 26 the single substitution which can generally be tolerated by all T cells is an arginine. Again, this conservative change has the most minor effect on binding in the competition assay.

In contrast to the P15-26-responders, the P15-26-non-responders have a pattern of reactivity to the peptides that is seemingly unrelated to their MHC-binding capacities. In fact at several different positions, strong preferences can be observed among all hybridomas for one amino acid substitution over others, but the particular preferred substitutions do not correlate with the

ability of the peptides to bind in the competition assays. At position 20, all of the cells accept only an alanine to serine change, whereas this peptide seems to bind MHC only slightly less well than the wild-type and equivalently to others substituted at the same position. At position 22, although a variety of substitutions are accepted by various T cells, a strong preference for an aspartate is observed. The peptide containing this substitution binds worst to E^k among the five substituted at this position. Finally, at position 26, a preference for a lysine to leucine change is observed. This is in marked contrast to the P15-26-non-responders which prefer the more closely related arginine and to the competition data suggesting that this peptide should bind less well than the wild-type or the arginine-substituted peptide.

The above data give several examples where a close correlation is observed between the MHC-binding capacity of the various peptides in the competition assay with their patterns of stimulation of P15-26-responders, but not P15-26-non-responders. Two possible explanations exist for this observation. The first and more classical is that two distinct groups of T cells, each containing conserved and distinctive features in their TCRs respond to 12-26. The second is that 12-26 is capable of binding to E^k in two distinct conformations, one of which induces P15-26-responder cells, and the other of which stimulates P15-26-non-responder cells. The strict distinction between the fine-specificities of the cells and the difference in the minimal peptide requirements led us to consider this second possibility.

Two groups have, in fact, reported indirect evidence that supports the idea that a single peptide could indeed bind MHC in more than one conformation (Bhayani and Paterson, 1989; Kurata and Berzofsky, 1990). Two different observations argue strongly against it in this case, however. First, if 12-26 could indeed bind to E^k in an alternative conformation, the critical residues should be those identified by the fine-specifity tests of the P15-26 non-responders. Thus, four residues should be essential, an ala or ser at position 20, a tyr or asp at position 22, a basic residue at position 25 and a lys or leu at position 26. Such a motif bears no resemblance to the pattern of critical residues identified in other good E^k-binding peptides (Figure 1.2). While it is possible that two quite disparate motifs exist for E^k binding, we would expect that other epitopes containing sequences resembling this second motif would have been identified, which is not the case.

An even stronger argument comes from analysis of the TCR genes from the 12-26-specific hybridomas studied here. We have determined the V_α and V_β gene usage of many of the hybridomas described above using PCR. All of six P15-26 non-responders and four of six P15-26 responders whose fine-specificity has been studied here employ the same combination of $V_\beta 1$ with closely related members of the $V_\alpha 2$ family (Table 2.3). This similarity in the TCRs of the two groups of cells strongly suggests that they are recognizing a similar peptide-MHC complex, and that the differences in responsiveness of the two groups of cells represent the particular structures within this complex that are chosen for recognition by each group. T cells specific for other peptides also restricted by E^k

use entirely different combinations of V_α and V_β (Hedrick, et al., 1988; Sorger, et al., 1990; Winoto, et al., 1986), indicating that this is not just a reflection of the fact that P15-26 responders and non-responders are restricted by the same MHC molecule.

Fine-specificity at position 22 correlates with usage of a conserved TCR α-chain sequence

A previous study of 12-26-specific T cells had described evidence linking recognition of position 22 alternatively to sequences in either the α- and β-chain junctions (Lai et al., 1990). However, in both cases the evidence was only indirect. As a means of further examining whether one chain of the TCR in particular was responsible for recognition of this position, and because all previous analysis had used cells from the P15-26-responder group, we determined the sequences of the TCRs from cells from the group of P15-26-non-responders. Cell 1.13 was chosen as a representative P15-26-non-responder, and cell 1.21 was also chosen because it was one of only two in the group which accepted only aromatic substitutions at position 22. Figure 2.5 shows the sequence of the α-chain for these cells, as well as the sequence of the β-chain for cell 1.21. The β-chain sequence for 1.13 has not yet been determined. Confirming the PCR analysis, the β-chain of 1.21 uses $V_\beta 1$ along with a member of the $J_\beta 1$ family. This result is significant in that most previously characterized E^k-restricted T cells specific for 12-26 (all P15-26 responders) also used $V_\beta 1$, but all used $J_\beta 2$.

The two T cells employ closely related members of the $V_\alpha 2$ family, as well as relatively closely related J_α elements (Figure 2.6). Strikingly, we observed that the sequence of the α-chain of 1.21 is identical to the α-chain of a previously studied P15-26-responder T cell, 4I (Lai et al., 1988). Several aspects of this observation are worthy of note. First, 4I uses $V_\beta 14$ and a member of the $J_\beta 2$ family paired with the α-chain described above, while 1.21 uses $V_\beta 1$ with $J_\beta 1.6$. This is not the first time that identical α-chains have been found to pair with different β-chains. Notably, cell 3I was found to share the identical α-chain with several other 12-26-specific T cells (Lai et al., 1990). However, for these cells, very closely related β-chains were used. Additionally, (Jorgenson et al., 1992) found that a variety of significantly different β-chains were able to pair with the identical α-chain to effectuate recognition of substituted MCC peptides. However, in their experiments the cells were derived from mice transgenic for this α-chain, and the cells were therefore constrained to using it. Our results therefore show that a very high degree of selection exists for the use of this particular α-chain, and that it apparently is capable of contributing to the recognition of 12-26 in concert with different β-chains.

Second, the fact that the identical α-chain is used by both cells along with related V_βs lends support to the idea that P15-26-responders and non-responders do not recognize 12-26 bound to E^k in different conformations. It has previously been shown that when T cells recognize different determinants, even within the same peptide they employ different V_α and V_β genes (Nanda, et al., 1992). Even relatively small changes in the fine-specificity of T cells are

often accompanied by changes in the V genes used (Jorgenson et al., 1992). Thus, it is difficult to imagine that these two T cells, 4I and 1.21 could be recognizing 12-26 bound to E^k in different conformations if they employ such closely related TCR genes.

Third, since these cells employ identical α-chains and the same V_β, this observation reinforces support for the hypothesis described above that differences in the β-chain junctions or J_β usage are at the origin of the gross differences in fine specificity between P15-26-responders and non-responders.

Finally, it is significant that 1.21 is one of only two P15-26-non-responders that have the typical fine-specificity at position 22 of P15-26-responders. This suggests that the conserved α-chain used by 4I and 1.21 might be primarily responsible for recognition of this residue. Such a conclusion would be dependent on 4I also having a similar fine-specificity at position 22. Previously, 4I was tested only against two analogs of 12-26 containing aromatic substitutions (Lai et al., 1988). We therefore verified that 4I responded to other substitutions at position 22 in a similar way as 1.21. Figure 2.6 shows that this is indeed the case. As is the case for 1.21 and most P15-26 responder cells 4I accepts no other substitution at position 22 up to concentrations 1000-fold higher than that required for stimulation by 12-26. These data, therefore, suggest that the conserved α-chain employed by 4I and 1.21 determines the fine-specificity of these T cells at position 22.

As mentioned above, data has been presented linking the fine-specificity of one group of T cells at position 22 to sequences in both the α- and β-chains of the TCR (Lai et al., 1990). For the β-

chain, this consisted of the observation that 6 out of 7 cells having identical α-chain junctions had identical fine-specifities at position 22 and all contained a glutamate residue at position 100 in their β-chain junctions. The seventh cell sharing the same α-chain junction (cell 51V), lacked the glutamate in its β-chain junction, and was the only one among the 7 which accepted a histidine substitution at position 22 (Figure 2.7). Evidence implicating the α-chain consisted of the identification of a partially overlapping set of 6 cells containing identical α-chain junctions which all accepted only a phenylalanine substitution at position 22. A seventh cell (24I) differed from the others in that it contained a single deletion in its α-chain junction, and that it was the only one in the group which did not accept a phenylalanine substitution at position 22. These data are reproduced in Figure 2.8.

These correlations between the fine specificity at position 22 and sequences in both the α- and β-chains could be the consequence of interactions with structures in the TCR selected for recognition of this residue, or of indirect consequences of modifications in structures that are principally involved in recognition of others. To determine whether the only correlation between the conserved glutamate in the β-chain junction was with fine-specificity at position 22, we examined the response of cell 51V to peptides substituted at other positions, and compared the results to those obtained for cell 3I. The most significant difference observed was that whereas 3I accepts only alanine and leucine substitutions at position 25, 51V also accepts arginine and tyrosine (Figure 2.9). In fact, 51V responds to P12-26(R25) approximately 4-fold better than

the peptide containing the original lysine at this position. Thus, as there is no response of 3I to concentrations of P12-26(R25) as much as 1000-fold greater than those required for stimulation by P12-26, there is at least a 4000-fold difference in the relative sensitivity of 51V and 3I to this substituted peptide, and at least a 1000-fold difference in relative sensitivity to P12-26(Y25). The effect on fine-specificity at this position may be therefore be greater than at position 22. This suggests that the conserved glutamate residue in the β-chain junctions of 3I may have been selected for recognition of other residues (perhaps residue 25), and that the different fine-specificities of 51V and 3I at position 22 may be an indirect consequence of this.

The two cells sharing the identical β-chain and differing by a single amino acid deletion in their α-chains, 24I and 7II, have also been compared for their acceptance of substitutions at positions other than 22 (Ming-Zong Lai, personal communication) In contrast to the results obtained for cells 3I and 5IV, no other differences in fine specificity have been found for these cells. Taking the ensemble of these results into consideration, therefore, it appears that conserved sequences in the α-chain junction are primarily involved in recognition of position 22 of 12-26, while variations in the β-chain junction may affect fine-specificity at this position only indirectly.

Discussion

We have characterized the set of E^k-restricted T cells specific for the immunodominant epitope of cI. Although previous studies had found that all cells specific for 12-26 could also respond to the truncated peptide P15-26, we identified a second set of cells produced only upon immunization with P12-26 which required a minimal peptide which was longer. The explanation for this difference appears to be that processing of cI protein produces a peptide which is capable of only inefficiently stimulating this second set of cells (referred to as P15-26-non-responders), because P15-26-non-responders appear to respond to cI protein significantly less well than P15-26-responders.

The fine-specificities of these two sets of cells are dramatically different. While P15-26-responders are extremely stringent with respect to substitutions at position 22, this is the position of lowest stringency for non-responders. At several positions the groups differed collectively in which amino acid substitutions were preferred. We reasoned that it should be possible to confirm the identification of MHC contact residues by examination of fine-specificity data. Substitutions at positions which contact MHC should, in principle, have a similar effect on stimulation of all cells which recognize the same peptide-MHC complex. A comparison of these results to the competition binding experiments described in the previous chapter revealed a close correlation between the probable MHC-contact residues predicted by the two methods, but only for the P15-26-responders.

This difference and the fact that a different minimal peptide was required led us to consider the possibility that P15-26-non-responders recognized P12-26 bound to E^k in an alternative conformation. Such a hypothesis is supported by two reports which have described T cells which appear to recognize the same peptide bound to the same MHC molecule in different conformations (Bhayani & Paterson, 1989; Kurata & Berzofsky, 1990). The most convincing evidence that this is not the case, however, is that almost all cells from both the responder and non-responder groups use the same $V\alpha 2$ and $V_\beta 1$ genes. Since T cells specific for other peptides bound to E^k employ different TCR genes (Hedrick et al., 1988; Sorger et al., 1990; Winoto et al., 1986), this coincidence would be difficult to explain if P12-26 were binding in two entirely different conformations. Furthermore, two hybridomas have been identified, one of them (4I) a P15-26-responder, and one (1.21) a P15-26-non-responder, which use identical α-chains. Again, this result is best explained by the groups of cells recognizing the same complex.

If the peptide-MHC complex recognized by P15-26-responders and non-responders is assumed to be the same, an important difference appears to exist between the two groups of cells in the number of peptide residues contacted by the TCR. Among the P15-26-responders we find that residues 16 and 17 can generally be freely substituted, while the two more N-terminal residues, 14 and 15, tend either to accept many substitutions, or none at all. Since residue 14 is not necessary for stimulation of these T cells, it can be concluded that the principal residues necessary for stimulation of these T cells lie between 18 and 26. The cells which do not

respond to substitutions at positions 14 through 17 may not mainly because these substitutions introduce negative interactions with the TCR, as opposed to causing the loss of an essential contact. If the probable MHC-contact residues 18 and 26 are excluded, the TCR would be expected to make essential contacts with residues 19-25. This is good agreement with the number of residues with which CDR3 is predicted to interact, if the peptide assumes an extended conformation (Claverie et al., 1989).

Surprisingly, both the fact that the minimal peptide for P15-26-non-responders includes at least residue 14, and the fact that these cells accept virtually no substitutions at positions 14 and 15 indicates that the TCRs of these cells make essential contacts with these residues. Positions 24 and 25 also seem to be critical for these cells. P15-26-non-responders, therefore, seem to interact with residues which are much more widely dispersed in the peptide than for P15-26-responders. T cells from these two groups employ the same set of V_α and V_β genes, which, if conventional models for TCR structure are correct, suggests that their interaction with the E^k molecule is similar. If this is the case, it would imply that the CDR3 of P15-26-non-responders extends over a much longer surface, and contacts many more residues than that of the responders. This issue will require further investigation.

