Role of Recurrent Hydrophobic Residues in Catalyzing Helix Formation by T Cell-Presented Peptides: a Thesis

Shan Lu
University of Massachusetts Medical School

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ROLE OF RECURRENT HYDROPHOBIC RESIDUES IN CATALYZING
HELIX FORMATION BY T CELL-PRESENTED PEPTIDES

A THESIS PRESENTED

BY

SHAN LU

Submitted to the Faculty of the
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Lu, S., and Humphreys, R.E. Evidence for the hypothesis that Ii148-164 peptide binds to both α and β chains of class II MHC molecules. (in preparation)
This thesis is dedicated to my parents,
Hui-shian C. and Pei-chiao Lu
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Finally, I would like to share the joy of this moment with my wife, Yigong Xing, whose understanding and support have made it all worthwhile.
ROLE OF RECURRENT HYDROPHOBIC RESIDUES IN CATALYZING HELIX FORMATION BY T CELL-PRESENTED PEPTIDES

December 1990

Shan Lu

Thesis Advisor: Robert E. Humphreys, M.D., Ph.D.

ABSTRACT

The overall objective of this study was to understand the mechanisms that control antigen processing and binding of peptides to major histocompatibility complex (MHC) molecules. Towards this goal I investigated (a) the structural features of T cell-presented peptides with a focus on the role of recurrent hydrophobic residues in catalysis of helix formation by these peptides and (b) the biochemical events that determine the fates of the invariant chain molecule (Ii) in its various post-translational processing pathways.

In the structural studies, I tested the hypothesis that the recurrence of hydrophobic amino acids in a polypeptide at positions falling in an axial, hydrophobic strip if the sequence were coiled as an α-helix can lead to helical nucleation on a hydrophobic surface. For a series of HPLC-purified peptides, including some T cell-presented peptides varying considerably in primary sequence, percentage helicity in the presence of lipid vesicles correlated with strip-of-
helix hydrophobicity index (SOHHI), as shown by circular
dichroism (CD) analysis. A prototypic helix peptide PH-1.0
(LYQELQKLTQTLK) was designed with a strong axial hydrophobic
strip of 4 leucine residues. PH-1.0 formed about 38% helical
structure in 10 mM phosphate buffer at pH 7.0 with di-O-
hexadecyl phosphatidylcholine (DHPC) lipid vesicles, but no
helical structure was detected when the peptide was in
phosphate buffer alone.

The helix-forming tendencies of 9 analogs of PH-1.0 with
one or two amino acid variations from the parent peptide were
examined in the presence of lipid vesicles and the results
showed that (a) decreasing the strip-of-helix hydrophobicity
by substituting even one of the four leucine residues in the
axial hydrophobic strip with a less hydrophobic threonine
residue reduced the helix-forming tendency of a peptide in
the presence of lipid vesicles; (b) the placement of
recurrent hydrophobic residues within the axial hydrophobic
strip appeared to be critical for a peptide to be induced to
form an \( \alpha \)-helix by a hydrophobic surface; (c) there was an
orientation preference for these peptides to interact with
lipid vesicles and to form helical structure; (d) extra
hydrophobic residues in other positions appeared to compete
with the hydrophobic residues within the axial hydrophobic
strip for interaction with the lipid vesicles and therefore
to decrease the helix-forming tendency of peptides.
For the biochemical studies of the function of Ii, a 17-residue peptide, Ii-3 (Ii 148-164), was synthesized. The CD analysis of Ii-3 showed mainly an α-helical conformation when the peptide was examined in the presence of lipid vesicles. [125I]-labeled Ii-3, after coupling at the N-terminus with a photoactivatable, heterobifunctional crosslinker N-hydroxysuccinimidyl-4-azidobenzoate (HSAB), was able to bind to both α and β chains of class II MHC molecules, indicating that this region of Ii might cover the desetope of class II MHC molecules from the time of their synthesis until their charging with foreign peptides at an endosomal compartment.

The biosynthesis of a chondroitin sulfate proteoglycan-form of Ii (CS-Ii) was examined in a class II MHC-negative cell line P3HR-1. [35S]sulfate-labeled microsomal membrane proteins of P3HR-1 were immunoprecipitated with anti-Ii monoclonal antibody and the results of SDS-PAGE analysis demonstrated that P3HR-1 could process Ii to CS-Ii in the absence of class II MHC molecules and the chondroitin sulfate identity of this molecule was confirmed by chondroitinase-ABC treatment. We conclude that there might be a class II MHC-independent pathway to process Ii to a chondroitin sulfate proteoglycan form as compared to the pathway in which Ii was associated with class II MHC and later cleaved by proteases residing in the endosomal compartment.
In an effort to demonstrate in vitro that the class II MHC-associated Ii was eventually dissociated from class II MHC molecules by a proteolytic cleavage process, it was found that cathepsin B could completely remove Ii without damage to class II α and β chains. In order to identify those cleaved Ii fragments, three polyclonal anti-Ii peptide sera were produced by immunizing rabbits with keyhole limpet hemocyanin (KLH)-conjugated Ii peptides. Anti-Ii(146-169) was shown to be able to precipitate a p18 molecule only in cells expressing Ii. Anti-Ii(148-164) and anti-Ii(78-92) were specific for their respective antigenic peptides as tested by enzyme-linked immunosorbent assay (ELISA).
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<td>ABTS</td>
<td>2,2'-azino-di-(3-ethyl-benzthiazoline-sulfonate)</td>
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<td>APC</td>
<td>antigen presenting cells</td>
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<tr>
<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>CS-II</td>
<td>a chondroitin sulfate proteoglycan-form of Ii</td>
</tr>
<tr>
<td>DHPC</td>
<td>di-O-hexadecyl phosphatidylcholine</td>
</tr>
<tr>
<td>EDAC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HSAB</td>
<td>N-hydroxysuccinimidyl-4-azidobenzoate</td>
</tr>
<tr>
<td>Ii</td>
<td>the invariant chain associated with class II MHC molecules</td>
</tr>
<tr>
<td>ka</td>
<td>association constant</td>
</tr>
<tr>
<td>kd</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>K_B</td>
<td>binding constant</td>
</tr>
<tr>
<td>KD</td>
<td>kilo-dalton</td>
</tr>
<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NRS</td>
<td>normal rabbit serum</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SHA</td>
<td>structural helix algorithm</td>
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<td>SOHHA</td>
<td>strip-of-helix hydrophobicity algorithm</td>
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<tr>
<td>SOHHI</td>
<td>strip-of-helix hydrophobicity index</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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<tr>
<td>TFE</td>
<td>trifluoroethanol</td>
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CHAPTER I

INTRODUCTION

A. Specific aims

The overall objective of this study has been to understand better the mechanisms that control antigen processing and binding to major histocompatibility complex molecules. The following specific questions were asked:

1. What structural features determine the selection of T cell-presented peptides?
   a. What is the role of recurrent hydrophobic residues in catalysis of helix formation by T cell-presented peptides?
   b. How does the strength and placement of recurrent hydrophobic residues in these peptides affect the coiling of a helical structure?

2. What are the roles of the invariant chain (Ii) in the regulation of antigen processing and presentation?
   a. Where is a potential decapeptide-binding sequence on the invariant chain and does a peptide from that sequence bind to class II MHC molecules?
B. Literature Review

1. T cell antigen

Introduction. Although antibodies and some of their biological effects were described by the late 1800s, the importance of the thymus and thymus-derived lymphocytes in antibody production and in the whole immune system was not generally recognized until the mid 1960s. Lethally irradiated mice reconstituted with both thymus and bone marrow cells together with antigen produced a much greater antibody response than when given either cell source alone (Claman et al., 1966). Other studies showed that lymphocytes were divided into two categories: B cells which secreted the antibodies and T cells which were necessary to "help" B cells make antibody (Davis et al., 1967; Miller & Mitchell, 1967). The discovery of T-B synergism was consistent with the phenomenon known as the "carrier" effect, i.e., the antibody response to a hapten was dependent on the carrier. In order to obtain a secondary response the hapten must be conjugated to the same carrier as used for the primary immunization (Ovary & Benacerraf, 1963). Thus the T cell was shown to be carrier-specific and the B cell was hapten-specific.

However, T cells and B cells differed fundamentally in the ways in which they recognized antigens. The B cell
receptor for antigen (immunoglobulin) bound soluble antigens. In contrast, the T cell antigen receptor could recognize antigens only in association with cell surface molecules encoded in the major histocompatibility complex (MHC) gene (Katz et al., 1973). In addition, T cells generally did not recognize native protein antigen, but only antigens that were processed and subsequently displayed in association with MHC molecules (Chestnut et al., 1982).

**MHC restricted T cell antigen presentation.** Class I and Class II MHC molecules are expressed on the cell surface and, being part of a ternary complex (T cell receptor, nominal antigen and MHC molecule), serve as accessory restricting structures in T cell recognition of protein antigens (Katz et al., 1973; Zinkernagel, 1976). Class I MHC molecules consist of a highly polymorphic, integral membrane glycoprotein (α chain) noncovalently bound to β₂-microglobulin (Orr et al., 1979). Class II MHC molecules consist of two noncovalently bound, highly polymorphic membrane glycoproteins (α and β chains) (Humphreys et al., 1976; Kaufmann et al., 1984). Crystallographic analysis of the structure of class I MHC revealed a groove at the top surface of the molecule formed by the two amino-terminal domains, α₁ and α₂ (Bjorkman et al., 1987). The three-dimensional structure of the class II molecule is not available but a similar antigen binding site
was modeled by aligning conserved elements between class I and class II molecules (Brown et al., 1988).

Identifying class I and class II MHC as antigen-presenting molecules led originally to a division of T cells into two classes, cytotoxic T cells (T\textsubscript{C}) and helper T cells (T\textsubscript{H}) (Cantor et al., 1975; Huber et al., 1978). T\textsubscript{C} eradicate intracellular pathogens and tumors by directly lysing cells and secreting potentially toxic cytokines such as \(\gamma\)-interferon. The primary function of T\textsubscript{H} is to secrete cytokines that promote the activities of B cells and other T cells. T\textsubscript{H} enhance antibody responses and thus help eradicate extracellular antigens susceptible to antibody-targeted immune mechanisms. The tissue distribution of MHC molecules is also consistent with these different functions of T\textsubscript{C} and T\textsubscript{H}. Class I MHC molecules were found on virtually all cell types. Class II molecules were constitutively expressed largely by cells of the immune lineage that either secreted cytokines necessary for T\textsubscript{H} stimulation (such as macrophages) or required T\textsubscript{H} cytokines for their own stimulation (B cells).

Although it was likely that residues from MHC molecules directly interacted with the T cell receptor, the differential recognition of class I and class II molecules by T cells appeared to stem largely from the interaction of CD8 and CD4 adhesion molecules on the T cell surface with class I and class II molecules on the antigen presenting cells (APC),
respectively (Cantor & Boyse, 1975; Long, 1989a). Of particular interest was Braciale's finding of both class I and class II-restricted, influenza-specific CTL clones (Morrison et al., 1986). Analysis of the differences in antigen presentation to these two types of CTLs suggested the close association between the MHC restriction of an antigen-specific T lymphocyte and the pathway of antigen presentation to that T lymphocyte. Therefore, the distinction between class I- and class II-restricted antigen presentation might be made by the source, or processing pathway of T cell antigens: exogenous antigen would be presented by class II MHC, whereas endogenous antigens would be presented by class I MHC (Morrison et al., 1986; Germain, 1986).

T cell antigen processing. Antigen processing constitutes the structural modification and trafficking of protein antigens that enable the determinants which will be recognized by T cells to interact with MHC molecules in the proper subcellular compartments. The dichotomy of class I and class II MHC restriction for T cell antigens led to the hypothesis that there are two different T cell antigen processing pathways (Long, 1989b).

The requirement for processing of T cell recognized antigen was first discovered in studies on class II MHC restricted antigen presentation. Ziegler and Unanue (1981),
using the bacterial antigen *Listeria monocytogenes*, discovered two major features of antigen processing. Firstly, if the antigen presenting cells were fixed after a processing period had occurred, they were still able to present antigen to T cells. If the antigen presenting cells were fixed before the processing period, then no presentation was observed. Secondly, treatment of the antigen presenting cell with lysosomotropic agents, such as ammonium chloride or chloroquine, completely inhibited the processing and presenting capacity of the treated cells. It was concluded that during processing the antigen must encounter an acidic intracellular compartment.

Shimonkevitz et al. (1983) further demonstrated that glutaraldehyde-fixed antigen presenting cells could present a tryptic digest of ovalbumin even though they could not present native ovalbumin. Some peptides in the digest were identified and synthesized, and were able to substitute for the native antigen in presentation (Shimonkevitz et al., 1984). Using planar membranes containing MHC molecules, Watts et al. (1984) showed that the minimal requirements for T cell recognition were antigen-derived peptides and MHC molecules.