Previous studies of the fine-specificity of 12-26-specific T cells have been limited to the use of peptides containing aromatic substitutions at position 22. One of the goals of this work was to identify other positions where correlations could potentially be found between fine-specificity and TCR sequence. Interestingly,

all cells tested to date that recognize a minimal peptide of P15-26 focus their response on residue 22, in the sense that this residue appears to be the most restrictive with respect to substitutions. This does not appear to be simply a minor consequence of differences in the way individual T cells recognize the peptide-MHC complex. Indeed, we would argue that the tyrosine at position 22 is particularly favored for interaction with the TCR. Lai et al. (1990) identified cells having three different types of fine-specificities with respect to substitutions at position 22: Those that accepted no aromatic substitutions, those that accepted only phenylalanine, and those that accepted phenylalanine and histidine. We have shown here that among the cells that accept no aromatic substitutions, some (8I and 1.11), do accept other substitutions to some degree. Thus, despite the fact that position 22 appears to be the single most important position interacting with the TCRs of cells of this group, several different means appear to be used to effectuate recognition. This suggests that the 12-26-E^k complex selects T cells bearing TCRs focussing their interaction on this position.

The identification of the P15-26-non-responder cells for which position 22 is the single position of lowest stringency with respect to substitutions is somewhat difficult to understand in this context. However, T cells of this group have a sensitivity for 12-26 which is on the average six-fold less than for P15-26-responders. They are also much more stringent with respect to substitutions at virtually every other position. Therefore, it may be that TCRs of the highest affinity are those which interact with position 22. Those

which cannot, must make up for this loss by relatively tenuous interactions with other residues.

If position 22 is indeed the most critical residue for the recognition of the 12-26 epitope, one may ask whether this is because of the particular amino acid at this position, i.e. tyrosine, or because this position interacts most strongly with the TCR because of its localisation within the MHC groove. One way to answer this question is to ask whether residues at the equivalent position in other E^k-restricted peptides have a similar importance. One previous study examined the response of two hybridomas specific for MCC 93-103 to peptides substituted at positions 98, 99, 101, 102 and 103 (Fox et al., 1987). Indeed, in this study, residue 99, which would be equivalent to residue 22 in 12-26 was found to be the position most stringent with respect to substitutions. In MCC 93-103, the amino acid at this position is lysine, suggesting that it is the localisation of the residue within the MHC groove, and not the particular amino acid found at this position that is responsible. Figure 2.3 shows a schematic representation of the results of this study and compares them to the results we have obtained. Such an alignment indicates that not only at position 22, but at all others where comparisons can be made, the importance of the residue for TCR recognition is similar. Position 98 in MCC 93-103 and position 21 in 12-26 both accept many substitutions, and are therefore classified as spacer residues. Position 24 and 25 and their equivalents in MCC 93-103 are of similar, although lesser, importance than position 22 for TCR recognition. These observations, therefore, strongly suggest that that the positioning

of a given residue more than the particular amino acid class to which it belongs determines the importance of its interaction with the TCR.

If this is in fact the case, one can ask whether the interaction of MCC 93-103-specific and 12-26-specific TCRs with their respective antigens are similar. Using mice transgenic for either the α- or β-chain from an MCC 93-103-specific T cell, (Jorgenson et al., 1992) showed that recognition of MCC position 99 seemed to be predominantly the result of interactions with the α-chain. This led them to propose a model with the TCR aligned across the peptide-MHC complex such that CDR1 and 2 interacted with the MHC molecule, while the junctional residues of the α-chain interacted with the N-terminal end of the bound peptide and the junctional residues of the β-chain with the C-terminal end. Here we present data indicating that despite the use of different TCR genes the alignment of the TCR with E^k-peptide complexes may be similar. Lai et al. (1990) originally identified 7 T cells bearing TCRs which differed primarily in that one, 24I, contained a single alanine deletion in its α-chain junction. This cell was the only one in the group incapable of recognizing P12-26(22F). This evidence, therefore, implicated the α-chain in position-22 recognition. However, correlations could also be made between position-22 recognition and the β-chain. Seven T cells were identified with closely related β-chains, all containing a conserved glutamate at position 100 in their junctions, and all incapable of recognizing a histidine substitution at position 22. An eighth, which lacked the glutamate at position 100, did respond to the histidine-substituted

peptide. Thus, for this group of 12-26-specific T cells, both chains of the TCR appeared to affect recognition at position 22. By examining the response of these T cells to a larger set of peptides, we have found that while the deletion in the α-chain junction appears to affect primarily fine-specificity at position 22, changes in the β-chain junction appear to affect recognition at other positions (e.g. position 25) just as significantly. Therefore, for both MCC 93-103-specific T cells and this set of 12-26-specific T cells, recognition of equivalent residue may be by the α-chain.

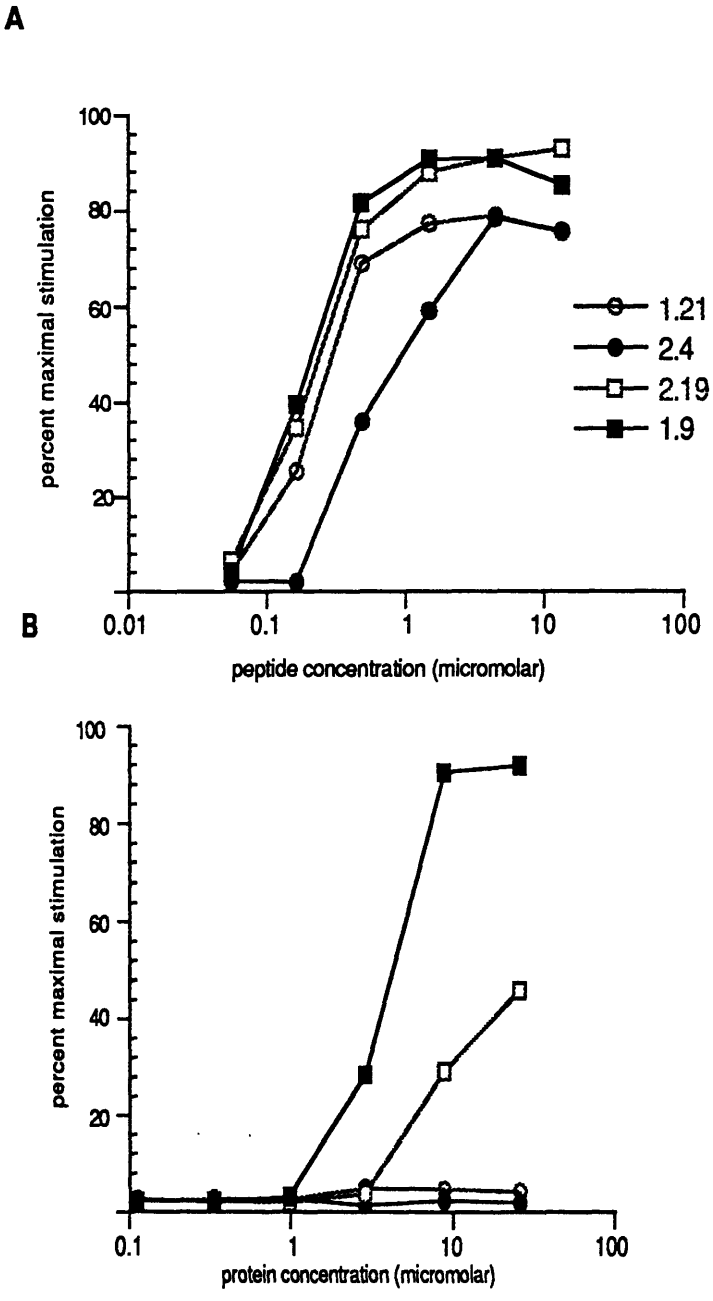
A particularly striking discovery that also supports this hypothesis was the finding that cell 1.21 employs the identical α-chain to one used by a previously described T cell, 4I (Lai et al., 1988). Lai et al. (1990) found one α-chain was found paired with closely related β-chains in 12-26-specific hybridomas, and Davis and colleagues have found that transgenic α-chains can pair with a variety of different β-chains. However, to our knowledge, this is the first example of an identical α-chain combining with widely different β-chains in a non-transgenic animal. This indicates, therefore, that a very high degree of selection exists for the use of this α-chain sequence, which suggests that it is responsible for recognition of elements of structure in either the bound peptide, or the E^k-molecule, or both. A likely contact residue for this α-chain is the tyrosine at position 22. Cell 1.21 is one of only two P15-26-non-responders which accept only a phenylalanine substitution at position 22. Thus despite the differences in minimal peptide requirement and fine-specificities at other positions for 1.21 and 4I, the primary points of comparison of these two cells are a similar

responsiveness to substitutions at position 22 and usage of the identical α-chain. Cell 1.13, which like most of the other members of the P15-26-non-responder group accepts preferentially an aspartate at position 22 uses a different α-chain. These data, therefore, support the view that three different α-chains, that of cells 4I and 1.21, that of 3I, 51V and others in this group described by Lai et al. (1990), and that studied by Jorgenson et al. (1992), are principally responsible for recognition of the amino acid occupying the equivalent position in the MHC groove.

A final piece of evidence supporting this possibility was the contrasting response of cells 51V and 3I to substitutions at position 25. These two cells differ primarily in that 51V contains a glycine residue at position 100 in its β-chain junction, whereas 3I contains a glutamate that is conserved among several other 12-26-specific cells. This difference was originally correlated with variations in fine-specificity at position 22 (Lai et al., 1990), but the more extensive analysis described here found that correlations could also be found at other positions, in particular with position 25. Most interestingly, 3I was found to be at least 4000-fold more selective than 51V against 12-26 containing an arginine substitution at this position in place of the normal lysine. This suggests that the conserved glutamate residue at position 100 of the β-chain may be involved in recognition of position 25 of 12-26, perhaps via a salt bridge. (Jorgenson et al., 1992) found that when α-chain transgenic mice were immunized with MCC 93-103, all resulting hybridomas analysed contained asparagines at the same position 100 of their β-chain junctions. A similar result was previously obtained in non-

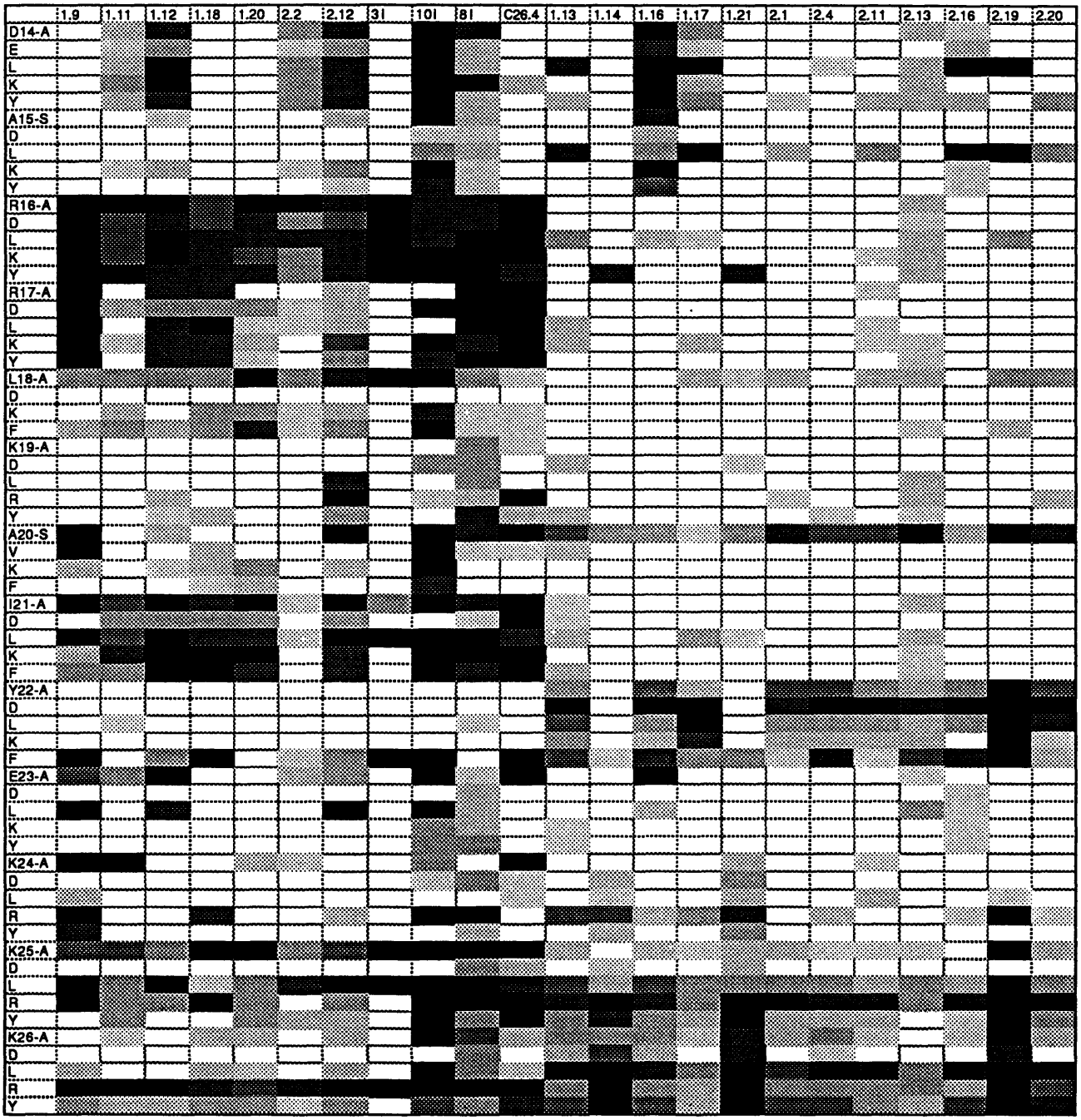
transgenic mice by Hedrick and colleagues (1988). When the mice were immunized with an MCC 93-103 analog containing a lysine instead of the normal threonine at the residue equivalent to position 25 of 12-26 (MCC position 102), the responding T cells often contained glutamates or aspartates in their β-chain junctions. The authors interpreted the results to indicate that these residues made direct contacts with position 102 of MCC. These parallels suggest the intriguing possibility that despite the usage of different V_α and V_β genes, TCRs specific for different peptides restricted by the same MHC molecule may have a largely similar interaction with the peptide-MHC complex.