Processing of antigen for class I MHC-restricted presentation also appeared to involve proteolysis and recognition of antigen-derived peptides bound to class I MHC
molecules. The reports of class I MHC-restricted T cell recognition of the expressed products of truncated viral genes (Gooding & O'Connell, 1983; Townsend et al., 1985) and sensitization of target cells by synthetic oligopeptides corresponding to specific portions of a viral polypeptide (Townsend et al., 1986) led to the hypothesis that, like class II MHC-restricted T cells, T cells restricted by class I MHC products also recognized "processed" and possibly degraded forms of protein antigens (Townsend et al., 1985, 1986). However, processing of class I MHC-restricted antigens was less sensitive to chloroquine and was usually not secondary to endocytosis of antigen (Morrison et al., 1986).

Therefore, it appeared that there might be two distinct pathways to process T cell antigens. The two pathways differed in three major features: sensitivity to agents such as lysosomotrophic amines that raise endosomal pH and/or interfere with endosomal trafficking; the subcellular portal for entry of antigen into the pathway; and the MHC molecules used to present processed antigens (Yewdell & Bennink, 1990).

The endosomal pathway was sensitive to lysosomotrophic amines. Endosomal processing of many antigens was also sensitive to specific inhibitors of endosomal and lysosomal proteases (Takahashi et al., 1989). Determinants produced by this pathway exclusively associated with class II molecules,
which explains why soluble antigens generally elicit TH and not TC responses. Antigens might enter the endosomal pathway by either fluid-phase pinocytosis or absorptive endocytosis, following the interaction of antigen with receptors on the surface of the antigen presenting cell. The considerable increase in efficiency provided by absorptive endocytosis had an important consequence in vivo, where antigens were commonly presented to TH by B cells expressing antibodies specific for the antigen. This enabled TH to provide specific help to B cell clones of the appropriate specificity (Taussig, 1974).

The cytosolic pathway was resistant to lysosomotropic amines and was accessed through the cytosol, either by the synthesis of proteins or the penetration of plasma or endosomal membranes by extracellular proteins. Penetration might occur naturally through the fusion of virus and cell membranes (Yewdell et al., 1988), or artificially by osmotic lysis of antigen-containing endosomes (Moore et al., 1988). Determinants produced by the cytosolic processing pathway typically associated with class I MHC molecules.

There were several examples of class II MHC-restricted presentation of endogenously synthesized antigens (Jin et al., 1988; Weiss & Bogen, 1989; Eisenlohr & Hackett, 1989), but they all involved transmembrane proteins expressed at the surface of target cells and thus it was possible that these
antigens were targeted into endosomes either during transport to the cell surface or by recycling from the cell surface (Long, 1989).

It is not clear what molecular mechanism determines whether a T cell antigen is presented by class I or class II MHC molecules, but it was suggested that the invariant chain might play a role in such a different restriction patterns (Long, 1989; also see detailed review about the function of invariant chain on page 24).

Biochemical studies of T cell-presented peptides. The first direct biochemical evidence of a specific T cell antigen/MHC complex came from equilibrium dialysis studies in which an I-\(\alpha^k\)-restricted immunogenic peptide derived from hen egg lysozyme (HEL 46-61) was bound to affinity-purified I-\(\alpha^k\) but not I-\(\alpha^d\) molecules in a non-ionic detergent solution (Babbitt et al., 1985). The binding of this peptide to I-\(\alpha^k\) was specific and had a dissociation constant of 2-4 \(\mu\)M.

This finding was subsequently confirmed in other similar studies (Buus et al. 1986a, 1986b, 1987; Guillet et al. 1987). Buus et al. reported that binding of 12 immunogenic peptides to purified class II MHC molecules correlated well with the known MHC-restrictions of these T cell antigens. The capacity of a peptide to inhibit the binding of an antigen to purified class II MHC also correlated with the
capacity of the peptide to inhibit presentation of the antigen by APC (Buus et al. 1987). The thermodynamics of peptide-MHC interactions showed a relatively low affinity interaction (K_D of 5-50 μM), a rate of association (k_a) of approximately 1 M^{-1}s^{-1} and a rate of dissociation (k_d) of approximately 7 x 10^{-5} s^{-1} (Buus et al. 1986b).

These studies indicated that synthetic peptides from unrelated protein antigens manifesting the same MHC restriction could compete with each other for MHC binding. The interaction of I-A^k with HEL(46-61) was competed not only with various HEL(46-61) derivatives but also with unrelated peptides which included HEL(34-45) and peptides derived from other unrelated proteins presented by I-A^k, such as ribonuclease A (Allen et al., 1987b). It was concluded that there might be only a single binding site on any given class II MHC molecule, but this site could probably recognize broadly defined motifs (Buus et al., 1987, Sette et al., 1987) because attempts to define a primary binding sequence on immunogenic peptides have not been very successful. This also suggested that in binding to MHC molecules, a T cell antigen might have created a novel and stable conformation. Such a novel conformation could be generated by a T cell-presented peptide along with portions of the MHC molecule, which would then serve as the substrate for recognition by the T cell receptor (Werdelin et al., 1988).
Structural features of T cell-presented peptides. In contrast to antibodies, the antigenic structures seen by T cell receptors did not depend on an intact configuration of the immunizing protein, but could be defined sufficiently by primary amino acid sequence, as suggested over 20 years ago (Gell & Benacerraf, 1959; Sela, 1969). The ability of antigen presenting cells to present either proteolytic digests and cyanogen bromide fragments of antigenic proteins or synthetic peptides to T cells supported this old theory (Shimonkevitz et al. 1983; Livingstone & Fathman, 1987).

Because (a) fragments of the antigenic protein can stimulate a T cell response that is as great as the response to native antigen, and (b) peptide technology has advanced, a new strategy for defining T cell epitopes has been developed. Now one can synthesize sets of overlapping, or "nested" peptides, serially removing one amino acid from either end and testing for their ability to stimulate specific T cell clones or hybridomas. This strategy was applied to fine specificity mapping of T cell epitopes of a number of globular protein antigens (Watts et al. 1985; Allen et al. 1985; Schwartz et al. 1985; Berkower et al. 1986). Some T cell epitopes could be as short as 7 amino acid residues (Livingstone & Fathman, 1987).
Nevertheless, accurate definition of a T cell epitope could be rather more complicated. The residues outside the minimal T cell epitope sequence might not be essential for stimulation whereas they could still play an important role in determining the antigenic potency of the peptides. One possibility was that these residues stabilized a particular secondary structure for peptides or contributed directly to the affinity of the interaction among nominal antigen, class II MHC and T cell receptor.

Since a large number of substituted analogs of antigenic peptides appeared to bind to class II MHC molecules and each to stimulate antigen specific T cell response (Sette et al., 1987), the requirements for peptide-MHC interaction might be highly permissive. This finding was consistent with the determinant selection hypothesis which required that relatively few MHC molecules had the capacity to bind a large universe of foreign antigens. The critical question was what were the structural requirements for these antigenic peptides to be selected during antigen processing and presentation?

The search for structural features intrinsic to the T cell-recognized determinants which contributed to immunodominance has been aided by the ability to focus on just the primary and secondary levels of peptide structure. Since T cells only saw antigen after its processing by antigen presenting cells, in a process which involved
proteolysis or at least unfolding of the native structure (Unanue, 1984; Allen, 1987a), it would appear that the native, tertiary structure of a protein antigen did not contribute much to the final structure of the antigenic determinants which were seen by T cells. Thus, in contrast to antibodies, T cells did not appear to recognize assembled topographic (or discontinuous) sites.

The first attempt to find a structural feature of an immunodominant T cell site was a series of studies of a single such site at the carboxyl terminus of pigeon cytochrome c (Pincus et al., 1983; Schwartz et al., 1985; Carbone et al., 1987; Fox et al., 1988;). It was observed that the potency of a series of peptides of different lengths and variant sequences from this region for stimulation of T cell clones and hybridomas correlated with their predicted and measured ability to fold as α-helices.

These and other similar observations (Berkower et al., 1986), combined with theoretical considerations, led to the hypothesis that T cell sites tended to be amphipathic α-helices, meaning α-helices that have hydrophilic amino acids on one face and hydrophobic amino acids on the opposite face (DeLisi & Berzofsky, 1985). Later, Spouge et al. (1987) made a statistical analysis of a database of 23 T cell antigenic sites in 12 proteins and concluded that as a group these 23 sites (a) tended to be amphipathic α-helices; (b) consisted
of residues that had a propensity to form helices and tended not to form random coils, and (c) tended to have lysine at or near the carboxyl terminus.

Based on this amphipathic helix hypothesis, Margalit et al. (1987) developed an algorithm to predict the location of potential T cell antigenic sites. It was reported that the computer program AMPHI, based on this algorithm, could predict 18 of 23 immunodominant helper T cell sites noted above.

Another approach to identify T cell sites was taken by Rothbard and Taylor (1988) who found a sequence pattern common to the primary sequences of known T cell antigenic sites and formulated a predictive template based on this pattern. They defined a motif of either four or five consecutive residues. The four-residue motif was the pattern: charged or glycine, hydrophobic, hydrophobic, polar or glycine; and the five-residue motif was the pattern: charged or glycine, hydrophobic, hydrophobic, hydrophobic or proline, polar or glycine. In their motif, the amino acids threonine and tyrosine were treated as being both hydrophobic and polar due to the physical properties of their side chains. It was found that 48 of 57 known helper and cytotoxic T cell antigenic sites contained one of their motifs and that the patterns were useful in identifying eight new helper and three new cytotoxic T cell epitopes in four
different proteins. This method, however, could not give the ranking order for T cell epitopes.

Dr. Humphreys and his colleagues proposed another approach to identify sequences closely related to T cell epitopes (Stille et al., 1987; Reyes et al., 1988). They developed a different helix hypothesis through the analysis of the invariant chain, a glycoprotein which associated with the class II MHC molecule at the time of MHC synthesis and was dissociated from class II MHC later probably in an endosomal compartment (Elliott et al. 1987a, 1987b). They computed only the hydrophobicity down one side of the helix as a measure of its potential antigenicity by using strip-of-helix hydrophobicity index (SOHHI), the mean hydrophobicity (in the Kyte-Doolittle scale) of sets of amino acids in longitudinal axial strips down one side of helices for 3-6 turns, at positions, n, n+4, n+7, n+11, n+14, and n+18. Such residues would form a strip along one face if the segment from n to n+18 were folded into α-helix, and it was hypothesized that if these residues were sufficiently hydrophobic, the segment would be selected as a T cell-presented epitope. A computer algorithm, the strip-of-helix hydrophobicity algorithm (SOHHA) was produced based on this hypothesis and took into account the presence of proline and the presence of lysine near the carboxyl terminus of a segment. This algorithm could be used to identify both class
and class II MHC-restricted T cell epitopes with ranking order for these sites within an antigenic protein.

The sites predicted on the basis of any of the above three methods did not correspond in every case while there was considerable overlap. So they all may have some structural basis but at the same time none of the three methods could predict all the T cell sites. Therefore the real challenge was to understand why T cell antigens need to have certain proposed structural features.

It was noteworthy that while differing widely in function, the antigenic determinants recognized by Tc and Th were chemically and structurally similar (Reyes et al., 1988). Most determinants recognized by T cell receptors could be substituted by synthetic peptides corresponding to continuous sequences in the intact proteins. Although certain features of peptide sequences favor binding to MHC molecules in general, or a specific class I and class II allele in particular, there are no obvious properties that favor binding to class I versus class II molecules. In fact, many synthetic peptide antigens can bind multiple class I and class II alleles (Perkins et al., 1989; Hickling et al. 1990).

Biophysical studies of T cell-presented peptides. Generally, there were very limited studies using biophysical tools to characterize the secondary structures of T cell-
presented peptides. By using synthetic peptide analogs of the carboxy-terminal sequence of moth cytochrome c, Schwartz et al. (1985) tested the hypothesis that increased antigenic potency could be contributed to the stabilization of the secondary structure of the molecule in an \( \alpha \)-helical configuration. They found that the minimum-sized peptide capable of stimulating a full T cell response was moth cytochrome c fragment 97-103. Addition of more amino acids at the amino terminal end increased the antigenic potency in uneven increments, with a large contribution being made at residue 95 which can only accept hydrophobic or non-charged residues. The degree of helix formation of the larger analogs was assessed by circular dichroism (CD) measurements. A good correlation was found between antigenic potency and percentage of \( \alpha \)-helicity for peptides of increasing chain length. These results suggested that secondary structure might play an important role in determining the potency of antigenic determinants involved in the activation of T lymphocytes.

Further studies (Carbone et al. 1987) in this system showed that the presence of an amino-terminal leader sequence generated by helically constrained amino acids (\( \alpha \)-aminoisobutyric acid and alanine) had a general effect on enhancement of T cell recognition and a strong preference for
the \( \alpha \)-helical conformation in nonpolar solvents as shown by CD measurements.

Lark et al. (1989) reported the biophysical properties of a series of peptides from sperm whale myoglobin (SWMb) which were already characterized with respect to activation of T cell clones from mice immunized against the whole protein. Their CD studies suggested that peptides 132-146 and 102-118, corresponding to immunodominant T cell antigenic sites from SWMb, tended to fold as amphipathic \( \alpha \)-helices in helix-promoting environments, such as trifluoroethanol (TFE) or sodium dodecylsulfate micelles (SDS). Control peptides 93-102 and 115-130 did not have T cell stimulatory activity and took up little ordered structure by comparison.

Collawn et al. (1989) analyzed the peptides' physical properties that influence the T cell response. Although the B10.A T cell proliferative response was directed to pigeon cytochrome c 95-104, residues added to the amino-terminal side of this determinant could influence antigen-specific T cell recognition. A series of analogs were synthesized containing the core determinant, residues 95-104, plus various seven-residue, non-native, leader sequences which were designed, in comparison with the native determinant, to make the whole peptide more or less helical, amphipathic, or lipid binding. The structure of each analog in aqueous, non-polar (TFE) or lipid environments was determined by CD. In
addition, the ability of each analog to bind to phospholipid membranes and to stimulate two different pigeon cytochrome c specific T cell hybridomas, 2B4 and 22.D11, was investigated. It was found that in a group of 15 analog peptides, only two peptides with the potential amphipathic, leader sequences could coil as an α-helix in lipid vesicles and they bound membranes better than other peptides. Therefore, a non-determinant leader sequence might stabilize the core determinant in a helical conformation against membrane structure before the peptide could be further charged to the desetope of MHC molecules.

The data were consistent with the possibility that even T cell-presented sequences which were not helical in the native protein could still fold as helices when freed of the constraints of the native protein, during antigen processing and binding to MHC molecules. One such example was hen egg-white lysozyme (HEL) 52-61 which in the native structure of HEL was located in a series of β-pleated sheets. In solution HEL (52-61) had no detectable ordered conformation, but the results of peptide binding and T cell stimulation suggested that HEL (52-61) could fold as an α-helix structure during the process of binding with class II MHC molecules (Allen et al., 1987c).
2. The invariant chain (Ii)

_**Structure.**_ Humphreys et al. (1976) found a 31,000 dalton, third chain which was associated with human class II MHC \( \alpha \) and \( \beta \) chains and was labeled with \([^{35}S]\)methionine but not radioiodinated by the lactoperoxidase method. Jones et al. (1978) found the comparable chain in the mouse non-covalently associated with class II MHC molecules. They designated this protein invariant chain because of its lack of electrophoretic polymorphism. The existence of Ii was again confirmed in the human system (Charron & McDevitt, 1979). This molecule has been called various names, the invariant chain, In chain, Ii, \( \gamma \) chain, and CD 74 (Dorken et al. 1989).

After cloning of the complementary DNA (cDNA) for Ii (Claesson et al., 1983; Strubin et al., 1984; Singer et al., 1984), Ii was found to be encoded by a single copy gene which was located on human chromosome 5 and murine chromosome 18 whereas the MHC molecules were encoded from human chromosome 6 and murine chromosome 17 (Yamamoto et al., 1984; Cleasson-Welsh et al., 1984).

The Ii gene encoded a type-II glycoprotein with its N-terminus on the cytoplasmic side of the membrane. Ii proteins experienced a broad post-translational processing. There were two N-linked glycosylation sites at positions Asn\(^{114}\) and Asn\(^{120}\) while two O-linked oligosaccharide units
were found at unknown locations (Machamer & Cresswell, 1982; Claesson-Welsh et al., 1986). Both of the N-linked carbohydrates were processed to a complex form with sialic acid on their side chains (Rudd et al., 1985). Ii could be fatty acylated by palmitic acid at Cys\textsuperscript{28} adjacent to the cytoplasmic part of the membrane (Koch & Hammerling, 1985; Koch, 1988). Spiro and Quaranta (1989b) demonstrated that Ii could be phosphorylated at one serine residue in the cytoplasmic tail, possibly at positions 9, 26 or 29 in the N-terminal portion of Ii.

Ii was also the core protein of the class II MHC-associated chondroitin sulfate proteoglycan molecules (CS-Ii). Ii was degraded by proteolytic cleavages with multiple proteases in more than one degradative pathways. (The detailed literature on these two types of Ii processing will be reviewed on page 26 and 29.)

The single gene for Ii had multiple protein products, utilizing alternate initiation sites for translation and alternative RNA splicing. Alternate use of two in-phase AUGs resulted in two different forms of the Ii protein, p31 and p35 (or γ2/γ3) (Strubin et al. 1986; O'Sullivan et al., 1987). The p35 form had 16 additional amino acids from a sequence encoded between the two AUGs. If an additional cysteine-rich exon (exon 6b) was included in the final transcript, the Ii gene would produce the species called p41 (Strubin et al.,
1986; O'Sullivan et al., 1987; Koch et al., 1987) rather than the regular p31 form.

Ii was expressed in all cell types expressing class II MHC molecules (Hammerling & Moreno, 1990) and induction of class II α and β chains by lymphokines, e.g. IFN-γ, was accompanied by induction of Ii (Mombeurg et al., 1986). While class II MHC molecules were clearly expressed on the cell surface, the answer for whether Ii was also expressed on the cell surface was not very clear for long time. Recent studies (Wraight et al. 1990) using several new monoclonal antibodies which recognized an extracytoplasmic determinant of Ii reinforced previous suggestions that Ii could be expressed on the cell surface (Koch et al. 1982). The cell surface expression of Ii was much weaker than class II MHC as shown by flow cytometry and most Ii on the cell surface did not appear to be associated with class II MHC molecules.

Class II MHC molecules (both α and β chains) and Ii associated very soon after their biosynthesis in the endoplasmic reticulum (ER) (Kvist et al. 1982). This complex was transported through the Golgi apparatus into an endosomal compartment (Machamer & Cresswell, 1982; Claesson & Peterson, 1983) where class II molecules presumably met endocytosed antigens (Hammerling & Moreno, 1990). At the same time Ii dissociated from class II MHC molecules as result of Ii cleavage into various small molecular weight fragments (Blum
& Cresswell, 1988; Nguyen et al., 1989; Reyes et al., unpublished observation). Class II MHC molecules complexed with the exogenously derived peptides then moved to the cell surface. The coexpression and physical association of class II MHC molecules with Ii led to the assumption that the function of Ii must be tightly connected with the function of class II MHC molecules (Hammerling & Moreno, 1990).

Hypothesized Functions of Ii. It is still unclear whether Ii has any function in antigen-processing and presentation. Stockinger et al. (1989) showed that cotransfection of I-E\textsuperscript{d} and Ii genes to L cells led to more efficient processing and presentation of the C5 antigen to C5-specific T cell clone T58C1 compared with cells transfected with only I-E\textsuperscript{d} genes. However, other studies (Peterson & Miller, 1990), using L cells, COS cells, rat-2 and CHO cells transfected with I-A\textsuperscript{d} or I-E\textsuperscript{k} as antigen presenting cells and ovalbumin, insulin and cytochrome C as antigens, showed that the presence of Ii did not improve processing and presentation to the respective T cell hybridomas. Different results were reported even in the same type of antigen presenting cells. Hammerling & Moreno (1990) reported that the processing and presentation of hen-egg lysozyme (HEL) by a set of I-A\textsuperscript{k}- and Ii-transfected, rat-2 fibroblasts was improved when stimulation of a panel of T
cell hybridomas specific for different HEL determinants was used as the readout system. However, with the same set of transfectants, no influence of Ii on the presentation of another antigen, RNase, was found. It appeared that for some but not all antigens, Ii-positive cells might be more potent antigen presenting cells than Ii-negative ones.

Currently, there are several hypotheses to explain how the invariant chain might exert its function in the cases where antigen presentation was clearly improved.

First, Ii might block the peptide binding groove early in the biosynthetic pathway, protecting class II MHC from association with endogenous peptides that could compete with foreign antigens (Elliott et al., 1987). Preventing such binding until the class II MHC molecules entered an endosomal compartment could maintain the functional dichotomy between class I and class II MHC molecules. Roche & Cresswell (1990) reported that the isolated HLA-DR5 α/β dimers effectively bound radiolabelled influenza haemaglutinin (HA) peptides while Ii-associated forms of DR5 did not.

As an alternative of this blocking hypothesis, the invariant chain was suggested to keep class II MHC molecules in a conformational state which bound peptides with low affinity (Hammerling & Moreno, 1990). The removal of Ii in endosomes would allow the class II MHC molecules to snap into another conformation with high affinity for certain antigenic
peptides. One evidence for this theory was the observed conformational changes of class II MHC molecules. Peterson and Miller (1990) reported that certain antibody determinants on I-Ad molecules on transfected cells were strongly influenced by the presence or absence of Ii in these cells.

The third hypothesis suggested that Ii might play a role in sorting class II molecules into the endosomes where exogenous antigen was met (Hammerling & Moreno, 1990). There was little evidence yet for this hypothesis. An earlier hypothesis similar to "sorting hypothesis" suggested that Ii, as a transporting vesicle, was required for cell surface expression of class II MHC molecules. However, this transport hypothesis was later dismissed because transfection studies showed that class II MHC could be fully expressed on the cell surface in the absence of Ii molecules (Miller & Germain, 1986; Sekaly et al., 1986).

The various hypotheses about the function of Ii do not exclude each other and they are testable. Detailed studies of these hypotheses would offer a molecular mechanism to explain why Ii has a regulatory effect in some antigen presentation systems.

CS-II. In 1983, another component in the murine class II MHC/Ii complex was reported (Sant et al., 1983; 1984). It was a sulfate-bearing molecule with considerable molecular
weight heterogeneity (46-69 kD by SDS-PAGE analysis). This component was immunoprecipitated with monoclonal antibodies specific for I-A, I-E and Ii glycoproteins but it was not detected in control precipitates employing normal mouse serum or an irrelevant monoclonal antibody, nor in association with class I MHC molecules. This component was characterized to be a chondroitin sulfate proteoglycan in nature. The core protein of this murine class II MHC-associated proteoglycan was found to be the murine invariant chain (Sant et al., 1985a), and thus established the proteoglycan to be an alternatively processed form of invariant chain (CS-Ii) associated with class II molecules. This result was confirmed by the finding that the human invariant chain and some of its related components were the core protein(s) of the human class II MHC-associated proteoglycan (Giaccoletto et al., 1986). The site of glycosaminoglycan addition to CS-Ii core protein was Ser201 in the murine Ii protein sequence, as shown by site-directed mutagenesis (Miller et al., 1988).

Treatment of antigen presenting cells with a selective inhibitor of proteoglycan synthesis, p-nitro-phenyl β-D-xyloside (xyloside), prevented the addition of glycosaminoglycan (GAG) to Ii and also depressed antigen presentation to responsive T helper cells (Sivak et al., 1987). This phenomenon was reproduced with a class II MHC-restricted, virus specific, cytotoxic T lymphocyte line
(Rosamond et al., 1987). At xyloside concentrations of 2.5 and 5.0 mM, CS-II synthesis was completely inhibited with marginal inhibition of protein synthesis. The inhibitory effect on CS-II synthesis was completely reversible. The number of class II MHC molecules on the cell surface was not affected by xyloside, but sensitization of target cells was markedly inhibited when target cells were exposed to virus in the presence of xyloside. It was not known why xyloside inhibited antigen processing and presentation events associated with class II MHC molecules.

Understanding the function of CS-II was complicated by insufficient information about the biosynthesis of CS-II and the association between CS-II and class II MHC molecules. Sant et al. (1985b) found that $[^{35}S]$sulfate-labeled CS-II molecules were detected in association with class II MHC immediately after a 15-min pulse, but were barely detectable after a 30-min chase, and were completely undetectable after a 60-min chase. Similarly, newly synthesized $[^{3}H]$leucine-labeled class II MHC molecules associated with the CS-II were detectable immediately after a 20-min pulse, but could not be detected in association with the CS-II after a 300-min chase. Virtually no CS-II was found free in the cells or was secreted into the media, but at any time point, only 2-5% of class II MHC molecules were associated with the CS-II. These findings led to the suggestion that class II MHC-associated
II was converted to the proteoglycan during transport. However, these results could not distinguish whether some or all of the class II MHC molecules associated transiently with the CS-Ii, or only a small fraction of class II MHC associated permanently with the CS-Ii in a short-lived complex.

Such biosynthetic intermediates of class II MHC/Ii/CS-Ii complex were isolated from detergent extracts of human B lymphoblastoid cell lines (B-LCL) by Kelner and Cresswell (1986) who found that the complex contained the α,β chains of class II MHC and Ii in 1:1:1 ratio plus a proteoglycan molecule with a Stokes mean radius of a 180 kD globular protein. Treatment of B-LCL with 10 mM monensin for 24 hr could enrich the population of such complexes before purification. However, both this study and the studies of Sant et al. (1985b) could not show whether class II MHC was necessary for the biosynthesis of CS-Ii.

Proteolytic Cleavages of Ii. It was shown that there were at least two degradative pathways for Ii molecules which were associated with class II MHC (Thomas et al., 1988; Blum & Cresswell, 1988; Nguyen et al., 1989).

The cleavage of Ii to p25 occurred intracellularly and was shown by both anti-Ii and anti-class II MHC antibodies (Koch & Hammerling, 1982; Thomas et al., 1988). The
relationship of p25 to Ii was suggested by Giacoletto et al. (1986) who demonstrated that Ii and p25 derived from SDS electrophoretic gels of immunoprecipitates had similar tryptic peptide maps.

p25 was shown to be an exomembranal, C-terminal fragment of Ii because (a) reprecipitation of denatured, anti-Ii mAb-immunopurified Ii-containing complexes by two rabbit antisera to C-terminal Ii peptides could immunoprecipitate p25, while an antiserum to an N-terminal Ii peptide failed to precipitate p25; (b) VIC-Y1, an anti-Ii mAb, immunoprecipitated [35S]methionine-labeled p25 but not [35S]cysteine-labeled p25, consistent with the loss of a portion of Ii containing the only cysteine in Ii, Cys²⁸ (Thomas et al., 1988).