Figure 2.1 Reactivity of 15-26-responders and non-responders to cI protein



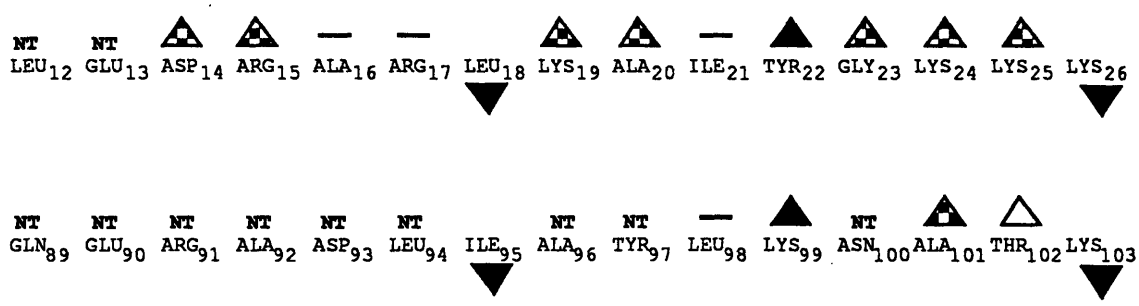
Comparison of the response of a representative 15-26-responder (cell 1.9) and three 15-26-non-responders to 12-26 peptide, (A) and cI protein (B).

Figure 2.2 Response of 12-26-specific T hybridomas to single-substituted analogs



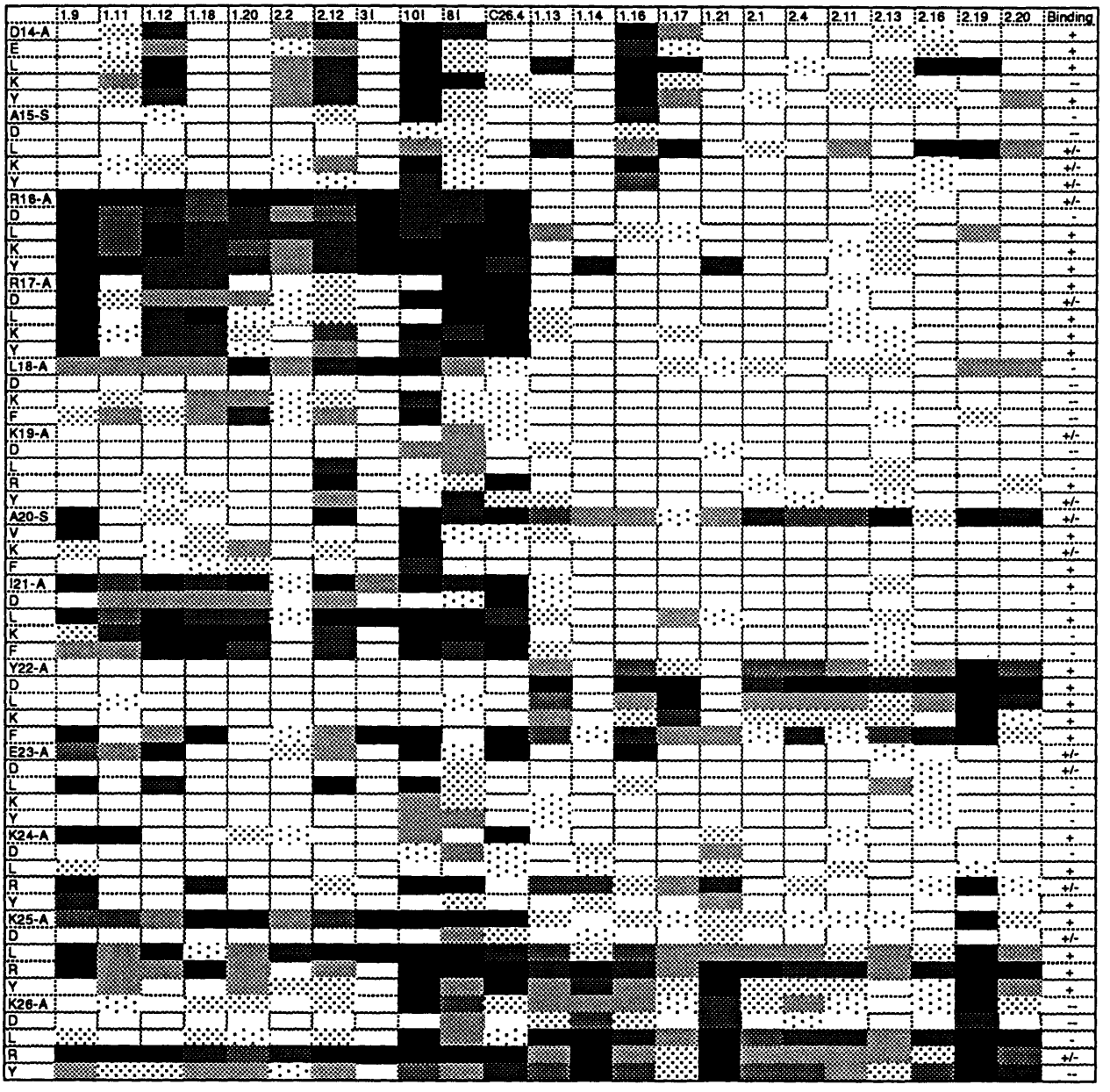
Responsiveness of T hybridomas to analogs of 12-26 containing the single substitutions indicated along the left side of the figure. Hybridomas were tested against serial 3-fold dillutions of peptides from an initial concentration of 900μ g/ml. Darkest motifs indicate the lowest concentration of peptide tested (3.7μ g/ml) induces a response of a given hybridoma which is greater than the half-maximal response to 12-26. Successively lighter motifs indicate 3-fold increasing concentrations of peptide are necessary to achieve half-maximal stimulation. Blank rectangles indicate half-maximal stimulation was not achieved at the highest concentrations tested. Results are the average of duplicate experiments.

Figure 2.3 Equivalent residues in two E^k-restricted peptides play similar roles in their interaction with the TCR



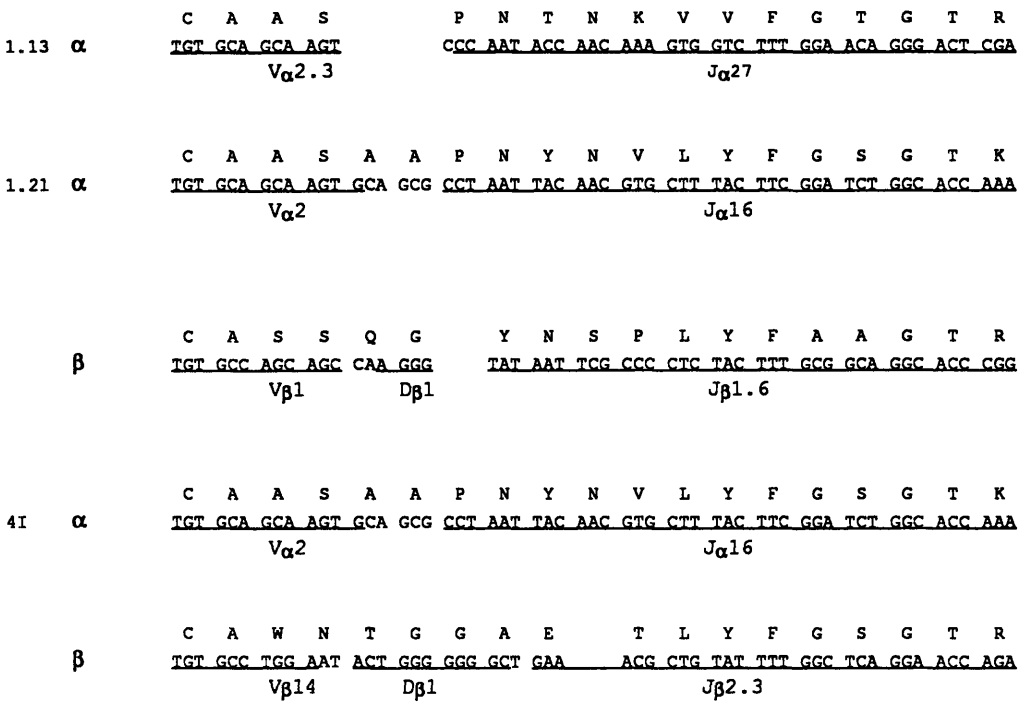
Comparison of the role of each residue in 12-26 in interacting with the TCR of 15-26-responder cells with the role of the equivalent residues in another E^k-restricted peptide, MCC 93-103. Roles of each residue in 12-26 are based on the results presented in Figure 2.2. The roles attributed to each residue in MCC 93-103 are based on the fine-specificity analysis two T hybridomas by (Fox, et al, 1987), using the classification system of (Jorgensen, et al, 1992). Alignment of the peptides is the same as in Figure 1.2. Probable MHC-contact residues are indicated by downward-pointing filled triangles. Upward-pointing filled triangles indicate residue where responsiveness of T cells is affected by conservative substitutions. Upward-pointing checked triangles indicated residues where responsiveness is affected by conservative replacements, but some non-conservative replacements are accepted. Upward-pointing empty triangles indicate residues where responsiveness is affected by non-conservative substitutions, and conservative changes have no effect. A dash above a residue indicates that even non-conservative substitutions generally have little effect. NT, not tested.

Figure 2.4 Comparison of competition binding results to fine-specificity data



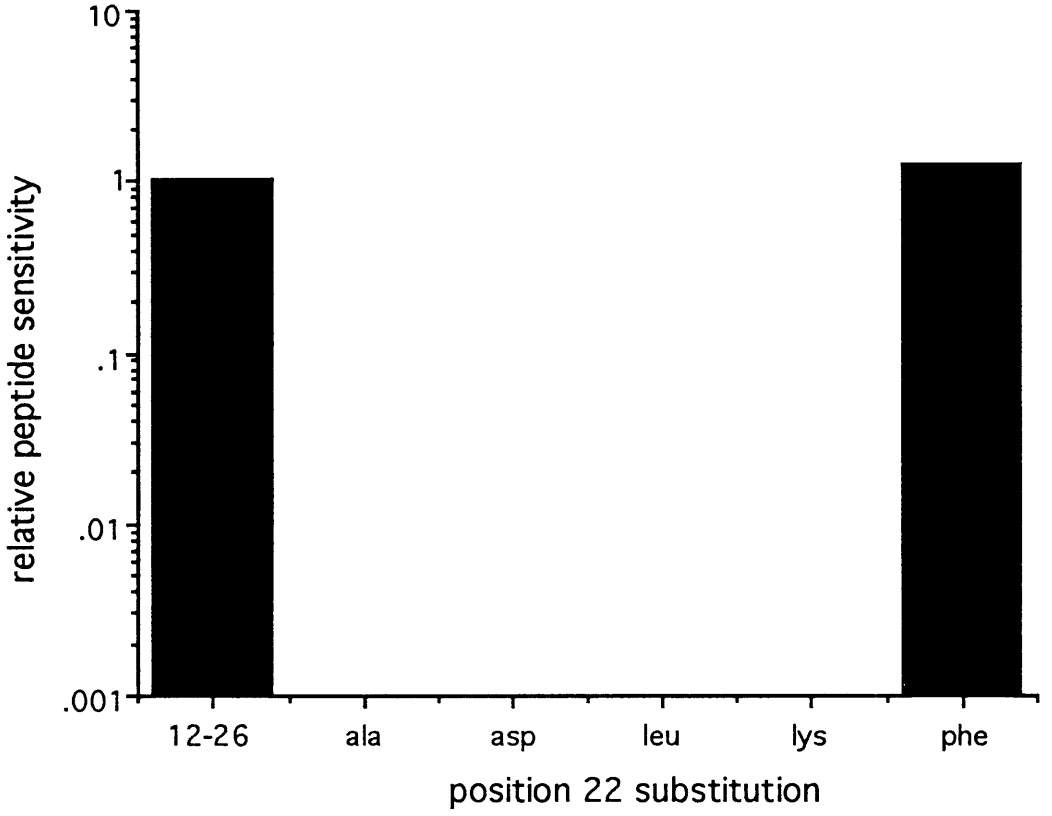
Comparison of fine-specificity experiments (Figure 2.2) to competition binding experiments from Table 1.1. Results of competition binding experiments are expressed as: +, changes which generally reduce the effectiveness of the peptide in the competition experiments by less than 4-fold; +/-, changes having an effect less than 10-fold; -; changes having an effect less than 100-fold; and --, changes having a greater than 100-fold effect.

Figure 2.5 Sequences of T cell receptor genes from 15-26-non-responders



α- and β-chain sequences of the TCRs from two 15-26-non-responders are shown. The previously reported sequences of cell 4I (Lai, et al, 1988) are shown for comparison. Spaces are introduced for alignment. Assignment of V elements is based on Wilson, et al 1988. Assignment of J elements is from Koop, et al, 1992. D element assignments are from Siu, et al, 1984.

Figure 2.6 Response of cell 4I to substitutions at position 22



Relative sensitivity of T hybridoma 4I to substitutions at 12-26 position 22. Half-maximal stimulatory concentration with un-substituted 12-26 is 0.3μ g/ml.