Cleavage of Ii to p25 appeared to occur in an ER or cis-Golgi compartment (Thomas et al., 1990). Since p25 was completely sensitive to treatments with endoglycosidases F and H, its carbohydrate side chains might be derived from a high mannose form of Ii rather than from the complex sugar forms. The maximal labeling of p25 was seen at 20-40 min chase time in a pulse-chase experiment using [35S]methionine-labeled, polyclonally activated B lymphocytes. The appearance of p25 in an ER or cis-Golgi compartment suggested that there could exist an early Ii degradative pathway to destroy incomplete complexes of class II MHC proteins and
associated molecules or that Ii could retard the class II MHC molecules to be further transported to meet the foreign peptides at an endosomal compartment.

The second, or late, Ii degradative pathway was a protease inhibitor-sensitive pathway. Incubation of cells with leupeptin or antipain, but not with chymostatin or pepstatin, revealed two proteolytic intermediates p21 and p10, at 2-5 hr after synthesis of the class II MHC/Ii complex (Blum & Cresswell, 1988; Nguyen et al., 1989). These leupeptin-induced proteins were named LIP (Blum & Cresswell, 1988). They were determined to be derived from the N-terminal region of Ii by peptide mapping and precipitation with various anti-Ii peptide antisera specific for either C-terminal or N-terminal portion of Ii protein.

Monensin, which blocked transport of class II complexes past the medial-Golgi compartment, prevented the generation of these molecules. Chloroquine also blocked the leupeptin-induced appearance of LIP. Treatment of LIP with endoglycosidases F and H showed that both of its N-linked oligosaccharides were in the complex form, indicating that proteolysis of Ii to generate LIP occurred in a late-Golgi or post-Golgi compartment (Blum & Cresswell, 1988).

One interpretation for this result was that Ii dissociation from class II MHC required at least two proteolytic steps. Cleavage of the processed Ii by a
leupeptin-insensitive protease generated LIP which remained associated with the class II MHC. A second cleavage with a leupeptin-sensitive protease generated fragments that no longer associated with class II MHC molecules. Since leupeptin and antipain generated LIP and both of them inhibited the intracellular protease cathepsin B, cathepsin B was suggested to be the enzyme responsible for the second proteolytic step. More work is needed to identify the enzyme at the first step which might be critical to completely remove Ii from class II MHC and open the desetope for foreign peptide charging.
CHAPTER II

MATERIALS AND METHODS

A. Cells

The EBV-transformed lymphoblastoid cell lines Jesthom (HLA-A2, B27, DR1, DP4) and Vavy (HLA-A1, B8, DR3, DP1, DQ2) were provided by Dr. Charles Carpenter (Brigham and Woman's Hospital, Boston).

Raji was a Burkitt's lymphoma cell line (Pulvertaft, 1965). Jijoye and its mutant, daughter cell line, P3HR-1 were established from a Burkitt's lymphoma patient (Hinuma, 1967). CEM was a human acute lymphoblastic leukemia cell line (Foley et al., 1965).

Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM glutamine. Cultures were maintained at 37°C in a 5% CO2 humidified air atmosphere. Cultures were used in logarithmic growth phase.

B. Antibodies

The anti-human II mononclonal antibody (mAb) VIC-Y1 (Quaranta et al., 1984) was the gift of Dr. W. Knapp (University of Vienna, Austria). The anti-chondroitin sulfate mononclonal antibody 7D4 was the gift of Dr. Bruce Caterson (University of North Carolina at Chapel Hill).
The hybridoma cells which produce anti-HLA-DR, DP mAb IVA-12 (Capra & Giles, 1985) and anti-human Ia mAb L243 (Lampson, 1980) were purchased from American Type Culture Collection (ATCC). Ascites fluids were aspirated from mice primed with pristane and injected with these hybridoma cells. The antibody titers were tested before use.

Rabbit anti-Ii(183-193) serum E1 was generated in the Lab (Thomas et al., 1988).

C. Metabolic Radiolabeling

Standard procedures with slight modification were used (Spiro et al., 1985). Cells in logarithmic growth phase with over 95% viability were washed once in RPMI 1640 medium supplemented with 10% FCS. For [35S]methionine labeling, 1X10⁸ cells were incubated for 3 hr in 5 ml methionine-free RPMI 1640 (GIBCO) with 0.5-1.0 mCi [35S]methionine (NEN, Cat.No.: NEG-009L). For [35S]sulfate labeling, 2X10⁷ cells were labeled for 6 hr in 5 ml Earle's balanced salt solution, with MgCl₂ substituted for MgSO₄, supplemented with amino acids and vitamins from an RPMI 1640 selectamine kit (GIBCO) and 2 mCi [35S]sulfate (NEN, Cat.No.: NEX-041). Cells were labeled at 37°C in a humidified atmosphere containing 5% CO₂.

D. Microsomal Membrane Preparation

For most of the experiments, a two-step lysing procedure was used. Cells were first pelleted and lysed in 10 mM Tris-
HCl buffer, pH 8.1. After removal of nuclear debris by centrifugation, the supernatant was decanted to a 30 ml polycarbonate ultracentrifuge tube. Membrane pellets were isolated by centrifugation of the above supernatant at 100,000 x g for 1 hr. Membrane proteins were then solubilized in PBS containing 1% Triton X-100. Insoluble material was removed by centrifugation at 100,000 x g for 1 hr. Both lysing buffers contained 2 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM N-ethylmaleimide (NEM). For some experiments, radiolabeled cells were directly solubilized in PBS at pH 7.2 containing 1% Triton X-100, 2 mM PMSF and 10 mM NEM. The supernatant was collected after the cell extracts were centrifuged at 100,000 x g for 1 hr. All the steps were done at 4°C.

E. Immunoprecipitation

The detergent-solubilized cell lysate was incubated at 4°C for 2 hours with 50 μl of normal rabbit or normal mouse serum (NRS or NMS). Proteins bound nonspecifically by NRS or NMS were then cleared by incubating above mixture with the pellet from 800 μl of 10% formalin-fixed Cowan I strain S. aureus suspension which had been washed and pelleted three times in a 0.05% (w/v) Triton X-100 buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.02% sodium azide, pH 7.4).
Immunoadsorbents were prepared separately by incubating specific antibodies with 100 μl of preswollen protein A-Sepharose (Sigma) for 2 hr at 4°C. After washing twice with 0.05% Triton X-100 buffer, the immunoadsorbents were incubated with precleared, solubilized lysate overnight at 4°C. On the following day, the complexes were washed five times with 0.05% Triton buffer, and the immunoprecipitated material was eluted in SDS sample buffer.

F. Electrophoresis

The one-dimensional (1-D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system of Laemmli (1970) was used. A 10% or 14% polyacrylamide slab gel was made with a Tris buffer (pH 8.8) containing 0.1% SDS. The Tris-HCl buffer (pH 6.8) with 0.1% SDS was used in 5% polyacrylamide stacking gel. The gels were run electrophoretically at 20 mA per gel until the dye front reached the lower edge of the gel. Gels were either directly stained or fixed for 30-60 min in 15% acetic acid followed by 60 min incubation in autofluor prior to autoradiography.

G. Peptides

MT11-A was the gift of Dr. T. Ciardelli (Dartmouth Medical School, Hanover). The other peptides in this study were synthesized by the University of Massachusetts Medical Center peptide synthesis facility and I did the desalting and
purification work. The peptides were desalted by Sep-Pak C18 cartridges (Waters, Milford, MA) and were purified by a preparative C18 column (Vydak, Milford, MA) in a reverse phase HPLC system (Waters) with a gradient of 0 to 75% Acetonitrile in 0.1% Trifluoroacetic acid. The compositions of most peptides were confirmed by amino acid analysis (AAA) using a WISP HPLC system (Waters) with a standard PICO-TAG procedure as suggested by manufacturer's manual. Ii-2 and Ii-3 peptides were sequenced in Dr. John Mole's lab (University of Massachusetts Medical School).

H. Circular Dichroism Studies

Peptides were analyzed in 0.01 M sodium phosphate buffer at pH 7.0, in that buffer with 45% TFE, or in that buffer with di-O-hexadecyl D,L-α-phosphatidylcholine (DHPC) vesicles. The samples with the vesicles were prepared for CD measurements by the method of Vogel (1987). About 300 µg of peptide was dissolved in 300 µl of HPLC-grade methanol. DHPC (10 mg) (Sigma, Cat No. P-3777) was dissolved in 1.0 ml of HPLC-grade methanol. The lipid solution was divided into two equal parts, to one of which was added 150 µl (=150 µg) of the peptide solution. Both samples were evaporated in vacuo in a speed evaporating centrifuge for 1 hr without heating, followed by 2 hr of high vacuum in a desiccator. Each sample was added with 500 µl distilled water and warmed at 37°C for
3 hr with occasional vortexing. The samples were lyophilized overnight. Distilled water (375 µl) was added to each sample. After vortexing briefly, each sample was sonicated for about 5 min until an optically translucent suspension was obtained. Each sample was diluted with water to 750 µl. The peptide-vesicle sample was added to a 1 mm CD cell, and ultraviolet absorbance was read from 190 to 255 nm, being less than 1.0 unit through that range. Far UV CD spectra were obtained on a Jobin Yvon Mark 5 Circular Dichrograph (Instruments SA, Metuchen, NJ) calibrated with (+) 10-camphorsulfonic acid. Each plotted spectrum represented an average of 3 to 5 spectra measured after subtracting the buffer blank.

An Apple IIe computer and program CD MARK V (Instruments SA, Metuchen, NJ) was used to control the CD measurement and data processing, including converting raw data (observed ellipticity, θλ) into peptide concentration adjusted data (molar ellipticity, [θ]λ). Peptide concentrations were determined by the same amino acid analysis method as described on page 37. Aliquots of the peptide samples were taken directly from the CD cell to determine the actual peptide concentration. The results of different aliquots from the same sample showed that the error of peptide concentration measurement was less than 3% (data not shown).
For peptides with CD spectra of α-helix, indicated by minima at 208nm and 222nm, percentage helicity was calculated from $[\theta]_{222}$ according to the following formula:

$$\% \alpha\text{-helix} = \left(\frac{[\theta]_{222} - 3000}{-39000}\right) \times 100.$$ 

This equation (Taylor & Kaiser, 1987) assumed limiting values of $[\theta]_{222} = 3000 \text{ deg cm}^2/\text{dmol}$ for 0% α helix and $[\theta]_{222} = -36000 \text{ deg cm}^2/\text{dmol}$ for 100% α helix. These values were derived from the spectra of standard peptides which were known to adopt particular secondary structures (Greenfield & Fasman, 1969). This method assumed the validity of the standard spectra and was certainly subject to error, but its value lies in the use of the results for general, comparative purposes (Taylor & Kaiser, 1987). In our studies, the pattern of CD spectra was first examined and the percentage helicity was only calculated as a relative indicator to describe the different helix-forming tendencies of those peptides which showed α-helical CD spectra.

I. Anti-peptide Antisera

The reverse phase-HPLC (RP-HPLC) purified peptides were coupled to keyhole limpet hemocyanin (KLH) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC). The peptide (4.8 mg) in 600 μl H2O (pH 3.0, adjusted with HCl) was incubated with 2.8 mg of EDAC in 5.4 ml H2O (pH 3.0) for 15 min at 0°C. A solution of 24 mg carrier protein (KLH) in
0.5 ml of H2O (adjusted to pH 9.0 with (NH4)2CO3) was added, and with constant stirring was incubated for 3 hr at 0°C (Tamura et al., 1983). The peptide-carrier conjugate was then dialyzed for 2 days against PBS, with frequent solution changes.

Rabbits were bled twice before immunizations began. Peptide conjugates, containing 150 µg of peptide, were injected with complete Freund's adjuvant into New Zealand albino rabbits, boosted with peptide conjugate in Freund's incomplete adjuvant and bled at periodic intervals.

J. Dot Blots

Peptide conjugated to bovine serum albumin (BSA) was deposited with vacuum on nitrocellulose membrane using a "Minifold" blotting apparatus (S & S, Keene, NH). After the membranes were blocked by incubation in 3% BSA/Tris buffered saline (TBS) solution for 1 hr, they were incubated overnight with a serial dilution of the respective antisera. Following washing, the strips were incubated for 2 hr with [125I]protein A in 0.05% solution of Tween 20 in TBS, allowed to dry, and exposed to film.

K. ELISA

Between 0.25 and 8.0 µg of peptide was deposited on each well of a 96-well microtiter plate. After washing, the remaining surface was blocked with BSA. Antisera, diluted to
various levels, were incubated in the wells at room temperature for 40 min. After washing, a dilution of horseradish peroxidase-coupled goat anti-mouse antibodies was added to each well and incubated at room temperature for 1 hr. After washing again, the substrate solution, 2,2'-azino-di-(3-ethyl-benzthiazoline-sulfonate) (ABTS) was put in the wells. After 10 to 20 min, a green color was developed in the wells and the intensity of each well was read directly by a microplate reader MR600 (Dynatech, Alexandria, VA) at wavelength 410 nm.