Figure 2.7 Fine-specificities at positions 22 and 25 of12-26-specific T cells bearing related β-chains

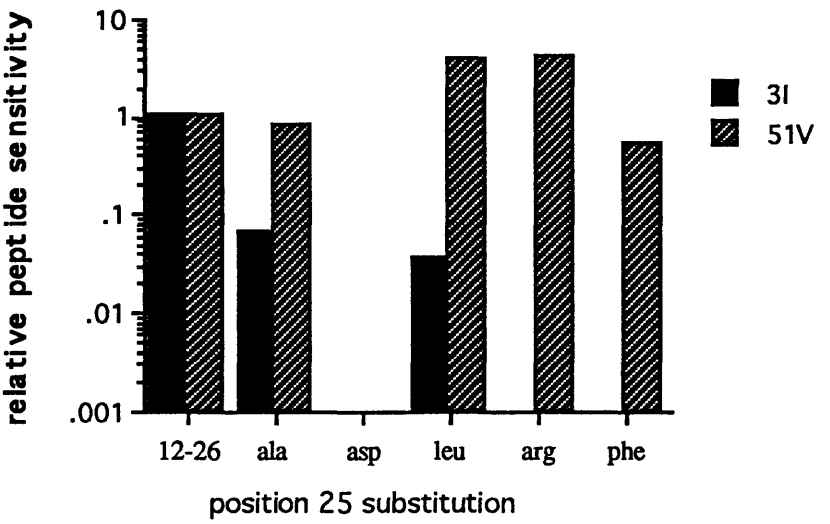
Cell	Vβ sequence	Response to 12-26(Phe22)	Response to 12-26(His22)	Response to 12-26(Arg25)/(Tyr25)
3I	C A S S Q **E** G G D Y A E Q F F TGT GCC AGC AGC CAA GAG GGG GGG GAC TAT GCT GAG CAG TTC TTC Vβ1 Jβ2.1	+	-	-
7II 24I	C A S S Q **E** T G D Y A E Q F F TGT GCC AGC AGC CAA GAG ACT GGG GAC TAT GCT GAG CAG TTC TTG Vβ1 Jβ2.1	+	-	NT
22I 26IV 31II	C A S S Q **E** T A N Y A E Q F F TGT GCC AGC AGC CAA GAA ACA GCA AAC TAT GCT GAG CAG TTC TTC Vβ1 Jβ2.1	+	-	NT
14I	C A S S Q **E** T I Q Y A E Q F F TGT GCC AGC AGC CAA GAG ACT ATA CAA TAT GCT GAG CAG TTC TTC Vβ1 Jβ2.1	+	-	NT
51V	C A S S Q G T F Q D T Q Y F TGT GCC AGC AGC CAA GGG ACT TTC CAA GAC ACC CAG TAC TTT Vβ1 Jβ2.5	+	+	+

Responsiveness of T cells bearing similar β-chains to aromatic substitutions at position 22, as originally reported by Lai, et al, 1990, and to arginine and tyrosine substitutions at position 25. T cells other than 51V do not respond to concentraions of P12-26(His22) 100-fold higher than that required for stimulation by P12-26. 51V is approximately equally responsive to 12-26 and to P12-26(His22), and approximately 4 times as responsive to P12-26(Arg25) as to P12-26. Conserved glutamate residue at position 100 of the β-chain junction is indicated in bold. The limits of V elements on the left and J elements on the right are based on the published germline sequences (Wilson, et al, 1988; Koop, et al, 1992) and are underlined.

Figure 2.8 Fine-specificities at position 22 of12-26-specific T cells bearing related α-chains

Cell	Vα sequence	Response to 12-26(Phe22)	Response to 12-26(His22)
3I	C A A S A G T G S K L S F G	+	−
7II	TGT GCA GCA AGT GCA GGC ACT GGG TCT AAG CTG TCA TTT GGG		
22I			
26IV			
31II			
51V			
24I	C A A S G T G S K L S F G	−	−
	TGT GCA GCA AGT GGC ACT GGG TCT AAG CTG TCA TTT GGG		

Figure 2.9 Relative sensitivity of cells 3I and 51V to position 25 substitutions



T cell 51V and 3I were tested for their sensitivities to substitutions at position 25. Results are expressed as the inverse of the concentration of each substituted peptide required for half-maximal stimulation, normalized with respect to the sensitivity of each cell to 12-26 containing no substitutions. Half-maximal stimulatory concentration by 12-26 was 0.2μg/ml for cell 3I, and 0.8μg/ml for 51V.

Table 2.1 Peptide specificity of hybridomas produced by immunization with 12-26 or cI protein

Immunogen	Number of hybrids screened	Response to 12-26	Response to 15-26
cI	27	13	13
12-26	45	45	25

Lymph node cells taken 7 days after immunisation with antigen from CBA (k haplotype) mice were fused with the thymoma BW5147$\alpha^-\beta^-$ (White, 1989) Hybridomas responsive to the immunogen were screened against peptide 12-26 and the truncated peptide 15-26. Cells classified as non-responsive to 15-26 did not respond to concentrations as high as 50μg/ml peptide.

Table 2.2 Peptide specificity of T hybridomas

Hybridoma	Immunogen	Sensitivity to 12-26	Sensitivity to 15-26
1.9	12-26	0.3µg/ml	0.2µg/ml
1.10	12-26	0.08	0.05
1.11	12-26	0.6	0.5
1.12	12-26	0.3	0.5
1.13	12-26	1	---
1.14	12-26	1	---
1.16	12-26	1	---
1.17	12-26	2	---
1.18	12-26	0.2	0.4
1.20	12-26	0.1	0.1
1.21	12-26	0.5	---
2.1	12-26	2	---
2.2	12-26	1	0.5
2.4	12-26	0.8	---
2.11	12-26	2	---
2.12	12-26	.06	0.05
2.13	12-26	2	---
2.16	12-26	2	---
2.19	12-26	0.4	---
2.20	12-26	2	---
C26.4	12-26	.2	.2
3I	cI	.2	.1
8I	cI	.1	.1
10I	cI	.1	.07

Table 2.3 T cell receptor gene usage among 12-26-specific T cells

Group	Cell	V_α	V_β
15-26-responders	3I, 1.9, 1.20, 2.12	$V_\alpha 2$	$V_\beta 1$
	8 1	$V_\alpha 2$	$V_\beta 6$
	10 I	$V_\alpha 2$	V_β 8.3
15-26-non-responders	1.13, 1.16, 1.21, 2.13, 2.16, 2.20	$V_\alpha 2$	$V_\beta 1$

TCR gene usage was determined by PCR analysis as described by Lai, et al, 1990. The TCR sequences of cells 3I, 8I and 10I were previously reported (Lai, et al 1990; Lai, et al 1988). PCR was performed in the laboratory of Ming-Zong Lai, Insitute of Molecular Biology, Taipei, Taiwan.

Chapter 3

Abstract

A variety of evidence suggests that residues outside a T cell epitope play a role in its antigenicity by affecting the efficiency with which it is processed from a protein by antigen presenting cells. In particular, the flanking residues have been hypothesized to contain target sites for proteases. A number of proteases, in particular, members of the cathepsin family, have been implicated in this process. Several types of indirect evidence have been produced to support this idea: 1) Treatment of a protein with one of these enzymes leads to the production of peptides that can be recognized by T cells without further processing, 2) Treatment of APC with inhibitors of various proteases reduces the efficiency of antigen presentation, and 3) Proteases have been identified in the intracellular compartments where processing is believed to be carried out. Nevertheless, no enzyme or set of enzymes has been demonstrated to be essential for processing for all different epitopes, and it has not been possible to identify consensus sequences in proteins that might be the sites recognized by proteases. In order to test the hypothesis that such consensus

sequences exist flanking T cell epitopes and to characterize them if they could be localized, we developed a system to screen cI variants containing substitutions in the flanking regions of the immunodominant epitope located between residues 12-26 for substitutions that would affect processing. We find that the wild-type amino acids can be replaced with significantly different sequences for several residues on either side of the minimal epitope without a decrease in the efficiency of processing. This result suggests that the immediately flanking residues do not direct the processing of 12-26. By contrast, two types of mutations were found during the course of the study which did appear to alter the processing of the protein. One, the substitution of an alanine at position 20 with either valine or glycine apparently decreased the efficiency of processing of 12-26. The other, a substitution in the C-terminal flanking region of 12-26 led to an *increased* efficiency of processing of both 12-26 and a second epitope within the protein. These observations are compatible with two alternative hypotheses, 1) That processing is controlled by motifs lying internal to the T cell epitope itself, or 2) That the mechanism of processing involves an initial cleavage occuring distant to the epitope followed by a trimming step to produce the mature peptide which binds MHC.

Introduction

As we have previously mentioned, T cells, unlike B cells, do not recognize protein antigens in their native conformation. For T cell stimulation to occur, the protein must first be processed by antigen presenting cells and the resulting fragments then appear on their surface in association with a molecule of the Major Histocompatibility Complex. This processing has not yet been well characterized, but clearly involves proteolytic degradation as peptides have been shown to substitute for intact protein antigen, and some of the actual peptides bound by MHC molecules have been purified and sequenced (Falk et al., 1991; Jardetzky et al., 1991; Rudensky et al., 1991). This work has led to the identification of peptide motifs which confer specificity of binding to different MHC molecules. For a sequence within a protein to become a T cell epitope, it must therefore be capable of binding to MHC. It is not clear, however, whether such a condition is sufficient. Processing may also play an important role in the selection of some epitopes and the loss of potential ones. Indeed, a great deal of evidence has accumulated indicating that this the case. Substitutions in proteins and peptides outside of class II-restricted T cell epitopes have occassionally been shown to alter the response to the epitopes (Gammon, et al., 1987; Liu, et al., 1991; Shastri, et al., 1986;

Vacchio, et al., 1989). Furthermore, treatment of antigen presenting cells with inhibitors of various proteases has been shown to modify the presentation of class II-restricted epitopes (Diment, 1990; Takahashi et al., 1989; Vidard et al., 1991). Most recently the presentation of a class I-restricted epitope has been shown to vary according to the site of its insertion into a heterologous protein (Del Val, et al., 1991). These data suggest that in both the class I and class II antigen presentation pathways particular structural features within a protein are necessary for proper processing and presentation. Such features may be local, for example specific sequences flanking the epitope necessary for its proper excision by proteases or may relate to the position of the antigenic sequence within the global structure of the protein. In the present work we describe a test of the hypothesis that flanking residues contain specific sequences that direct the processing of class II restricted epitopes.

The 12-26 epitope of cI is advantageous for this study for two reasons. First, this sequence is immunodominant in two disparate haplotypes (d and k) (Lai et al., 1987), which may be consequence of a conserved cellular processing machinery. Second, a system of cassette mutagenesis has been developed which permits the facile modification of the cI protein (Reidhaar-Olson and Sauer, 1988) and an exhaustive study has been conducted to determine the structural and functional consequences of substitutions at various positions (Reidhaar-Olson & Sauer, 1990), thus, the effects on the cI protein of many substitutions are already known.

Our assumption in conducting these experiments is that if there are specific sequences in the flanking residues of 12-26 which direct its processing, they should be relatively restrictive in terms of the number and types of substitutions they will accept. Most substitutions introduced into such sites should therefore lead to their destruction, and consequently, a diminished efficiency of processing of the 12-26 epitope. Using the cassette mutagenesis system described above and adding to it a simple method for screening the T cell response to the cI protein expressed in E. coli we have measured the effect of substitutions in the regions flanking the 12-26 epitope. This approach would provide an additional advantage in the event where a processing site could be localized. In this case, further mutagenesis of the identified sequence could be used to precisely characterize the motif recognized.

The results indicate that a wide variety of different amino acids can be substituted in the positions flanking 12-26 without significantly decreasing the effectiveness of processing. This indicates that the immediately flanking residues are unlikely to contain precisely defined sequences that are recognized by putative processing enzymes. Consequently, these data favor a model of processing where initial proteolytic cleavage of a protein occurs relatively distantly to the epitope, followed by trimming to produce the mature peptide which is bound by MHC, or a model whereby the processing of the epitope is controlled by sequences lying within the epitope itself. This second possibility is suggested by the identification during the course of the study of two proteins whose processing did differ from wild-type cI. Both proteins contained

substitutions for the wild-type alanine at position 20, in one case to glycine, and in the other to valine. These substitutions led to a decreased efficiency of processing of 12-26 relative to another epitope in the protein.

A second mutant whose processing was altered compared to the wild-type protein contained several substitutions at residues 24-27 which appears to lead to an increased efficiency of processing of at least two epitopes in cI. These mutants represent potential tools for the further analysis of antigen processing.

Results

The peptide corresponding to residues 12-26 of cI has been shown to stimulate T cells specific for cI in two different haplotypes (d and k). The "natural" peptide actually bound to the MHC molecule is not known however. Thus, in order to modify the residues flanking the epitope, the minimal peptide capable of stimulating two I-A^d-restricted T cells (denoted 24.4 and 26.2) was determined. As shown in Figure 3.1, 24.4 responds well to a peptide corresponding to residues 12-23, but the response is significantly diminished by the loss of either residue 12 (P13-24), or residue 23, (P12-22). Cell 26.2 has a slightly different minimal peptide requirement. It responds approximately ten-fold less well to P12-23 than to P12-26. Both cells therefore require residue 12 for maximal stimulation, although they differ in their C-terminal requirements. Peptide 12-23 appears to be capable of binding class II MHC because it maximally stimulates cell 24.4. The length of this peptide is slightly shorter than the size of peptides (13-17aa) that have been eluted from class II molecules (Rudensky et al., 1991). Residues which may influence processing should therefore be predicted to lie outside this sequence.