L. Class II-MHC Purification

Class II-MHC proteins were purified as described by Buus et al. (1986a, 1986b) with some modifications. Detergent-solubilized cell lysates were passed through an affinity chromatography column (Antibody Oriented Kit, Pierce) coupled with anti-human Ia mAb L243. Then, the column was washed with 15 column volumes of PBS containing 0.5% NP-40 and 0.1% SDS, followed by 2 column volumes of PBS containing 1% n-octyl-glucoside (OG). Finally, the class II MHC molecules were eluted with 0.05 M diethylamine in 0.15 M NaCl containing 1% OG (pH 10). A half-volume of 0.1 M Tris, 0.15 M NaCl (pH 6.8) was added to the eluate to reduce the pH to ~7.5. The fractions with eluted class II MHC were pooled, dialyzed against the PBS and lyophilized.
M. Peptide Crosslinking to Purified Class II MHC

The Ii-3 peptide was coupled with a crosslinker N-hydroxysuccinimidyl-4-azidobenzoate (HSAB) by established methods (Massague et al., 1981). Briefly, the peptides (325 mM) were incubated with HSAB (360 mM in 2% DMSO) in 0.01 M sodium phosphate buffer, pH 7.4, with 0.15 M NaCl for 30 min at 0°C in the dark. The reaction was quenched with 40 mM glycine.

The peptides were iodinated using the Enzymobead radiiodination reagent (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. The peptides were freshly iodinated immediately prior to each experiment.

HSAB-coupled and radiiodinated peptides (50 nM) were incubated in the dark at 20°C with affinity purified class II MHC (100 nM) in Tris-HCl buffer, pH7.5, containing 0.5% Triton X-100, 10 mM NEM and 2 mM PMSF. Samples were crosslinked by exposure to short wavelength ultraviolet light for 30 min and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.
CHAPTER III

STRUCTURAL STUDIES ON THE ROLE OF RECURRENT HYDROPHOBIC RESIDUES IN CATALYZING HELIX FORMATION BY T CELL-PRESENTED PEPTIDES.

A. Theoretical Analysis.

Sheet projection of an α-helical structure. The α-helix is a rod-like structure with the tightly coiled polypeptide backbone forming the inner part of the rod and the side chains extended outward in a helical array. Each amino acid is related to the next one by a translation of 1.5 Å along the helix axis and a rotation of 100°, which gives 3.6 amino acid residues per turn of helix. Therefore, amino acids spaced three and four apart in the linear sequence are spatially quite close to one another in this conformation while amino acids two apart in the linear sequence are situated on opposite sides of the helix and are unlikely to make contact (Stryer, 1981).

In order to have a simple and relatively efficient method to visualize the α-helical pattern of a short polypeptide sequence, a method known as the sheet projection was developed to present such a structural relationship among the residues of an α-helix (Fig.3.1).
Graphically, the $\alpha$-helix could be divided longitudinally into four quadrants. The sheet projection represented a flat display of the helix being cut arbitrarily between longitudinal quadrants one and four. This type of projection offered a better way to analyze the side chain interactions among the neighboring residues than the classical Edmundson wheel plot (Schiffer & Edmundson, 1967). The Edmundson projection, looking at a helix from the N-terminus, placed all of the residues from one longitudinal quadrant into a narrow arc of the wheel on a flat plane even though some "neighboring" residues in the wheel projection were actually 2-3 cycles away. The sheet projection easily demonstrated potential salt bridges between positively and negatively charged residues in close proximity. The positioning of charged residues in a sheet projection also offered information about whether they stabilized or opposed the macrodipole of an $\alpha$-helix. Most importantly, the sheet projection could be used to identify certain types of amino acids which had a tendency to recur within one or another quadrant. T cell-presented peptides, as shown below, tended to have recurrent hydrophobic residues within one longitudinal quadrant if the peptide coiled as an $\alpha$-helix. Quadrant III of the sheet projection in Fig.3.1 represented such an hydrophobic quadrant, or hydrophobic strip-of-helix as it was more commonly called in our articles.
Fig. 3.1  Sheet projection and other graphical methods to present an α-helical structure. The squares represent hydrophobic residues.
SOHHI/SHOA. In order to determine quantitatively whether a protein fragment would be selected as a T cell-recognized epitope, a new hydrophobicity index, the strip-of-helix hydrophobicity index (SOHHI), was proposed (Elliott et al. 1987). The SOHHI was defined as the mean hydrophobicity (from Kyte-Doolittle values) of set of amino acids in a longitudinal strip down one side of a helix for 3 to 6 turns, at positions of n, n+4, n+7, n+11, n+14 and n+18 in a protein sequence. The SOHHI was reported at position n. This computation yielded the mean hydrophobicity of residues lying along a strip within +/-45° of the indexed residue in up to six successive turns of a helix. This index evaluated only hydrophobicity in a strip along the side of a hypothetical α-helix and ignored the contributions to a hydrophobicity moment from hydrophilic or hydrophobic side chains elsewhere on the helix. Therefore this index measured periodic hydrophobicity and not amphipathicity as defined by DeLisi and Berzofsky (1985).

The strip-of-helix hydrophobicity algorithm (SOHHA), which ranked peptides according to SOHHI calculated over 3 to 6 turns of a helix, was packaged in a computer program, and was used to identify 10 of 12 known T cell-presented peptides in seven well-studied proteins available at that time (Stille et al., 1987). The SOHHA could be adapted to analysis of 310 helices (positions n, n+3, n+6, n+9, n+12, n+15) or beta-
pleated sheets \((n, n+2, n+4, n+6, n+8, n+10)\) (Reyes et al., 1988).

A derivative of the SOHHA, the structural helices algorithm (SHA), was further developed to test for the prediction of helices in 35 crystallographically defined proteins (Reyes et al., 1989). In comparison with other two leading predictive methods (Chou & Fasman, 1978; Garnier et al. 1978), the SHA was slightly less sensitive but more efficient in the prediction of protein helices (Reyes et al., 1989).

The above theories were established with the effort of many people in the laboratory during the past 4 years. In particular, I proposed to take into account the roles of certain specific amino acid residues in these algorithms. For SOHHA, I observed specific positioning of lysine residues at or near the C-terminus of the reported T cell peptides, i.e., in 7/12 cases such lysine residues were in +1 position from the last hydrophobic amino acid in a presumptive, axial hydrophobic strip and in +3 position in 4/12 cases. For the SHA, I initiated the data analysis to examine whether Pro, Gly and Asn had position preference within a helical structure. This work was finished by other members in the lab and led to the addition of "capping rules" in the SHA, i.e. the N-terminal preference for Asn and Pro and the C-terminal preference for Gly in an \(\alpha\)-helical structure. This
analysis was confirmed and extended in more detail by the studies of two other groups (Presta & Rose, 1988; Richardson & Richardson, 1988)

**Sensitivity and efficiency of SOHHA.** Predictions made with the SOHHA, the amphipathicity algorithm (DeLisi & Berzofsky, 1985) and the motifs algorithm (Rothbard & Taylor, 1988) were compared to peptides which had been experimentally determined to be T cell-presented (Table 3.1 and 3.2). Sensitivity was the fraction of known T cell-presented peptides which were predicted by each algorithm. Efficiency was the fraction of the predictions which correctly identify T cell-presented peptides by an algorithm.

The sensitivity and efficiency of each algorithm to predict T cell-presented sequences were determined in terms of the degree of two measures of proximity, varying in stringency. The more stringent level "overlapping" occurred when the intersection of the experimental and predicted segments was 50% or more of the union of the two segments. For example, if the true segment occupied sequence positions 1-10 and the prediction occupied positions 4-13, then the union of the two covered 1-13, while the intersection covered 4 through 10 (7 positions), and 7/13 exceeded 50%. At the less stringent level, "touching" was defined as a non-empty intersection of two segments, the predicted and the known.
The two levels of stringency were "nested" in the sense that touching included overlap.

Table 3.1 and 3.2 shows one of such analyses with 5 well-studied proteins. At the more stringent level of comparison, SOHHA was more sensitive (0.43) than the amphipathicity (0.29) and motifs (0.0 and 0.0) predictions, and more efficient (0.35) than amphipathicity (0.14) and motifs (0.0 and 0.0) predictions. At the less stringent, touching criterion, the amphipathicity (0.71) was as sensitive as Rothbard-5 (0.79) and more sensitive than SOHHA (0.57) and Rothbard-4 (0.43). At that criterion, the SOHHA was more efficient (0.47) than the amphipathicity (0.36) and motifs (0.25, 0.40) methods.
TABLE 3.1
Experimentally Determined and Predicted T-cell Epitopes
for Five Well-Studied Proteins

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### TABLE 3.2
Comparisons of Methods to Predict T-cell Epitopes, Evaluated with Five Well-studied Proteins

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</tr>
<tr>
<td>Overlapping</td>
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<td>0.29</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
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<tr>
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<td>0.71</td>
<td>0.79</td>
<td>0.43</td>
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<tr>
<td>Efficiency&lt;sup&gt;§&lt;/sup&gt;</td>
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<tr>
<td>Overlapping</td>
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<td>0.14</td>
<td>0.00</td>
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</tr>
<tr>
<td>Touching</td>
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<td>0.36</td>
<td>0.25</td>
<td>0.40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Chicken ovalbumin, chicken lysozyme, horse cytochrome, sperm whale myoglobin and staphylococcal nuclease.

<sup>oo</sup> Numbers shown in parenthesis are SD.

<sup>†</sup> Correct predictions/number of known epitopes.

<sup>§</sup> Correct predictions/number of predictions.
B. Structural Studies of a Group of HPLC-Purified Peptides

**Peptide description.** In order to test whether the axial, hydrophobic strip *per se* could promote helix formation, the secondary structures of a series of HPLC-purified peptides, which were prepared for different purposes and varied in likelihood for T cell presentation according to experimental reports and predictive algorithms, were evaluated by CD analysis in phosphate buffer, in phosphate buffer with 45% trifluoroethanol (TFE), and in phosphate buffer with di-O-hexadecyl phosphatidylcholine (DHPC) vesicles.

The sequences of these peptides varied greatly (Table 3.3) as did their SOHII and mean hydrophilicity (Table 3.4) values. Influenza virus matrix protein (57-72) and nucleoprotein (336-356) were presented by human class I MHC alleles to cytotoxic T lymphocytes (Gotch et al., 1987; McMichael et al., 1986). Influenza virus hemagglutinin (493-513) was predicted by the SOHHA to be a T cell-presented peptide and touched the sequence of the H-2K\(^d\)-restricted T cell epitope hemagglutinin (512-528).

Two peptides (MA-2 and MA-3) from the Epstein-Barr virus membrane antigen (EBV MA) gp350/220 were selected for development of neutralizing antibodies based on their high hydrophilicity in a Hopp-Woods analysis and the presence of prolines which might create stable \(\beta\)-turns in both the native
### TABLE 3.3
Sequence of Peptides

<table>
<thead>
<tr>
<th>PEPTIDE NAME</th>
<th>ABBREVIATION</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prototypic</td>
<td>PH-1.0</td>
<td>LYQELQKLTQTLK</td>
</tr>
<tr>
<td>Invariant chain (148-164, Tyr162)</td>
<td>li-3</td>
<td>ENLRHLKNTMETIDYKV</td>
</tr>
<tr>
<td>Invariant chain (Tyr + 78-92)</td>
<td>li-4</td>
<td>YRMKLPKPPKPVSFKMR</td>
</tr>
<tr>
<td>Influenza hemagglutinin (513-528)</td>
<td>HA</td>
<td>NREKVDGVKLESMSGIY</td>
</tr>
<tr>
<td>Influenza matrix protein (57-72, +Tyr)</td>
<td>MX</td>
<td>KGILGFVFTLVPSERY</td>
</tr>
<tr>
<td>Influenza nucleoprotein (336-355, +Yr)</td>
<td>NP</td>
<td>AAFEDLRVLSFIRGTVSPRY</td>
</tr>
<tr>
<td>EBV MA gp350/220 (607-816)</td>
<td>MA-2</td>
<td>STTPRPRYNA</td>
</tr>
<tr>
<td>EBV MA gp350/220 (718-729, +Yr)</td>
<td>MA-3</td>
<td>SSPEPPGTTSEY</td>
</tr>
<tr>
<td>Murine CD-2 (88-101, +Tyr)</td>
<td>MT11-A</td>
<td>GTNGMTRLEKDLDVY</td>
</tr>
</tbody>
</table>
protein and the KLH-conjugated peptides. Both MA-2 and MA-3 had low SOHHI values and multiple prolines which might be against helix formation.

Two peptides from invariant chain sequence were included. II-3 was synthesized to test the hypothesis that this segment of II was an amphipathic helix which might cover the class II MHC desetope until charging of a foreign peptide (Elliott et al., 1987a, 1987b). II-4 was synthesized to raise an anti-II antiserum because of its almost palindromic sequence highlighted by 6 positively charged residues on a tightly kinked structure induced by 4 prolines.

PH-1.0 was designed as a prototypic helix model to test the hypothesis of SOHII. It was constructed with a narrow and strong hydrophobic strip with 4 leucines along one side of a putative α-helix. MT11-A was synthesized from the murine CD2 sequence and used as a negative control for PH-1.0 because MT11-A had a mean hydrophilicity very similar to PH-1.0, but its maximal SOHII was low (Table 3.4).

Results of CD analysis. Structural order of the peptides are summarized in Table 3.5. In phosphate buffer all of the nine peptides in this group were in a random coil conformation. This was expected because none of the peptides here was end-blocked and unblocked peptides were reported to
### TABLE 3.4
SOHII and Mean Hydrophilicity of Peptides

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>SOHII*</th>
<th>MEAN HYDROPHILICITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH-1.0</td>
<td>3.80</td>
<td>+ 0.72</td>
</tr>
<tr>
<td>li-3</td>
<td>3.64</td>
<td>+ 1.01</td>
</tr>
<tr>
<td>li-4</td>
<td>1.40</td>
<td>+ 1.33</td>
</tr>
<tr>
<td>HA</td>
<td>1.72</td>
<td>+ 0.66</td>
</tr>
<tr>
<td>MX</td>
<td>1.80</td>
<td>- 0.49</td>
</tr>
<tr>
<td>NP</td>
<td>1.92</td>
<td>+ 0.02</td>
</tr>
<tr>
<td>MA-2</td>
<td>0.70</td>
<td>+ 1.39</td>
</tr>
<tr>
<td>MA-3</td>
<td>0.93</td>
<td>+ 1.67</td>
</tr>
<tr>
<td>MT11-A</td>
<td>1.56</td>
<td>+ 0.81</td>
</tr>
</tbody>
</table>

* The maximal SOHII through the most hydrophobic strip found in sheet projections of each peptide.
<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>Phosphate Buffer</th>
<th>TFE</th>
<th>Lipid Vesicles</th>
</tr>
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<tr>
<td></td>
<td>[θ]222nm</td>
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<td>[θ]222nm</td>
</tr>
<tr>
<td></td>
<td>Helicity (%)</td>
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<td>Helicity (%)</td>
</tr>
<tr>
<td>PH-1.0</td>
<td>-918</td>
<td>-19,660</td>
<td>58</td>
</tr>
<tr>
<td>Ii-3</td>
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<td>51</td>
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<td>Ii-4</td>
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<tr>
<td>HA</td>
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<td>MK</td>
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<tr>
<td>NP</td>
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<td>-22,070</td>
<td>64</td>
</tr>
<tr>
<td>MA-2</td>
<td>-4,227</td>
<td>-5,210</td>
<td></td>
</tr>
<tr>
<td>MA-3</td>
<td>-2,509</td>
<td>-3,722</td>
<td></td>
</tr>
<tr>
<td>MT11-A</td>
<td>-1,520</td>
<td>-1,600</td>
<td></td>
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</tbody>
</table>

* Percentage helicity was calculated only for peptides with CD spectra shown clear α-helical pattern.
not form stable α-helix in aqueous solution (Bierzynsky et al., 1982; Shoemaker et al., 1987).

In 45% TFE buffer, CD spectra showed three groups of peptides, with high, moderate or low helix-forming tendencies. By the formula of Kaiser et al. (1987), PH-1.0, Ii-3, and NP had 58%, 51% and 64% calculated helicities, respectively. CD spectra with helical pattern were also found with HA and MX peptides, with 23% and 18% calculated helicities, respectively. Peptides with multiple prolines (Ii-4, MA-2, MA-3) or with relatively low SOHHi (MT11-A) did not show recognizable α-helical CD spectra. Each experiment was repeated at least 3 times and the [θ]222nm values were collected as the average of 3-5 measurements. Fig.3.2 shows CD spectra of NP, HA and Ii-4 in both phosphate buffer and TFE solution, representing high, moderate and low helix formation respectively.

Structural order of peptides in the presence of lipid vesicles was further tested to assess the degree to which hydrophobic surface might catalyze formation of helices or other structures. The lipid vesicles were made by a simple technique described by Vogel (1987, 1988) and had negligible contribution to the CD spectrum when the lipid vesicles were measured alone (Fig.3.3). Excepting MX, all the other peptides showing helix formation in TFE solution maintained their helix-forming tendencies in the presence of lipid.
vesicles, but with a lower percentage of helicity (Table 3.5). The prototypic helix peptide PH-1.0 maintained 36% helix formation in lipid vesicles in comparison with 58% in TFE, while other peptides had less than half of the helicity formed in lipid vesicles than that in TFE solution. The CD spectrum of peptide MX in lipid vesicles was typical of β-sheet structure. Its pattern could not rule out a minor degree of helical content, but did indicate a stronger β-sheet component. Ii-4, MA-2, MA-3 and MT11-A still did not show a helical pattern in the presence of lipid vesicles. CD spectra of NP, MX and MA-2 in lipid vesicles are shown in Fig.3.3, representing α-helix, β-sheet and random coil structures, respectively.

The results from lipid vesicles experiments suggested that lipid vesicles, resembling natural conditions, might represent a more stringent helix inducing environment than TFE which, by decreasing the dielectric constant of the buffer, only enhanced intrapeptide interactions generally. In the presence of lipid vesicles, the peptides might depend more on their SOHII to form a helical structure than in TFE. Among peptides forming α-helices in the presence of vesicles, percentage helicities calculated from the CD spectrum were proportional to the SOHII (r=0.77) (Fig.3.4). With the sample size of 5, the p value came close to significance, p=0.07.
Fig. 3.2  Circular dichroism spectra of NP, HA, li-4 in 10 mM phosphate buffer (B) and that buffer with 45% TFE (T) at room temperature.
Fig. 3.3  Circular dichroism spectra of NP, MX, li-4 in the presence of lipid vesicles (V) at room temperature. The unmarked curves are the vesicles-only control spectra.
Fig. 3.4 Correlation between percentage helicity and SOHHi. Percentage helicity (Table 3.5) in the presence of vesicles is plotted for PH-1, II-3, HA, NP, and MT11-A as a function of maximal SOHHi (Table 3.4).
C. Structural Studies of Prototypic Helix Peptide PH-1.0

Peptide design. PH-1.0 was designed in order to establish a putative model to test the validity of SOHHI theory and to examine important biophysical principles governing the formation of peptide secondary structures when the peptide was in close contact with a hydrophobic surface. As the initial step, PH-1.0 was designed with simply 6 types of amino acids (Fig.3.5). If PH-1.0 could coil as an \( \alpha \)-helix, it would have an axial strip of maximal hydrophobicity made by 4 leucine residues. A potential salt bridge between Glu\(^4\) and Lys\(^7\) could link the helical loops of the peptide. Putting a negatively charged residue (Glu\(^4\)) close to the N-terminus and a positively charged residue (Lys\(^13\)) at the C-terminus could help stabilize the \( \alpha \)-helical structure of the peptide through favorable interactions with the macrodipole of the helix. The inclusion of a C-terminal lysine residue was also consistent with the finding that many T cell-presented peptides had a lysine at or near the carboxyl terminus (Spouge et al., 1987). Tyr\(^2\) could serve two purposes: (a) radioiodination of the peptide for other biochemical studies, such as the binding of PH-1.0 to purified MHC molecules; (b) spin-labeling of the peptide by a nitroxide-containing molecule to allow electron spin resonance (ESR) study of interactions between the peptide and
Fig. 3.5  Sheet projection of the sequence of prototypic helix peptide PH-1.0.
a hydrophobic surface. Relatively hydrophilic, uncharged residues, glutamine and threonine, were placed in other positions of the PH-1.0 sequence. We did not block N- and C-termini of the peptide because our major interest was in mechanisms for the scavenging of proteolytic fragments of protein antigen during antigen processing and presentation.

**Working hypothesis.** We hypothesized that the helix formation of a short peptide might be catalyzed by the helix stabilization effect of anchoring hydrophobic residues on the successive loops of a growing helix to a nearby hydrophobic surface. Fig.3.6 depicts such a process with PH-1.0 as the model peptide.

**PH-1.0 in phosphate buffer and in lipid vesicles.** The secondary structure of PH-1.0 was measured by CD in phosphate buffer and in phosphate buffer with di-O-hexadecyl phosphatidylcholine (DHPC) vesicles with MT11-A as the control peptide (Fig.3.7). PH-1.0 and MT11-A were similar in several aspects, including length (Table 3.3), mean hydrophilicity (Table 3.4), retention time in the same reverse phase-HPLC column (Fig.3.8), but differed in their SOHHI values (Table 3.4) as a result of the different distribution patterns of hydrophobic residues (Fig.3.9).
Fig. 3.6 Helix formation can be stabilized by recurrent hydrophobic residues which create an axial, hydrophobic strip when the helix is formed against a hydrophobic surface.
Fig. 3.7  Circular dichroism spectra of PH-1.0 and MT11-A in 10 mM phosphate buffer (B) and in that buffer with lipid vesicles (V) at room temperature. The unmarked curves are the vesicles only control spectra.
Fig. 3.8 Chromatograms of purified PH-1.0 (upper panel) and MT11-A (lower panel) analyzed by a same reverse phase HPLC system using 0.1% TFA as the starting buffer and increment of ACN at 1%/min, flow rate 1 ml/min.
Fig. 3.9  Wheel plots for PH-1.0 and MT11-A. The hydrophobic residues are shown in bold fonts.
In 0.01 M phosphate buffer alone both PH-1.0 and MT11-A could not form an α-helix while in the presence of lipid vesicles PH-1.0, but not MT11-A, demonstrated a distinct helical CD spectrum (Fig.3.7). Thus, the ability of a peptide to form an α-helical structure in lipid vesicles appeared to relate to the pattern of recurrent hydrophobic residues in the sequence.

The possibility of aggregation of PH-1.0 peptide was tested by measuring the molar ellipticity of PH-1.0 at high concentrations of salt and/or peptide. The results showed that no helicity was induced when PH-1.0 concentration was increased either from 82 μg/ml to 400 μg/ml in 0.2 M phosphate buffer or from 82 μg/ml to 200 μg/ml with the addition of 1 M KCl (Fig.3.10). Thus, helicity under these conditions could not be induced via self-association of PH-1.0 through their axial hydrophobic strips.

**PH-1.0 in TFE solution.** PH-1.0 could form α-helix in TFE solution (Fig.3.11). CD measurements were carried out immediately after the addition of various amount of TFE to the non-helical peptide sample in phosphate buffer and the CD spectra demonstrated that the transition from random coil to helix had been completed (Fig.3.11). TFE was used as a stabilizer to maintain the stability of intermediates in the peptide folding reaction and this stability might be
important in both helix initiation and propagation. The higher the concentration of TFE, the more peptides formed α-helix (Table 3.6).

**Temperature dependence of helicity.** Helix formation for PH-1.0 in TFE or in lipid vesicles was temperature-dependent with a higher percentage of α-helix formation at lower temperatures (Table 3.7, Fig.3.12). In TFE, there was a smooth decrease of helicity found in the CD spectra from 4°C (76% helicity) to 75°C (38% helicity). In the presence of lipid vesicles, PH-1.0 formed 32% helicity at 25°C while no helicity was observed when the temperature was raised to above 50°C, perhaps reflecting the changing organization of the lipid vesicles.

The clean isodichroic point at 202 nm in CD spectra of peptides in TFE or lipid vesicles (Fig.3.12) suggested that the transition from α-helical to random-coil was two-state, so that each amino acid was in either a helical state or a random-coil state.
Fig. 3.10  CD spectra of PH-1.0 in 0.2 M phosphate buffer with peptide concentration at 82 mg/ml (L), at 400 mg/ml (H) and at 200 mg/ml with addition of 1 M KCl (K).
Fig. 3.11  CD spectra of PH-1.0 and MT11-A in 10 mM phosphate buffer (B) and that buffer with 45% TFE (T) at room temperature.
TABLE 3.6
Percentage Helicity Formed in Different TFE Concentrations

<table>
<thead>
<tr>
<th>TFE*</th>
<th>PH-1.0</th>
<th>MT11-A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[θ]222 nm</td>
<td>% Helicity</td>
</tr>
<tr>
<td>0%</td>
<td>-1,849</td>
<td>-</td>
</tr>
<tr>
<td>30%</td>
<td>-21,530</td>
<td>63</td>
</tr>
<tr>
<td>45%</td>
<td>-22,240</td>
<td>65</td>
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</table>

* Expressed as percentage of TFE in 10 mM phosphate buffer.
Fig. 3.12 CD spectra of PH-1.0. Panel A: 10 mM phosphate buffer alone (B) and with PH-1.0 (PB) at 4°C. Panel B: PH-1.0 in 10 mM phosphate buffer with 30% TFE at different temperatures. Panel C: lipid vesicles alone (V) and with PH-1.0 at 4°C. Panel D: PH-1.0 in lipid vesicles at different temperatures. Spectra shown in panels A and C are raw data unadjusted by peptide concentration.
### TABLE 3.7
Percentage Helicity of PH-1.0 at Different Temperatures

<table>
<thead>
<tr>
<th>Temperature</th>
<th>30% TFE</th>
<th>Lipid Vesicles</th>
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<td></td>
<td>[θ]222 nm</td>
<td>% Helicity</td>
</tr>
<tr>
<td>40°C</td>
<td>-26,490</td>
<td>76</td>
</tr>
<tr>
<td>25°C</td>
<td>-21,510</td>
<td>63</td>
</tr>
<tr>
<td>50°C</td>
<td>-16,060</td>
<td>49</td>
</tr>
<tr>
<td>75°C</td>
<td>-11,860</td>
<td>38</td>
</tr>
</tbody>
</table>
D. Structural studies of PH-1.0 analogs.