If a motif required for processing exists bordering this epitope, its length and stringency at each position is not known. We therefore developed a technique to permit the rapid screening of different amino acid substitutions in the several residues adjacent to residues 12-23. Our goal was to derive a method to screen a

large number of protein mutants with a minimal level of purification in order to identify likely candidates for processing mutants that could subsequently be purified and studied in greater detail. Furthermore, such a technique would aid in the characterization of a processing site, in the event where one could be localized. First, a test was conducted to determine whether lysates from bacteria expressing cI could be directly assayed for the presence of the protein without the need for purification. Such lysates might, for example, contain either toxins or factors inducing non-specific stimulation of T cells that would interfere with a screen. E. coli carrying a plasmid for the expression of cI were grown in liquid culture, induced to express the protein and a crude lysate was prepared from the cells (See *Materials and Methods* for details). T cells in the presence of antigen presenting cells were then tested for stimulation by various dilutions of this lysate. Figure 3.2 shows that a crude lysate prepared in this manner can specifically stimulate both 26.2 and 24.4. At the highest concentrations, roughly 10% and above, the lysates are indeed toxic. At roughly 5%, however, maximal stimulation occurs. Neither cell is stimulated significantly by a lysate prepared from bacteria which does not express cI. Furthermore, based upon the extent to which the lysate can be diluted before stimulation drops to background levels, a 5% lysate contains approximately 10-20 times the amount of protein necessary for detectable stimulation (Figure 3.2). This therefore suggested that E. coli lysates containing cI mutants could be directly screened for stimulation of T cells without the need to purify the proteins individually.

A^d-restricted T cells have been found using peptides to be sensitive to substitutions at position 20 of 12-26 (A.S., unpublished). This observation was used to confirm that lysates could be screened for mutations in cI that lead to a decreased stimulation of 12-26-specific T cells. Random cassette mutagenesis was employed to create a bank of cI mutants containing various substitutions at position 20. Crude lysates from bacteria expressing these proteins were prepared as above and tested directly for the stimulation of T cell 26.2. The level of protein expression was confirmed by SDS gel and found to be similar for the mutants and wild-type cI (not shown). After DNA sequencing to determine the amino acid substitution at position 20, the results were compared to the stimulation induced by peptides containing the same substitutions. Figure 3.3 shows the stimulation by 5% lysates containing various cI mutants to the relative stimulation by peptides containing the same substitutions. Unexpectedly, there are significant differences between the results obtained. Peptides with two of the substitutions, glycine and valine, stimulate 26.2, while the corresponding proteins do not. The response to the former may reflect the limits of the screening process in that the same substitution in the peptide leads to a response which is diminished compared to P12-26. Significantly however, two different proteins containing valine substitutions were found not to stimulate, while the same substitution in the peptide does not diminish the response. This difference between the response to the protein and the peptide containing the same substitution could indicate the identification of proteins whose processing is modified. One report exists in the

literature of a mutation within the epitope which diminishes the efficiency of processing (Finnegan and Amburgey, 1989).

To investigate this further, these proteins and the wild-type were purified. While the response of 26.2 to 12-26 and 12-26(V20) is essentially the same (Figure 3.4A), the response to the purified protein containing the valine substitution is diminished approximately 50-fold compared to the wild-type (Figure 3.4B). To demonstrate that this diminished response is particular to the 12-26 epitope, the response of a second cell specific for an epitope located between residues 46-62 was also determined. As can be observed (Figure 3.4B), this cell responds equally well to the valine mutant as to the wild-type.

Based on the results presented in Figure 3.3, the mutant containing glycine at position 20 may also be processed less efficiently than wild-type. This is difficult to evaluate with cell 26.2, however, because it also responds less well to the peptide containing the glycine substitution. Cell 24.4 also responds poorly to this peptide. An E^k-restricted cell, C26.4, has been identified which responds approximately equally to P12-26(G20) and P12-26 (Figure 3.5A). This cell and A128 were tested against cI(G20). The results are displayed in Figure 3.5B. As was the case for cell 26.2 with cI(V20), cI(G20) has greatly reduced stimulatory activity for C26.4, despite the fact that the peptide containing the same substitution stimulates comparably, if not better than P12-26. Again, A128 responds equally well to the two proteins. These results, therefore, strongly suggest that these two substitutions alter the processing of the 12-26 epitope from cI protein.

Furthermore, it is significant that mutations at the same position appear to modify the processing of cI as measured by T cells of different haplotype restrictions (A^d and E^k). This implies that processing occurs by a mechanism independent of the interaction of the peptide with MHC.

Several alternative explanations for the results observed were considered, and experiments were conducted to eliminate them. First, the effects of the substitutions at position 20 are unlikely to be due to a degradation of the protein unrelated to antigen processing. The mutant and wild-type proteins elute from ion exchange columns at the same salt concentration during purification indicating that the structural and biochemical properties of the mutant are not significantly altered. Furthermore, cI has been the subject of an exhaustive mutagenic study (Reidhaar-Olson & Sauer, 1990). Position 20 has been found to accept a variety of amino acid substitutions, among them glycine, without impairment of its function as a repressor of λ-phage. We have confirmed this by testing the ability of the mutant cI proteins to protect E. coli from lysis by a series of λ-phage mutants varying in lysogenicity (Hecht and Sauer, 1985). The effectiveness of the mutants is indistinguishable from wild-type in this test (not shown). Finally, the difference between the behavior of the wild-type and mutants is not due to an error that might have intervened during the process of cloning and sequencing. The proteins themselves have been sequenced and contain no other differences from wild-type within the first 26 amino acids. We conclude that the effect seen is most likely due to a reduced efficiency of processing of the 12-26 epitope

in the mutant protein. Experiments aimed at determining the means by which these mutations affect the processing of the 12-26 epitope will be described below.

The identification of mutations affecting processing at position 20 of cI suggests the possibility that processing is controlled by motifs lying within the epitope. For example, the effect of the substitutions may be to destroy a sequence which is essential for the correct processing of the 12-26 epitope. Alternatively, substitutions may lead to the creation of a processing site which would cause the cleavage of the protein during processing and the consequent loss of the epitope. In the latter case, the normal processing of the epitope might still be controlled by the flanking residues. In order to evaluate this second possibility, we applied the same technique to screen the protein for mutations at positions N-terminal to the epitope that might affect processing. As the leucine at position 12 had been found to be essential for both cells 24.4 and 26.2, the effect of the four residues preceding this position was evaluated. In this case the mutagenesis was performed at pairs of positions, that is, substitutions were introduced at either positions 8 and 9, or 10 and 11. Lysates from bacteria expressing the mutant proteins were prepared and tested for the stimulation of cell 26.2. Table 3.1 gives the relative stimulation induced by 20 such mutants, along with the changes that have been made in the protein. In contrast to what was observed for mutations at position 20, although a wide variety of different amino acids have been introduced at positions 8-11, the majority of the mutants stimulate comparably to the wild-type protein. At positions 8 and

9, replacement of the the wild-type threonine-glutamine with pairs of amino acids as disparate as glycine-arginine, histidine-glycine or proline-proline does not seem to prevent presentation of the epitope. Similarly, at positions 10 and 11 a wide variety of replacement amino acids is allowed. If indeed a motif controlling processing is present in the wild-type sequence bordering the epitope one would expect the majority of these sequences not to conform to it and thus to impair proper processing. Initial comparison of this result, therefore, contrasts significantly with that obtained by mutagenesis at position 20, where dramatic differences occurred depending upon the substitution. This suggests that no such motif exists at these positions.

The range of acceptable amino acid substitutions at positions 8-11 which maintain the functional integrity of cI has previously been studied by the group of R.S. Sauer (Reidhaar-Olson & Sauer, 1990). Table 2 also includes the effect of the mutations we have introduced on the structure of cI based on this work. As is the case with the primary structure in the residues flanking the epitope, no correlation can be drawn between the effectiveness of presentation and whether a given set of substitutions affects the overall structure of the protein.

To eliminate the possibility that these results are the consequence of a lack of sensitivity of the screening technique, a number of the most likely candidates for processing mutants were purified and tested for stimulation of T cells 26.2 and A128. By comparing the dose-response curves for stimulation of these cells by the various mutants, we hoped to identify more subtle

differences in processing that may have escaped our initial screen. Among the proteins purified and tested were mutants number 14 and 16 which had been among the weakest stimulators in the screen. As can be seen in Figure 3.6 some variations do occur in the stimulation 26.2 by the various mutants. These are generally small, however, roughly a factor of 4 for the most significant effect. As these effects are small compared to those observed for changes at position 20, we conclude that no sequence motif necessary for processing exists in the N-terminal region immediately bordering the epitope.

A similar screen was carried out among proteins mutated in the C-terminal flanking region of the epitope, using a slightly different procedure. As the T cells 24.4 and 26.2 had been found to have different minimal peptide requirements, mutagenesis was carried out for positions 24-27 and the proteins produced were simultaneously screened for stimulation of both T cells. In this way the differential responsiveness of the two cells to substitutions at position 24 could be expected to serve as an internal control for the sensitivity of the screen. In this case all four residues were simultaneously randomized. Table 3.2 shows the relative stimulation in such a screen of 24.4 and 26.2 by 27 such mutants and the substitutions they contain. As was the case for the mutants at positions 8-11, the differences in the stimulation of the T cells are relatively minor and proteins containing vastly different substitutions stimulate one or both T cells essentially as well as wild-type. As expected, in some cases one T cell was stimulated significantly more stongly than another by a given mutant (for

example, mutants 7 and 12). Based on studies using peptides substituted at position 24, this can be accounted for by differences in the acceptance of replacement amino acids at this position by the two T cells (not shown). 26.2 does not respond to a peptide containing aspartate at position 24, while 24.4 does at nearly the same level as to the wild-type peptide. That similar results are obtained for the protein indicates that the naturally processed peptide probably includes residue 24.

Once again, several of the most likely candidates for processing mutants were purified and tested in parallel for stimulation of 26.2 and A128. As shown in Figure 3.7, all of the proteins except mutant 20 stimulate both 26.2 and A128 similarly when purified protein is used. These results, then, combined with those obtained with proteins substituted at positions 8-11 lead us to conclude that no strict motif that controls the processing of this epitope is located in either the N-terminal or C-terminal flanking residues. Interestingly, however, mutant 20 stimulates 26.2 roughly 5-fold better than wild-type protein. This effect is not likely to be due to an altered binding of the epitope to MHC or T cell receptor because the stimulation of A128 is similarly increased. Rather, it appears to be due to a modification of the processing of the protein as a whole.

Discussion

Antigen processing could occur by one of several conceivable mechanisms: 1) It could, for example, be a random process whereby non-specific proteases cut the protein with no sequence specificity whatever. Such a mechanism would require that the concentration of the enzymes are dosed so as to prevent the total digestion of the protein. Furthermore, the concentration of any particular peptide produced would be extremely low, which would have the ultimate consequence of limiting the sensitivity of the immune response to the protein. It would also be in contradiction with observations that cellular processing compartments contain proteases with sequence specificity (Guagliardi et al., 1990), and that specific inhibitors of proteases prevent the presentation of certain epitopes (Diment, 1990; Takahashi et al., 1989; Vidard et al., 1991). A second possibility has been suggested by the work of Allen and colleagues (Donermeyer and Allen, 1989), in which it was demonstrated that binding of a denatured protein to MHC protects the bound portion from digestion by a protease (chymotrypsin). In a model based on this observation, one can imagine processing proceeding by the denaturation of the protein under the acidic conditions of the endosome, followed by binding of certain segments to MHC, followed by trimming of all parts not protected by MHC, again by non-specific proteases. This model would be compatible with the idea that sequences within the epitope itself direct processing. However, it would not be compatible with our

identification of mutations at position 20 that alter processing of cI, because in this case the fact that the peptide containing the same substitution is presented indicates that this substitution does not affect binding to MHC. In a third model, protein antigens could first be digested by specific proteases into relatively long peptides, followed by binding to MHC and subsequent trimming by non-specific proteases to produce mature peptides. This model would be compatible with the existence of specific proteases in putative processing compartments (Guagliardi et al., 1990), could explain why specific inhibitors of proteases can block presentation of some epitopes (Diment, 1990; Takahashi et al., 1989; Vidard et al., 1991), and would be in agreement with the observation that peptides eluted from MHC class II proteins have different C-terminii indicating a trimming step has occurred (Falk et al., 1991; Jardetzky et al., 1991; Rudensky et al., 1991). Finally, in a fourth model one can envision that the flanking residues of T cell epitopes contain specific sequence motifs which are recognized by processing enzymes. This model would explain the observation that certain protein antigens digested *in vitro* by proteases implicated in processing (the cathepsins B and D) can stimulate T cells without the need for further processing (Takahashi et al., 1989; Van Noort, et al., 1991).

The present work was conducted to test the fourth hypothesis described above. In order to do so we have introduced random amino acid substitutions for several residues on either side of a minimal T cell epitope and have found that the presentation of the epitope remains comparable to that of the wild-type. We conclude,

therefore, that in the case of the 12-26 epitope proper processing is not determined by the immediately flanking residues.

This conclusion is based on several assumptions. First, if specific sites recognized by proteases do exist flanking T cell epitopes, they should have relatively restrictive sequence motifs. Therefore, the majority of substitutions introduced into these sites should lead to their destruction, and a consequent reduction in the processing of the epitope. We find that a large variety of different amino acids can be substituted at the several positions flanking an epitope and the epitope is still presented effectively. An alternative approach has been used by another group to study processing of a class I restricted epitope (Del Val et al., 1991). These workers inserted an epitope at different sites in a heterologous protein, and found that the efficiency of processing did indeed depend upon the site of insertion. This approach suffers from the limitation that it may be difficult to distinguish the effect of the protein environment on the fate (processing) of the epitope from the effect of the insertion of the epitope on the fate of the protein i.e. degradation, compartmentalization, etc. It was for this reason that we chose the approach of modifying only the surrounding residues, while keeping the overall protein environment the same. The different conclusions obtained by these authors and ourselves may reflect the use of the different approaches chosen, or, more probably, the different mechanisms of class I and class II processing.

A second assumption is that the sequences recognized by processing enzymes are located close to the epitope. The criticism

might be made that mutagenesis was not performed far enough from the epitope in order to identify processing motifs. However, as the length of peptides eluted from class II molecules has been between 13-17 amino acids (Kropshofer et al., 1992; Rudensky et al., 1992; Rudensky et al., 1991), the 20 amino stretch (residues 8-11 and 24-27) examined here should have identified them. Therefore, these results argue against processing occuring only by specific cleavage of protein antigens without subsequent trimming.

Finally, our conclusion is based upon the assumption that the effects of mutations that interfere with processing would cause a significant decrease in the stimulation of T cells. The initial screen employed using lysates of bacteria expressing variants of cI probably would not have been able to detect small effects on the efficiency of processing. However, a number of the proteins giving the lowest stimulation in the initial screen were purified and found to stimulate comparably to the wild-type protein. Some small effects on the response of T cells to these proteins were observed (on the order of a factor of 4). However in a number of cases, residues in a peptide outside a minimal epitope can be shown to affect the response of T cells. For example, hybridoma 24.4 does not require residue 24 for maximal stimulation, but responds significantly less well to a peptide containing a aspartate substitution at this position (not shown). Furthermore, the fact that the two mutants containing substitutions at position 20 were identified, suggests that the system is sensitive enough to detect mutations that do cause veritable reductions in processing efficiency. The effects of these two mutations, an apparent

reduction in processing efficiency of 20-50-fold, demonstrates that these effects are significant.

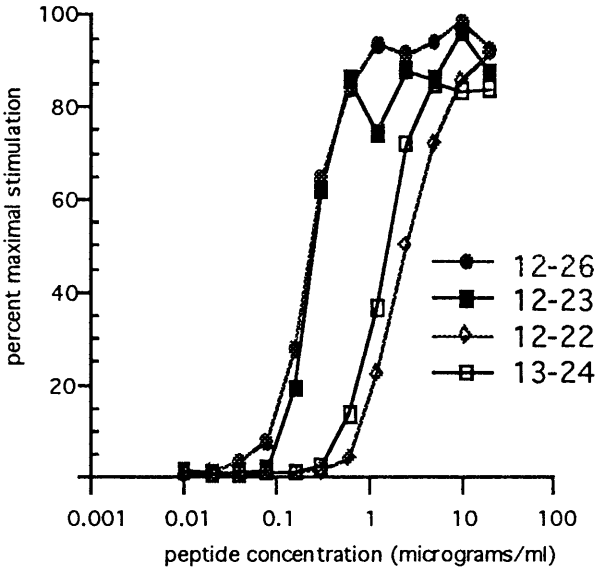
A surprising finding was the identification of these two proteins from which the 12-26 epitope is apparently processed less efficiently than from the wild-type protein. Both of these proteins contain changes at position 20 of cI, in one case from alanine to valine, and in the other to glycine. This conclusion is based upon the observation that peptides containing the identical substitutions stimulate T cells comparably to 12-26. These proteins represent useful tools for the characterization of the mechanism of processing.

If processing occurs by the recognition of specific sequences, these mutations would be presumed to modify processing either creating or destroying a cleavage site. A number of attempts have been made to identify such sites, both by sequence comparison and by experiment. The replacement of the alanine at position 20 with either a glycine or valine neither creates nor destroys an obvious site for either Cathepsin B, or Cathespsin D, the two enzymes most commonly implicated in processing. Cathepsin D cleaves preferentially between the amino acid pairs phe-phe, phe-tyr or leu-phe (Offerman, et al., 1983). The role of Cathepsin B in antigen processing is implied both by its presence in endosomes (Guagliardi et al., 1990), and the demonstration that myoglobin digested with the enzyme can be presented to different T cells without the need for further processing (Takahashi et al., 1989). It appears to cleave after an arginine (Bond & Butler, 1987), but has also been shown to have a peptidyldipeptidase activity, cleaving dipeptides from the C-

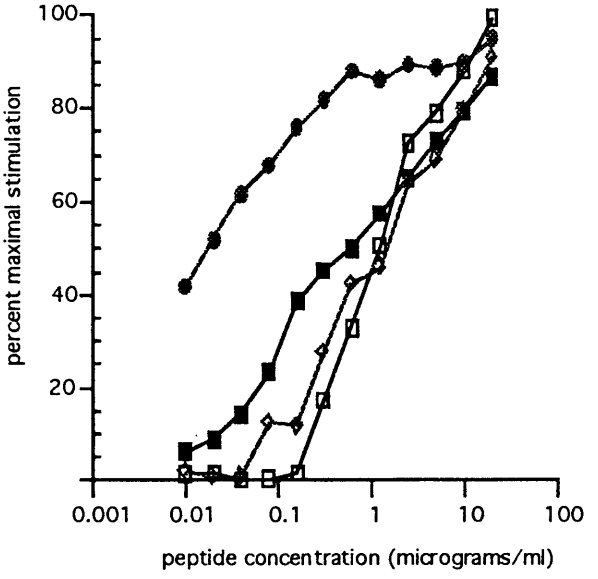
terminus (Aronson and Barrett, 1978; Bond and Barrett, 1980). A number of experiments have been conducted to evaluate the possibility that either Cathepsin B or D cleaves the wild-type and position 20 mutants differently. To date we have been unable to detect differences in the pattern of peptide fragments produced by either of these enzymes using SDS-PAGE. More recently, a third enzyme, Cathepsin E, has been shown by the use of specific inhibitors to be essential for the processing of ovalbumin (Bennett et al., 1992). Experiments are currently planned to test the possibility that the position 20 variants are differentially cleaved by this enzyme.

Figure 3.1

A

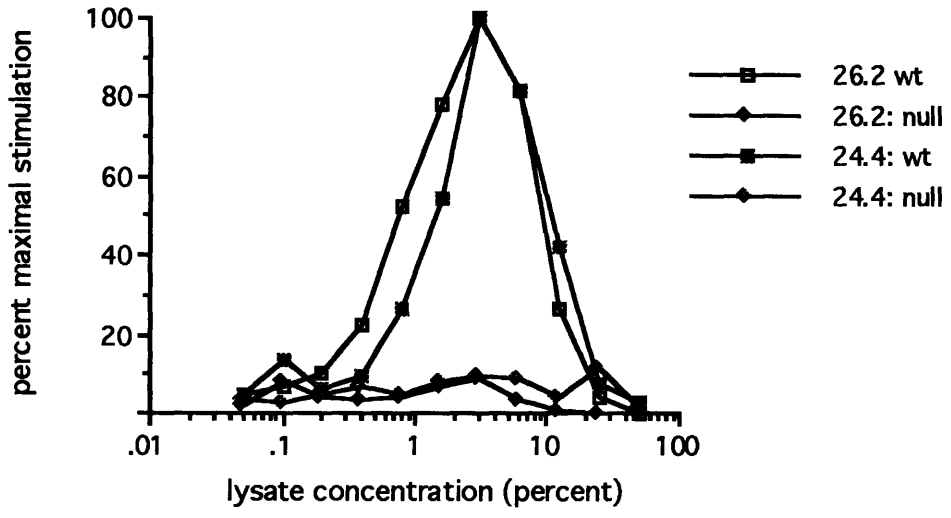


B



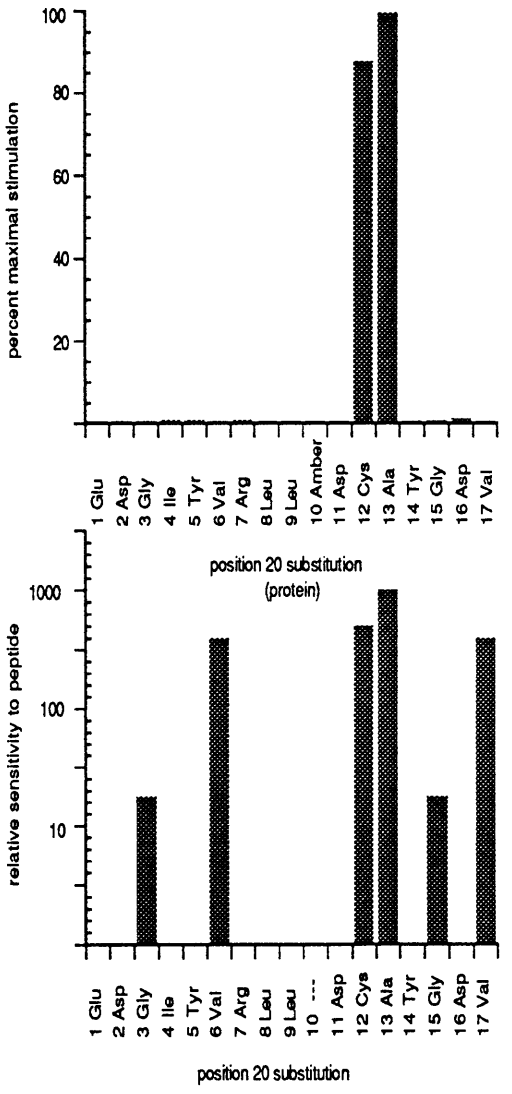
Response of cells 24.4 (A) and 26.2 (B)
to 12-26 and truncated analogs.

Figure 3.2



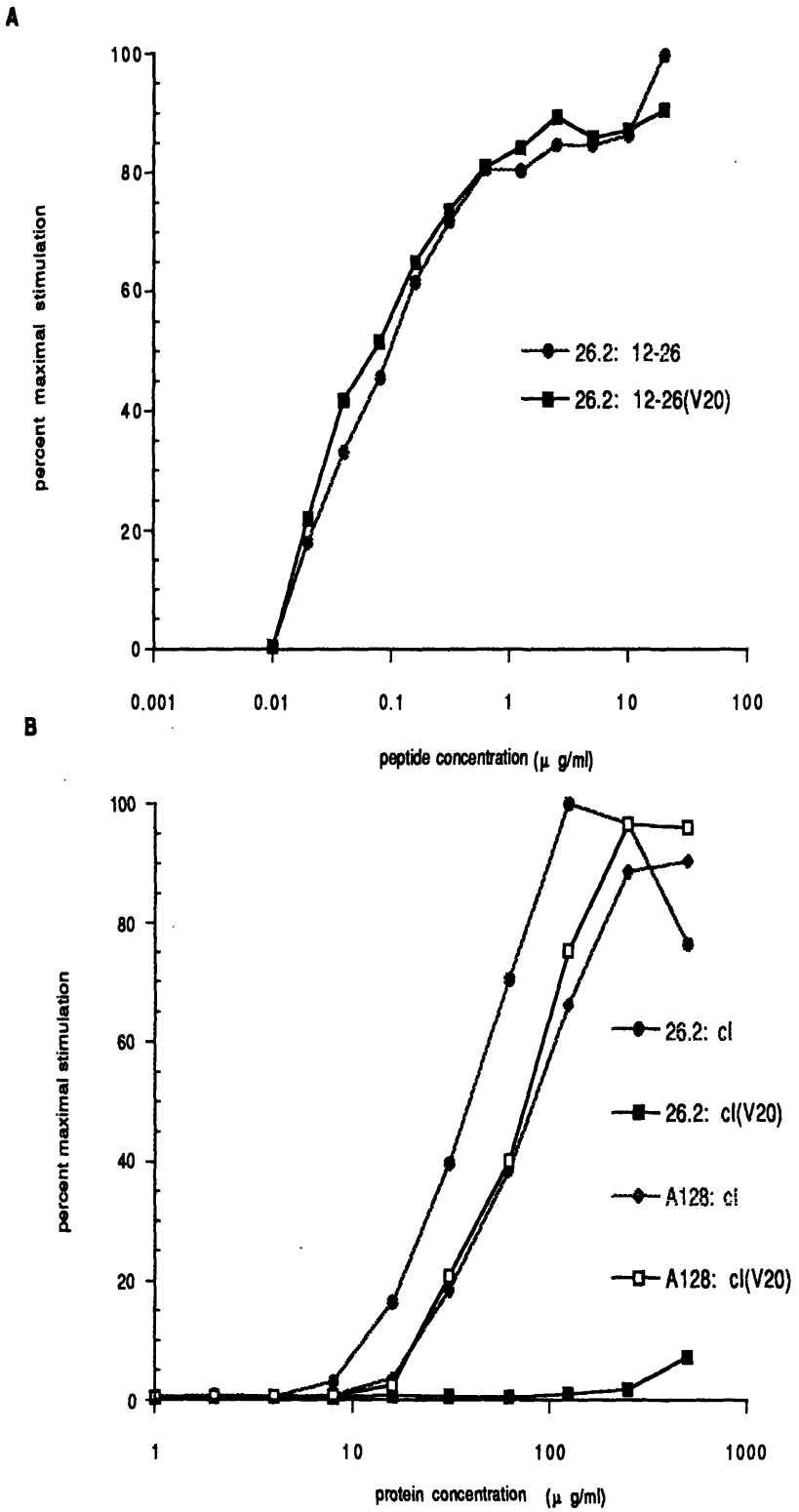
Response of cells 26.2 and 24.4 to serial dilutions of lysates of E. coli expressing wild-type cI (squares), or not expressing cI (diamonds). See *Materials and Methods* for lysate preparation.

Figure 3.3



Response of cell 26.2 in screen of E. coli lysates from bacteria expressing cI mutants containing substitutions at position 20. Relative sensitivity of the same cell to 12-26 analogs containing the same substitutions is shown below.