Analogs of PH-1.0. The strong helix-forming tendency of PH-1.0 prompted us to design more analogs of PH-1.0 to analyze systematically the contribution of each residue to the formation of a helical structure in the presence of lipid vesicles. As part of a general plan, at least the following analogs should be considered.

Leucine residues in the axial hydrophobic strip of PH-1.0 could be replaced with non-hydrophobic, non-bulky residues such as threonines (Fig.3.13). Such a replacement could be made simply at one leucine or jointly at two or more leucines at the same time. This type of change was expected to lead to a decrease in SOHII and thus to a decrease in the helix-forming tendencies of the peptides. It could also be shown whether the four leucines were equally important in catalyzing the formation of an α-helix in lipid vesicles.

The next set of analogs (Fig.3.14) was designed to test whether the proposed salt bridge Glu4-Lys7 did contribute to the α-helix formation for PH-1.0 and whether the positioning with the negatively charged residue at N-terminal and positively charged residue at C-terminal played an important role in stabilizing a helical structure.

Another way to show that the α-helix formation of PH-1.0 depended directly on SOHII was to keep the overall
<table>
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<tbody>
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<tr>
<td>II</td>
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</tr>
<tr>
<td>III</td>
<td>1. Leu</td>
<td>5. Leu</td>
<td>8. Leu</td>
</tr>
<tr>
<td>IV</td>
<td>2. Tyr</td>
<td></td>
<td>9. Thr</td>
</tr>
</tbody>
</table>

- 1. Thr-Tyr-Gln-Glu-Leu-Gln-Lys-Leu-Thr-Gln-Thr-Leu-Lys
- 2. Leu-Tyr-Gln-Glu-Thr-Gln-Lys-Leu-Thr-Gln-Thr-Leu-Lys
- 3. Leu-Tyr-Gln-Glu-Leu-Gln-Lys-Thr-Thr-Gln-Thr-Leu-Lys
- 4. Leu-Tyr-Gln-Glu-Leu-Gln-Lys-Leu-Thr-Gln-Thr-Thr-Lys
- 5. Thr-Tyr-Gln-Glu-Leu-Gln-Lys-Leu-Thr-Gln-Thr-Leu-Lys
- 6. Thr-Tyr-Gln-Glu-Leu-Gln-Lys-Thr-Thr-Gln-Thr-Leu-Lys
- 7. Thr-Tyr-Gln-Glu-Leu-Gln-Lys-Leu-Thr-Gln-Thr-Thr-Lys
- 8. Leu-Tyr-Gln-Glu-Thr-Gln-Lys-Leu-Thr-Gln-Thr-Leu-Lys
- 9. Leu-Tyr-Gln-Glu-Thr-Gln-Lys-Leu-Thr-Gln-Thr-Thr-Lys
- 10. Leu-Tyr-Gln-Glu-Leu-Gln-Lys-Thr-Thr-Gln-Thr-Leu-Lys
- 11. Thr-Tyr-Gln-Glu-Thr-Gln-Lys-Thr-Thr-Gln-Thr-Leu-Lys
- 12. Leu-Tyr-Gln-Glu-Thr-Gln-Lys-Thr-Thr-Gln-Thr-Thr-Lys
- 13. Thr-Tyr-Gln-Glu-Thr-Gln-Lys-Leu-Thr-Gln-Thr-Thr-Lys
- 14. Thr-Tyr-Gln-Glu-Thr-Gln-Lys-Thr-Thr-Gln-Thr-Thr-Lys

Fig. 3.13 Analogs of PH-1.0 with changes at the axial hydrophobic strip.
2.1. Leu-Tyr-Gln-Thr-Leu-Gln-Lys-Leu-Thr-Gln-Thr-Leu-Lys
2.2. Leu-Tyr-Gln-Glu-Leu-Gln-Thr-Leu-Thr-Gln-Thr-Leu-Lys
2.3. Leu-Tyr-Gln-Thr-Leu-Gln-Thr-Leu-Thr-Gln-Thr-Leu-Lys
2.4. Leu-Tyr-Gln-Glu-Leu-Gln-Thr-Leu-Gln-Thr-Leu-Lys

2.5. Leu-Tyr-Gln-Lys-Leu-Gln-Lys-Leu-Thr-Gln-Thr-Leu-Lys
2.6. Leu-Tyr-Gln-Glu-Leu-Gln-Glu-Leu-Thr-Gln-Thr-Leu-Lys
2.7. Leu-Tyr-Gln-Lys-Leu-Gln-Glu-Leu-Thr-Gln-Thr-Leu-Lys

2.8. Leu-Tyr-Gln-Glu-Leu-Gln-Lys-Leu-Thr-Gln-Thr-Leu-Thr
2.9. Leu-Tyr-Gln-Glu-Leu-Gln-Lys-Leu-Thr-Gln-Thr-Leu-Glu
2.10. Leu-Tyr-Gln-Glu-Leu-Gln-Thr-Leu-Thr-Gln-Thr-Leu-Thr
2.11. Leu-Tyr-Gln-Thr-Leu-Gln-Lys-Leu-Thr-Gln-Thr-Leu-Thr
2.12. Leu-Tyr-Gln-Thr-Leu-Gln-Thr-Leu-Thr-Gln-Thr-Leu-Thr
2.13. Leu-Tyr-Gln-Lys-Leu-Gln-Glu-Leu-Thr-Gln-Thr-Leu-Glu

Fig. 3.14 Analogs of PH-1.0 with changes made to break the potential salt bridge and to counteract with the macrodipole of peptide.
composition of the peptide but to exchange residues between the hydrophobic strip and its opposite hydrophilic strip, such as PH-3.1 shown in Fig.3.15.A. However, this analog involved two changes at the same time and thus it might be necessary to first test what would be the effect of each change alone (PH-3.2 and PH-3.3) to the structure of peptide (Fig.3.15.A).

Since we hypothesized that the axial, hydrophobic strip in PH-1.0 stabilized an α-helical conformation against lipid vesicles, it was worthwhile to test whether alternatively placed hydrophobic residues in other positions might compete to decrease the helical structure component of a peptide. In PH-1.0, such analogs (e.g., PH-4.2) could be produced by substituting glutamines at quadrant I with leucine residues (Fig.3.15.B). Some of these analogs might have to break the potential salt bridge Glu⁴-Lys⁷ in order to achieve a competitive effect and this could be tested by simultaneously making changes at position 4 or 7 with uncharged residues.

Nine peptides were synthesized from above possible analogs (Fig.3.16) with major interest on the number and placement of hydrophobic residues required to induce PH-1.0 to form a helical structure in lipid vesicles. Thus, only one or two changes were made with hydrophobic residues for each peptide, leaving the salt bridge and other important structural features untouched. We did not synthesize all the
### Analogs of PH-1.0 with alternately placed hydrophobic residues to compete with the axial hydrophobic strip.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
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<td>Tyr</td>
<td>Gln</td>
<td>Gln</td>
</tr>
<tr>
<td>2</td>
<td>Leu</td>
<td>Tyr</td>
<td>Gln</td>
<td>Gln</td>
</tr>
<tr>
<td>3</td>
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<td>Tyr</td>
<td>Gln</td>
<td>Gln</td>
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<tr>
<td>6</td>
<td>Leu</td>
<td>Tyr</td>
<td>Gln</td>
<td>Gln</td>
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</table>

**A.**

3.1. Leu-Tyr-Gln-Glu-Leu—**Leu**—Lys—**Gln**—Thr—Gln—Thr—Leu—Lys
3.2. Leu-Tyr-Gln-Glu-Leu—**Leu**—Lys—Leu—Thr—Gln—Thr—Leu—Lys
3.3. Leu-Tyr-Gln-Glu-Leu—Gln—Lys—Leu—Thr—Gln—Thr—Leu—Lys

**B.**

4.1. Leu-Tyr-Gln-Glu-Leu—**Leu**—Lys—Leu—Thr—Gln—Thr—Leu—Lys
4.2. Leu-Tyr—**Leu**—Glu-Leu—Gln—Lys—Leu—Thr—Gln—Thr—Leu—Lys
4.3. Leu-Tyr—Gln—Glu-Leu—Gln—Lys—Leu—Thr—**Leu**—Thr—Leu—Lys

4.4. Leu-Tyr—Gln—**Thr**—Leu—**Leu**—Lys—Leu—Thr—Gln—Thr—Leu—Lys
4.5. Leu-Tyr—**Leu**—Thr—Leu—Gln—Lys—Leu—Thr—Gln—Thr—Leu—Lys
4.6. Leu-Tyr—Gln—**Thr**—Leu—Gln—Lys—Leu—Thr—**Leu**—Thr—Leu—Lys

4.7. Leu-Tyr—Gln—Glu-Leu—**Leu**—Thr—Leu—Thr—Gln—Thr—Leu—Lys
4.8. Leu-Tyr—Leu—Glu—Leu—Gln—**Thr**—Leu—Thr—Gln—Thr—Leu—Lys
4.9. Leu-Tyr—Gln—Glu—Leu—Gln—**Thr**—Leu—Thr—**Leu**—Thr—Leu—Lys

4.10. Leu-Tyr—**Leu**—Glu—Leu—**Leu**—Lys—Leu—Thr—Gln—Thr—Leu—Lys

---

**Fig. 3.15** Analogs of PH-1.0 with alternately placed hydrophobic residues to compete with the axial hydrophobic strip.
peptides with two or more leucines substituted as originally proposed in Fig.3.13 because the current set should be sufficient to achieve same goal, and practically it would be too expensive to synthesize all the analogs. However, we did synthesize PH-4.2 as an initial step to test the competitive force against the axial hydrophobic strip to interact with lipid vesicles.

**CD spectra of analogs in phosphate buffer and in TFE.** None of the analogs, as with PH-1.0, formed α-helix in 10 mM phosphate buffer alone (Fig.3.17). In 10 mM phosphate buffer with 45% TFE, all the analogs exhibited 35-43% helicity (Fig.3.17, Table 3.8). Two of the analogs, PH-1.1 and PH-4.2, were further analyzed for their temperature dependent patterns of helix formation in TFE solution. For both peptides, the CD spectra showed a smooth decrease of helicity from 4°C to 75°C and again a clean isodichroic point at 202 nm was found (Fig.3.18). In comparison with PH-1.0 (Table 3.7), these two analogs consistently formed less helicity at same temperature (Table 3.9), suggesting they might not coil as well as PH-1.0 in these experimental conditions.
<table>
<thead>
<tr>
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<tr>
<td>PH-1.1</td>
<td>T Y Q E L Q K L T Q T L K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH-1.2</td>
<td>L Y Q E T Q K L T Q T L K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH-1.3</td>
<td>L Y Q E L Q K T T Q T L K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH-1.4</td>
<td>L Y Q E L Q K L T Q T T K</td>
<td></td>
<td></td>
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<td>PH-1.5</td>
<td>T Y Q E T Q K L T Q T L K</td>
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<tr>
<td>PH-1.6</td>
<td>T Y Q E L Q K T T Q T L K</td>
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<td></td>
</tr>
<tr>
<td>PH-1.7</td>
<td>T Y Q E L Q K L T Q T T K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH-1.10</td>
<td>L Y Q E L Q K T T Q T T K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH-4.2</td>
<td>L Y L E L Q K L T Q T L K</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3.16 Schematic drawing of the placement of hydrophobic residues (short bars) and the Thr substitutes (circles) for synthesized set of PH-1.0 analogs. Molecular weight of peptides:
- PH-1.0, 1605.7;
- PH-1.1 to 1.4, 1593.6;
- PH-1.5 to 1.7 and 1.10, 1581.5;
- PH-4.2, 1590.9.
Fig. 3.17  CD spectra of analog peptides in 10 mM phosphate buffer (B) and that buffer with 45% TFE (T) at room temperature.
Fig. 3.18  CD spectra of PH-1.1 and PH-4.2 in 10 mM phosphate buffer with 30% TFE at different temperatures.
### TABLE 3.8
Percentage Helicity of Analog Peptides in 45% TFE

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>[θ]_{222} nm</th>
<th>Percentage Helicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH-1.1</td>
<td>-13,770</td>
<td>43</td>
</tr>
<tr>
<td>PH-1.2</td>
<td>-13,640</td>
<td>43</td>
</tr>
<tr>
<td>PH-1.3</td>
<td>-11,220</td>
<td>36</td>
</tr>
<tr>
<td>PH-1.4</td>
<td>-10,800</td>
<td>35</td>
</tr>
<tr>
<td>PH-1.5</td>
<td>-10,810</td>
<td>35</td>
</tr>
<tr>
<td>PH-1.6</td>
<td>-11,390</td>
<td>37</td>
</tr>
<tr>
<td>PH-1.7</td>
<td>-12,890</td>
<td>41</td>
</tr>
<tr>
<td>PH-1.10</td>
<td>-11,110</td>
<td>36</td>
</tr>
<tr>
<td>Temperature</td>
<td>PH-1.1</td>
<td>PH-4.2</td>
</tr>
<tr>
<td>-------------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td></td>
<td>[θ]222 nm</td>
<td>% Helicity</td>
</tr>
<tr>
<td>4°C</td>
<td>-18,240</td>
<td>54</td>
</tr>
<tr>
<td>25°C</td>
<td>-15,080</td>
<td>46</td>
</tr>
<tr>
<td>50°C</td>
<td>-10,100</td>
<td>34</td>
</tr>
<tr>
<td>75°C</td>
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<td>26</td>
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</table>
CD spectra of analogs in lipid vesicles. The eight analogs with systematic substitution of threonine for either one or two leucines in the longitudinal hydrophobic strip demonstrated quite diverse CD spectra when the peptides were measured in lipid vesicles. While none of these eight analogs could show a detectable helical CD spectrum at room temperature, at least three of them formed helical structure when the experiments were performed at 4°C (Fig.3.19, Table 3.10). PH-1.4 clearly had helix formation with 34% calculated helicity. PH-1.1 could also coil as α-helix with 26% helicity. PH-1.7, with one minimum shifted to above 200 nm and a detectable minimum at 222 nm in comparison with other analogs, at least partially formed a helical structure with 21% helicity calculated from its [θ]222 value.