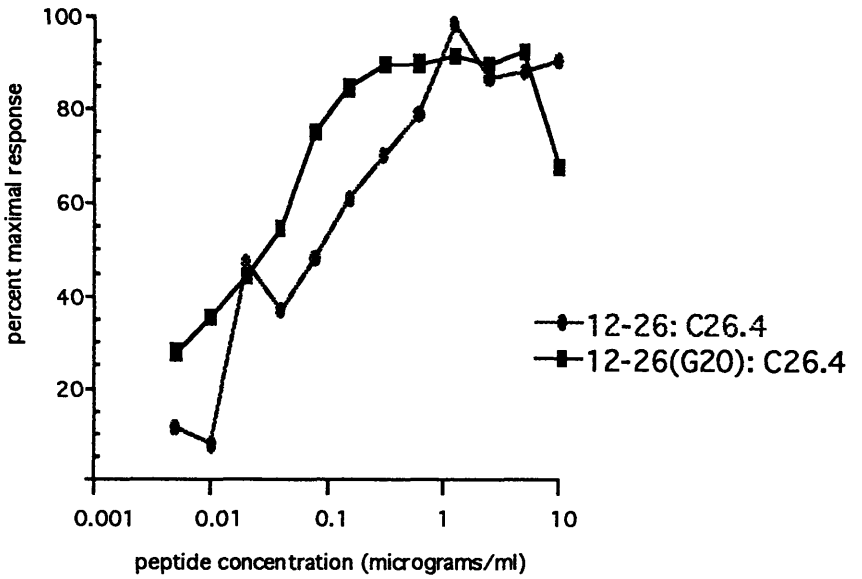
Figure 3.4



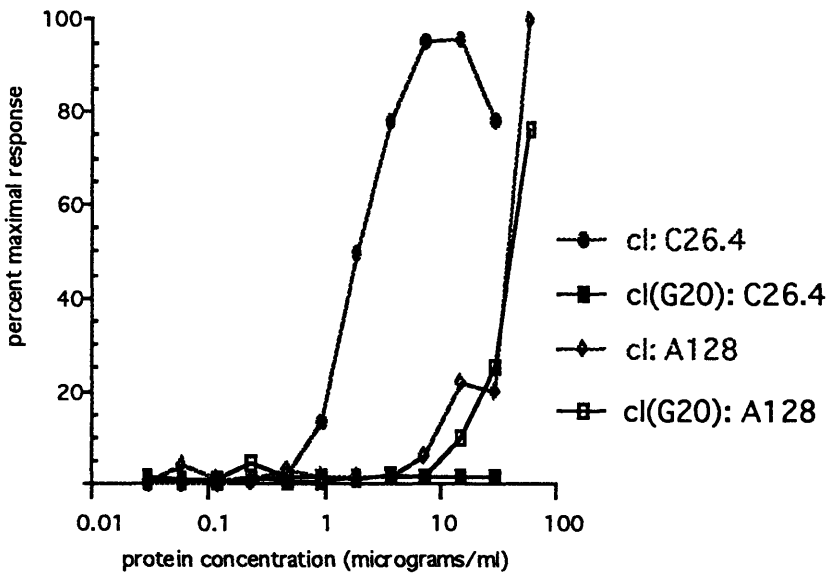
Response of hybridoma 26.2 to 12-26 and 12-26 containing a valine substitution at position 20 (A) and to cI protein and cI protein containing the same substitution (B). Also shown is the response hybridoma A128 specific for cI residues 46-62 to the same proteins.

Figure 3.5

A

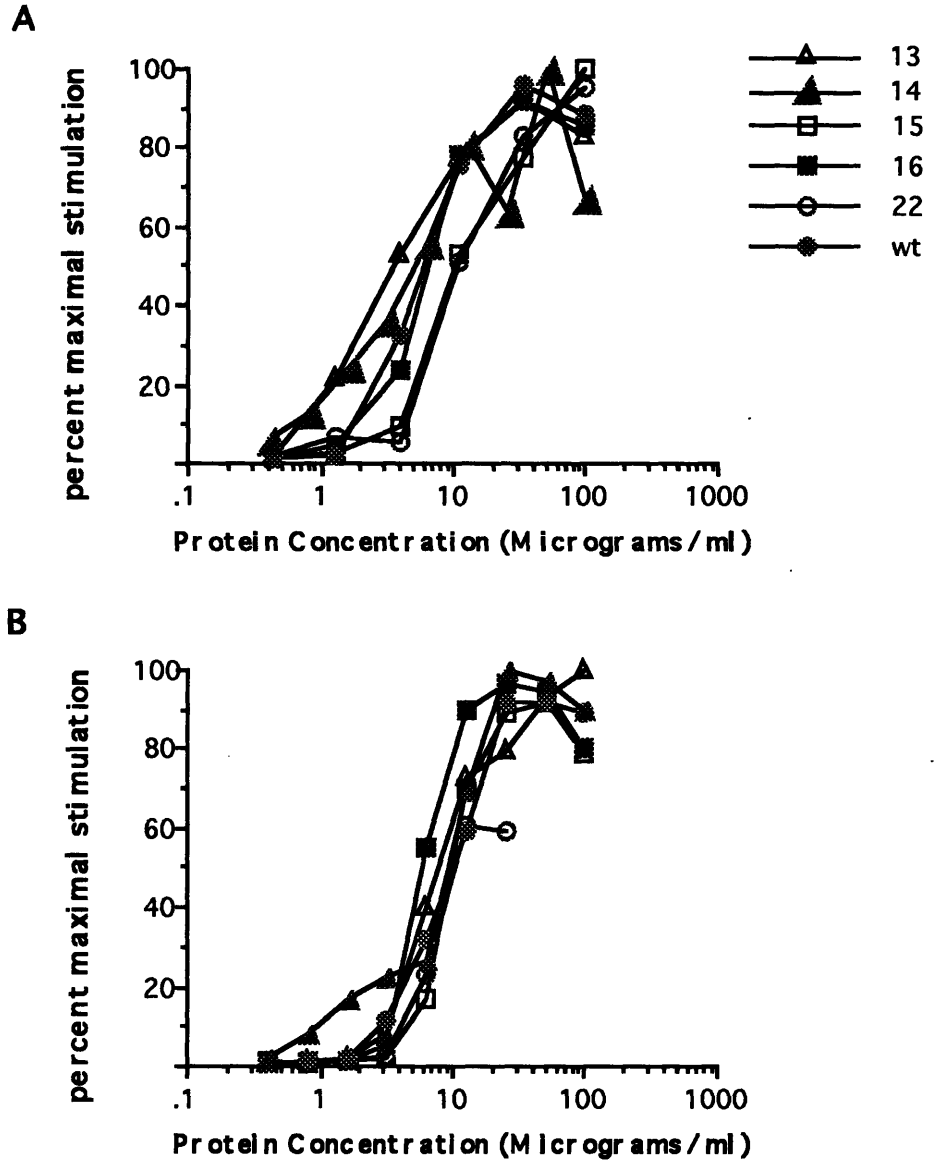


B



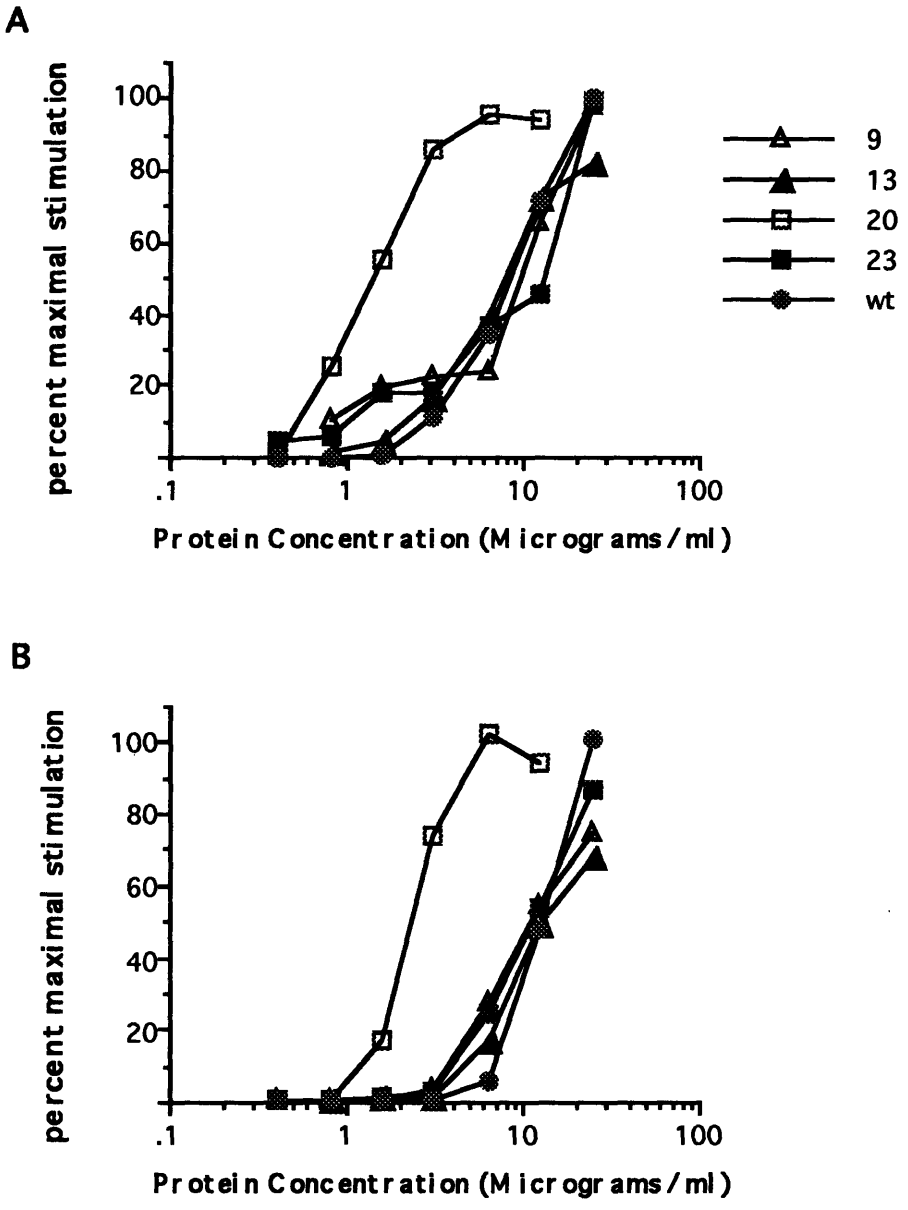
Response of 12-26-specific hybridoma C26.4 to peptides (A) and cI proteins (B). Response of the hybridoma A128 specific for cI residues 46-62 to cI and cI(G20) if shown for comparison (B).

Figure 3.6



Response of hybridoma 26.2 (A) and A128 (B) to purified cI mutants containing substitutions in residues 8-11. Data points represent the average of duplicates. The experiments have been repeated at least three times. See Table 3.1 for substitutions.

Figure 3.7



Response of hybridoma 26.2 (A) and A128 (B) to purified cI mutants containing substitutions in residues 24-27. Data points represent the average of duplicates. The curves shown are representative of experiments that have been repeated at least three times. See Table 3.2 for substitutions.

Table 3.1
Stimulation of T cell 26.2 by cI proteins containing substitutions at positions 8-11

	Position					Protein
	8	9	10	11	Relative Simulation	Conformation
1	STOP				0.06	--
2	Gly	Arg			0.23	++
3	His	Gly			0.68	--
4	Pro	Pro			0.99	--
5	Ile	Ser			0.78	--
6	Tyr	Arg			0.75	--
7	Pro	Pro			0.43	--
8	Trp	Arg			0.73	--
9			Trp	Trp	0.25	--
10			ND		0.29	ND
11			Leu	Glu	0.7	++
12			Phe	Glu	0.7	--
13			Ala	Thr	0.54	--
14			Ser	Phe	0.19	--
15			ND		0.48	ND
16			ND		0.15	ND
17			Gly	Glu	0.71	++
18			ND		0.05	ND
19			Cys	Phe	0.18	--
20			Arg	His	0.82	--
WT	Thr	Gln	Glu	Gln	1	++

Response of cell 26.2 in a screen of E. coli lysates containing mutants with substitutions at positions 8-11. Responses are relative to the response to a lysate from E. coli expressing wild-type cI. Protein conformation refers to whether the indicated substitutions are expected to inhibit the function of cI as a repressor of λ-phage based on the results of Reidhaar-Olson and Sauer, 1990.

Table 3.2

STIMULATION OF T CELLS BY cI MUTANTS CONTAINING SUBSTITITUTIONS AT POSITIONS 24-27

	SUBSITUTUTION AT POSITION:				RELATIVE STIMULATION OF T CELL:	
	24	25	26	27	24.4	26.2
1		ND			1.52	0.88
2	LYS	LEU	LYS	SER	0.49	0.78
3		ND			2.99	0.97
4		ND			3.28	0.96
5		ND			3.67	1.12
6		ND			2.08	1.01
7	ASP	LEU	ARG	MET	0.33	0.01
8		ND			1.02	1.02
9	SER	LYS	THR	CYS	0.04	0.03
10		ND			1.18	0.27
11		ND			0.46	0.7
12	ASP	LYS	VAL	LYS	1.89	0.01
13	LEU	LEU	LYS	HIS	0.01	0.01
14		ND			3.22	0.94
15		ND			0.26	0.41
16		ND			0.31	0.83
17		ND			0.16	0.57
18		STOP			0.01	0.00
19		ND			0.61	0.33
20	ASN	SER	GLN	TYR	2.93	0.87
21	GLY	THR	ALA	CYS	0.45	0.01
22	GLY	ARG	PRO	LYS	1.01	0.06
23	ILE	PRO	THR	PRO	1.19	0.81
24	ARG	GLY	ARG	VAL	0.83	0.86
25	ARG	SER	ARG	PHE	2.84	1.04
26	PRO	ARG	THR	ASN	0.28	0.36
27		ND			2.96	1.08
28	ASN	GLN	VAL	VAL	1.53	0.06
WT	LYS	LYS	LYS	ASN	1	1
	NEGATIVE CONTROL				0.01	0.00

Results represent the response of T hybridomas 26.2 and 24.4 to a screen of 5% lysates from E. coli bacteria expressing the indicated cI mutants. Responses are the average of duplicates, and are normalized with respect to the response to a lysate containing wild-type cI.

Concluding Discussion

Since the experiments described here were performed, a number of important findings relating to antigen presentation have been made. The most important of which is the crystallization of the human HLA-DR1 class II antigen (Brown et al., 1993). Despite the numerous differences in primary structure between the HLA-DRAI molecule and E^k, the crystal structure illuminates a number of the findings described here. First, the structure explains the more heterogenous length of class II-restricted peptides compared to their class I-restricted counterparts. While the amino terminus of the class I α_1 domain contains two turns which effectively block the N-terminal end of the peptide binding groove, in the equivalent portion of the class II structure these are replaced with extended chains that leave the groove open. Another difference in structure leaves the C-terminal end of the groove open as well. The direct consequence of these differences is to allow larger peptides to bind, extending out from the ends of the class II molecule much as a hotdog in a bun. This explains the observation that the average length of peptides eluted from class II molecules is 14-15 amino acids(Rudensky et al., 1991; Rudensky et al., 1992) Furthermore, as many as 15 amino acids of the peptides bound in the class II groove contact HLA-DR, although the tightness of the interactions at each position is variable. This supports the observation made here that at least some substitutions at every position of 12-26 could affect binding (Chapter 1). Furthermore, a number of contacts appear to exist between conserved residues of the class II molecule and

peptide backbone, again supporting the observation that substitutions at a number of positions could affect binding by subtle alterations in the conformation of bound peptide.

Only a single side chain pocket is found in the structure. This is in agreement with the observation that an HLA-DR1-restricted peptide with all residues but ones substituted to alanine retains MHC binding(Jardetzky et al., 1990). The E^k structure should therefore contain a pocket to stabilize the hydrophobic residue corresponding to the leucine at position 18 of 12-26. A second pocket containing a negatively charged residue is likely to exist in the E^k structure to account for the preference for positively charged residues at position 26.

Contrary to the case in the class I HLA-A2 and HLA-Aw68, where peptides fit into the structure by bulging out in the middle(Silver et al., 1990; Madden et al., 1991), there is no weak electron density in the middle of the class II-bound peptide. If this proves to be general for all class II molecules, it would be in disagreement with the E^k-binding motif proposed in Chapter 1 which invoked a variable 7-8 space between the two anchor residues. However, in the class I B27 structure, no apparent bulging of the peptide was observed(Madden et al., 1991). Thus, it may be the tendency of peptides to adopt a bulging conformation is not unique to class I. The strong electron density from the bound peptide indicates that the peptide is held in a unique conformation and supports the conclusion reached here that peptides are not likely to bind to class II in multiple conformations. Since the bound material is a mixture of endogenous peptides, it represents an effective

sampling of a variety of peptides. Thus, if an appreciable number of peptides were oscillating between alternative conformations, one would expect to see a correspondingly lowered electron density. Additional support for this conclusion comes from an NMR study of a cytochrome C peptide bound to E^k. Although the resolution of this technique is not yet equal to that of crystallography, because it is much less time consuming to apply to each new structure, it probably represents the wave of the future for for understanding of peptide MHC interactions. In this study, the cytochrome C peptide was ^{13}C-labelled at each position along the backbone. Definitive assignments of the peptide residues accounting for binding could not be made in all cases, but single cross-peaks were obtained for each residue, indicating that the peptide was bound in a unique conformation (Driscoll et al., 1993).

The manner in which the peptide is bound in the class II structure also lends some support to one proposed mechanism of antigen processing (Donermeyer and Allen, 1989). As mentioned in Chapter 3, antigen processing could occur by an initial denaturation of proteins, followed by binding to the class II molecule, followed by the proteolytic digestion of unprotected portions. Such a model is not possible for class I-restricted peptides because the closed form of the binding groove prevents longer peptides from binding. It is important to note, however, that this model could not explain the antigen processing mutants identified here. If this model were true in its simplest form, all peptides capable of efficiently binding MHC would also be efficiently processed. Since the mutations we have identified lie within the epitope do not significantly affect the

capacity of synthetic peptides to bind MHC, they would, according to this hypothesis, still be properly processed. These observations could, however, be explained by a model involving first an initial cleavage step distant from the epitope, followed by binding and trimming to a final size.

One particularly puzzling observation made in this work was that there were several cases of T cells which gave maximal responses to peptides which are predicted by the competition binding experiments to bind weakly to E^k. For example, T cells C26.4, 1.21, and 2.19 all respond well to 12-26(Y26), even though this peptide has no detectable activity as a competitor. These results are paradoxical in that the peptide should ordinarily be incapable of stimulating all T cells if it cannot bind MHC. Such an observation could have a variety of explanations. The simplest is that these substitutions simply increase the affinity of the the TCR for the peptide-MHC complex, either by adding a productive interaction with the TCR or removing an unproductive one. As the T cells were originally selected upon immunization only for having a minimal affinity for the complex, it is probable that some changes in the sequence of the peptide or MHC structure could augment this affinity. Such a situation would be analogous to the process of somatic mutation that occurs to refine the affinity of antibodies after an initial immune response. However, in this case, it would not be the immune receptor (the TCR), but the ligand which was modified.

Alternatively, this result may be explained by differences between the experimental protocols used to measure MHC binding

(competition) and T cell stimulation. In the former, fixed APC are used. Binding of peptide occurs on the surface of the cells at the neutral pH of the tissue culture medium. In the latter, binding may occur either on the surface of the cells, or intracellularly, after endocytosis. The pH of the compartment where peptide binding to MHC is believed to occur is estimated at 4.7 to 5.2 (Mellman et al., 1986). The binding of some peptides to purified MHC has been shown to be a maximum at a pH in this range(Wettstein et al., 1991). Other factors besides the pH may also affect the intracellular binding of peptide to MHC. One particularly intriguing possibility is suggested by the detection of PBP72/74, a member of the hsp70 molecular chaperone family in endocytic vesicles (Lakey et al., 1987; Vanbuskirk et al., 1989). These proteins, all of which identified to date are ATPases, have been shown to be involved in the assembly of newly synthesized proteins in the cell, as well as in protein transport and perhaps protein degradation(Rothman, 1989). PBP72/74 has been suggested to function at the site of antigen processing to capture peptides and facilitate their with MHC molecules. It is therefore possible that the binding of peptide antigen to class II molecules in living cells is an active process that occurs with greater efficiency than on the surface of cells. Thus, if intracellular binding of a given peptide occurs more readily than on the exterior of cells, the competition experiments may underestimate the binding capability of the peptide under physiological conditions. Experiments were performed to consider the possibility that such enhanced binding efficiencies are at the origin of the contrasts in the peptide competition and stimulation

data. If this hypothesis is correct, this should be revealed by a difference in the titration curves of T cells for some peptides when fixed or un-fixed APC are used. However in experiments of this type, no difference was observed in the sensitivity of T cells to fixed or un-fixed cells. The original conditions of fixation used in the competition binding experiments were such that the same dose-response curves were obtained using 12-26 with either fixed or un-fixed APC.

A further possibility is that the presence of the peptide-specific T cell in the stimulation experiments may actually facilitate the binding of the peptide to MHC. In the stimulation experiments 12-26-specific T cells, APC and 12-26 analogs were added simultaneously. Antibodies specific for MHC molecules have been shown to enhance their interaction with peptides, and it has been proposed that a weak interaction can be established between TCR and MHC, facilitating peptide binding (Bodmer et al., 1989). If such an interaction exists, it is conceivable that it could accelerate the kinetics of binding of peptides to MHC on the surface of the APC. In contrast, in the competition binding experiments, the T cell used is not specific for the competitor peptide used, and should therefore not facilitate its binding to MHC in any way. Thus, in this case the stimulation experiments would overestimate the real physiological binding capability of a peptide to MHC that would normally occur intracellularly, and in the absence of T cells. In order to evaluate the this hypothesis, the following experiment was performed. T cells were preincubated for several hours with APC to allow possible weak interactions between the TCR and MHC molecules to

develop. A dilution series of 12-26 was then added to these cells. As controls, the same dilution series was added to APC at the same time as T cells. If indeed weak interations between the TCR and MHC can facilitate the binding of peptides to MHC, one would expect a lower concentration of peptide to be required for stimulation when T cells are preincubated with APC. However, this was not the case. Similar titration curves were obtained whether T cells were preincubated with APC before the addition of peptide or simultaneously.

Finally the observation, mentioned above, that MHC class II molecules crystallize as dimers (Brown et al., 1993), may shed light on this phenomena. This observation, supported by a growing body of functional data, suggests a mechanism by which the affinity of T cell receptors for peptide-MHC might be enhanced. According to the hypothesis proposed by these authors, TCRs recognizing identical peptide-MHC complexes would be drawn together by the dimer interactions on the class II molecule. These interactions may be supplemented within the plane of the TCR membrane by additional weak interactions between the TCR molecules and co-receptor CD4 molecules. The combined action of these forces would then lead to a sort of aggregate at the site of interaction between the T cell and APC that would have a greatly enhanced avidity with respect to the simple interaction between a single TCR and class II-peptide complex. As receptor dimerization plays an important role in intracellular signalling, the nature of the signal sent may be highly dependent upon the avidity of this complex. The formation of such a proposed three dimensional complex may be qualitatively different

in the presence of competitors. Competitor peptides could, for example, "poison" some class II molecules in the aggregate leading to less efficient receptor interactions in the plane of the membrane and consequently less efficient signalling.

The above-described caveat is particularly important in view of the observation that analogs of a given peptide can inhibit T cells not by competition at the level of MHC, but by competion of the analog-MHC complex for binding to the TCR(De Magistris et al., 1992). Thus, the nature of the antigen recognized by the TCR is more sophisticated than previously thought. It is becoming more and more clear that the TCR is does not simply signal or fail to signal depending upon whether it binds antigen or not. De Magistris and colleagues showed that some peptides closely related to that for which a given T cell is specific can act as much more effective inhibitors of T cell activation than more distantly related peptides that bind MHC with the same affinity. In this case, the more effective inhibitors appear to be acting as antagonists of the T cell receptor itself. Thus, while the complex of these peptides with MHC is bound by the T cell receptor, the receptor fails to signal. The reason for this more effective inhibition has to do with the minimum number of peptide-MHC complexes that must be recognized by the T cell for stimulation to occur. Because this number is small - on the order of 50-300 (Demotz et al., 1990), corresponding to approximately 0.1% of MHC molecules on the surface of the antigen presenting cell, peptides that compete by blockading MHC must occupy nearly 100 % of MHC molecules. By contrast, it is found empirically that a ten-fold excess of peptides that compete by

acting as antagonists of the TCR can prevent T cell activation. Thus, in this case, only 1 % of MHC molecules need be occupied. Thus, competitors of this type are more powerful, are likely to be more specific for a given type of T cell, and potentially more clinically useful.

A similar, but mechanistically distinct phenomena has been studied by Allen and co-workers (Evavold and Allen, 1991; Sloan-Lancaster et al., 1993). In their initial study, they found that peptides differing from the immunogenic peptide by some single amino acid substitutions induced cytokine secretion, but not proliferation. This "partial activation" appears to be the result of a differential signalling capacity of the TCR depending upon the nature of the ligand recognized. While these peptides appeared to have a reduced affinity for the TCR, simply increasing the concentration of peptide did not lead to proliferation. Thus, the authors speculated that this differential signalling capacity is due to an altered balance between the signal delivered by the TCR and other co-receptors such as CD4 or CD3. These results were enlarged upon in a second paper where it was shown that T cells treated with some peptides that induced partial activation subsequently became unresponsive to the original immunogenic peptide for extended periods. The identification of partial activating peptides would therefore be potentially even more powerful than that of TCR-antagonist peptides because it would not require the continuous presence of the peptide.

In their study, De Magistris *et al.* found the TCR-antagonist peptides with relatively high frequency. Among a set of 54 analogs tested, at least 4 were active. Thus in the set of peptides tested in

Chapter 2, we can presume that a certain number also can act as TCR antagonists. In light of these findings number of significant questions can be asked. First, for the moment, the capacity of peptides to act as TCR antagonists has been demonstrated only for T cell clones. As the co-stimulatory signals that are required for clones and T cell hybridomas are different (Schwartz, 1992), it will be important to determine whether TCR antagonists can be found for the hybridomas studied here. It will be interesting to return to the panel to see if correlations can be found between the location of substitutions permitting an analog to act as a competitor for a given T cell and the group (15-26-responder or non-responder) to which the cell belongs. For example, 15-26-responders were particularly sensitive to substitutions at position 22. It will be important to determine whether these substitutions preclude interaction with the TCR of these cells, or permit an antagonistic interaction, or whether different substitutions at the same position can have different effects. Notably, the studies by Allen's group (Evavold and Allen, 1991; Sloan-Lancaster et al., 1993) which were performed using E^k-restricted T cells did not find any substitutions at the position corresponding to 12-26 residue 22 which had a differential effect on interleukin production and proliferations. And they observed that within the limited set of peptides they identified, changes in more permissive residues were more likely to lead to differential signalling than those at less permissive residues. Some cells belonging to the 15-26-responder group were nonetheless unable to respond to analogs containing substitutions at position 14, so one would like to know whether substitutions at this position can create

antagonistic interactions. Perhaps the most striking result of the fine-specificity analysis carried out in Chapter 2 was that the 15-26-nonresponders accepted almost no substitutions at nearly all positions. It will therefore be interesting to determine whether these cells represent a class that is particularly susceptible to antagonistic substitutions.

These recent results and future prospects show the potential applications of experiments using synthetic peptides to study the T cell response. Ultimately, studies of this kind may lead to the rational design of peptides designed to specifically prevent the stimulation of a given T cell, a prospect with great clinical importance.

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