PH-4.2 can form α-helix structure in lipid vesicles at both room temperature and 4°C (Fig.3.20). However, less helicity was formed for PH-4.2 in comparison with PH-1.0 in lipid vesicles (Table 3.11).
Fig. 3.19 CD spectra of analog peptides in the presence of lipid vesicles at room temperature (left panels) and at 4°C (right panels).
TABLE 3.10  
Percentage Helicity of Analog Peptides in Lipid Vesicles

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>[θ]222 nm</th>
<th>Percentage Helicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH-1.1</td>
<td>-7,264</td>
<td>26</td>
</tr>
<tr>
<td>PH-1.4</td>
<td>-10,280</td>
<td>34</td>
</tr>
<tr>
<td>PH-1.7</td>
<td>-5,259</td>
<td>21</td>
</tr>
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</table>
Fig. 3.20  CD spectra of PH-4.2 in the presence of lipid vesicles at room temperature (upper panel) and 4°C (lower panel).
<table>
<thead>
<tr>
<th>Temperature</th>
<th>PH-1.0</th>
<th>PH-4.2</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>[θ]222 nm</td>
<td>% Helicity</td>
</tr>
<tr>
<td>4°C</td>
<td>-12,490</td>
<td>40</td>
</tr>
<tr>
<td>25°C</td>
<td>-9,422</td>
<td>32</td>
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</table>
E. Functional Studies on PH-1.0

Once we found that PH-1.0 could coil as α-helix in lipid vesicles, we asked whether PH-1.0 have the function to compete with other known T cell-presented peptides to block the antigen presentation. Dr. Dolores Jaraquemada in Dr. Eric Long's lab (NIH) tested the PH-1.0 peptide I prepared in a DR 1-restricted cytotoxicity test. EBV-transformed cell line 45.1 (DR 1) was used as target cell. T cell lines 109.2B2 and 130.1C6 were HLA DR 1-restricted and could recognize M1 17-31, an influenza M1 matrix protein derived peptide (Jaraquemada et al., 1990) (Fig. 3.21). CTL 109.2B2 could lyse target cells treated with antigen peptide M1 17-31 (Fig. 3.22.A). Such antigen specific lysis was inhibited by another DR 1-restricted peptide HA 307-318 but not by a negative control peptide ATIII (Fig. 3.22.B). HA 307-318 inhibited original lysis by 60%, which was the maximum level expected (Shimojo et al., 1989). PH-1.0 inhibited the lysis at the same level as H3 307-318 (Fig. 3.22.B) even though treatment of the target cells with PH-1.0 alone could not induce a significant lysis (Fig. 3.22.C). Similar results were produced by using a different CTL line, 130.1C6 (Fig. 3.23).
M1 17-31

I   PRO   ALA   GLN   GLU
II   GLU   ARG   ASP
III  SER   LEU   ISO   LEU   VAL
IV   GLY   LYS   ALA

Fig. 3.21  Sheet projection of the sequence of M1 17-31.
Fig. 3.22  Competitive inhibition of presentation of M1 17-31 by PH-1.0 peptide. DR1-restricted M1 17-31 specific CTL 109.2B2 was assayed on 45.1 targets. In the control condition (A,C), targets were either not exposed to peptide (○), or exposed to 1 mM M1 17-31 (□), 1 mM negative control peptide ATIII (○) or 1 mM PH-1.0 (△) for 18 hr at 37°C, washed three times, and assayed for their ability to be lysed by an DR1-restricted CTL 109.2B2. Under the competitive inhibition conditions (B), targets were exposed to combinations of 1 mM M1 17-31 peptide plus 450 mM of either PH-1.0 (△), or another DR1-restricted peptide HA 307-318 (△) as a positive control, or the negative control peptide ATIII (○) for 18 hr at 37°C, washed three times, and assayed for their ability to be lysed by the CTL line 109.2B2.
Effector to target ratio

% Lysis

0 2 1 5 0.2 1 5

Panel A and C: no peptide (●), 1μM M1 17-31 (□), 1μM negative control peptide ATIII (○) or 1 μM PH-1.0 (▲). Panel B: combinations of 1 μM M1 17-31 peptide plus 450 μM of PH-1.0 (▲), plus positive control peptide HA 307-318 (△), or plus ATIII (○).

Fig. 3.23 Competitive inhibition of presentation of M1 17-31 by PH-1.0 peptide. Similar as Fig.3.21 except the DR1-restricted M1 17-31 specific CTL 130.1C6 was used instead of 109.2B2. Panel A and C: no peptide (●), 1μM M1 17-31 (□), 1μM negative control peptide ATIII (○) or 1 μM PH-1.0 (▲). Panel B: combinations of 1 μM M1 17-31 peptide plus 450 μM of PH-1.0 (▲), plus positive control peptide HA 307-318 (△), or plus ATIII (○).
CHAPTER IV

BIOCHEMICAL STUDIES OF INVARIANT CHAIN

A. Binding of II(146-164) to Purified Class II MHC Molecules.

**Hypothesized function of II(146-169).** In an analysis of the primary sequence of II, a hypothetical, amphipathic α-helix sequence was found from residue Phe\(^{146}\) to Met\(^{169}\) (Fig. 4.1). It had a distinctive strip of hydrophobic amino acids along one side of the helix and otherwise generally hydrophilic amino acids. Because it is quite unusual to have such a long (5-cycle), narrow hydrophobic strip, we hypothesized that this 24-residue region of II might be a sequence which bound to class II MHC molecules until dissociation in endosomes where processed foreign antigen could be charged onto the desetope (Elliott et al., 1987a, 1987b).

When the whole II protein sequence was subjected to screening by three different algorithms which selected T cell-presented peptides, the results further supported the above hypothesized function of this short II sequence. Although several sequences in II were selected by each method, this region in II was selected by all three methods (Fig. 4.2). This 24-residue sequence was well conserved between human and murine II sequences (Fig. 4.3), accepting
**Invariant Chain 146-169 (11-2)**

<table>
<thead>
<tr>
<th>I</th>
<th>GLU</th>
<th>ARG</th>
<th>ASN</th>
<th>THR</th>
<th>TRP</th>
<th>GLU</th>
<th>MET</th>
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<tr>
<td>II</td>
<td></td>
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<td>HIS</td>
<td>THR</td>
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<tr>
<td>III</td>
<td>PHE</td>
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<td>LEU</td>
<td>MET</td>
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<tr>
<td>IV</td>
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<td></td>
<td>LYS</td>
<td>GLU</td>
<td>ASP</td>
<td>PHE</td>
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**Invariant Chain 148-164 (11-3)**

<table>
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<td>THR</td>
<td>LYS</td>
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<tr>
<td>III</td>
<td>LEU</td>
<td>LEU</td>
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<td>LYS</td>
<td>GLU</td>
<td>ASP</td>
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</table>

Fig. 4.1 Sheet projection of peptides II-2(146-169) and II-3(148-164).
Fig. 4.2 Analysis of invariant chain sequence by various computer algorithms. The printed short lines represent the selections made by the respective algorithm.
**HUMAN li 146-169**

FPENLRLKNTMETIDWKVFESWM

**MURINE li 100-123**

---------K---------SDGVNI-----

Fig. 4.3  Comparison of proposed helical sequences in human and murine li
structurally similar residues among 7 positions which differed between these species. Thus, this segment might be important in maintaining some function of Ii.

**Ii-2 and Ii-3 peptides.** Two peptides from the putative helical sequence Ii(146-169) were synthesized (Fig.4.1). First, a 24-residue peptide Ii-2 was synthesized. However, the peptide yield was very low and only 1-2 mg of Ii-2 was purified, just enough for rabbit immunizations. A shorter version, Ii-3(148-164), was then synthesized based on the following rationales:

(a) Ii-3 started from Glu\(^{148}\) instead of Phe\(^{146}\) since putting a negatively charged residue closer to N-terminus might favor the \(\alpha\)-helix formation through its dipole. Since proline usually was a helix breaker, removal of Pro\(^{147}\) might also have a positive effect on the helix formation of the peptide as an immunogen.

(b) Ii-3 ended at Val\(^{164}\) with a positively charged Lys\(^{163}\) in longitudinal quadrant II and very close to the C-terminus. Thus, it both fitted the statistical finding of the positioning of C-terminal Lys in a T cell-presented peptide and had a favorable interaction with the helix dipole.

(c) Two pairs of potential salt bridges remained in Ii-3, i.e., Glu\(^{148}\)-His\(^{152}\), Lys\(^{154}\)-Glu\(^{158}\).
(d) A shorter peptide with the removal of several C-terminal residues with unknown roles (Phe\textsuperscript{165} to Met\textsuperscript{169}) would be easier to synthesize, as well as bringing focus up on the helical portion of the sequence.

(e) Since Trp\textsuperscript{162} was the only aromatic residue in Ii-3, it was replaced with a tyrosine for future radiiodination or spin-labeling with a nitroxide moiety. Again, because we were interested in antigen processing and presentation, Ii-3 was not end-blocked.

\textbf{CD spectra of Ii-3 peptide.} As shown by Table 3.5, Ii-3 did not coil as an \(\alpha\)-helix in 10 mM phosphate buffer, but it formed 51% and 18% helicity in 45% TFE and lipid vesicles, respectively. Fig. 4.4 shows the CD spectra of these experiments including the control CD spectrum with lipid vesicles alone.

\textbf{Ii-3 binds to purified class II MHC molecules.} The general plan of this experiment was shown in Fig. 4.5. By using a technique similar to that of Buus et al. (1986b), sufficient quantities of class II MHC molecules were purified to assess their binding of Ii-3. Several important modifications were made to ensure a high quality binding result.
Fig. 4.4 CD spectra of li-3 in 10 mM phosphate buffer (B), in that buffer with 45% TFE (T) and with lipid vesicles (V) at room temperature. The unmarked spectrum in the lower panel shows lipid vesicles alone.
Fig. 4.5 Experimental steps of Ii-3 binding to affinity purified class II MHC molecules from Jesthom cells.
First, anti-DR mAb L243 was selected because it could immunoprecipitate class II MHC molecules free of associated invariant chains. It was suggested by Shackelford et al. (1981) that L243 antibody could recognize a DR epitope that became exposed as these molecules matured. Immunoprecipitation of Raji cells with L243 antibody showed clear α and β chains of class II MHC with very little Ii (unpublished data). Before using L243, other anti-DR antibodies were tested in this type of experiment, but without success. One problem was that those antibodies could only precipitate the class II MHC/Ii complex which did not bind Ii-3 unless some mechanism could be found to deplete the Ii molecules from the complex.

Another major modification on the purification of class II MHC molecules was that an "antibody orientation kit" (Pierce) was used to make the immunoaffinity column. By conventional techniques, antibodies were randomly immobilized to activated beads with multiple-site attachment and possible misorientation of the immunoglobulin molecules, which reduced the efficiency of the Ab/Ag reaction. With the orientation kit which uses protein A-agarose, the Fc portion of the antibody molecules first bound to Protein A, allowing the antigen binding sites to be pointed outwards, and then the antibodies were crosslinked to immobilized Protein A.
The immunoaffinity column thus made with L243 mAb could have a higher binding efficiency to purify class II MHC molecules.

These methods produced a good purification of class II MHC molecules. After running L243-affinity column-purified materials in SDS-PAGE, the Coomassie blue staining of the gel showed a large amount of purified \( \alpha \) and \( \beta \) chains of class II MHC without detectable Ii molecules (Fig. 4.6).

The photoactivatable and heterobifunctional crosslinker N-hydroxysuccinimidyl-4-azidobenzoate (HSAB) was used. It was first coupled to Ii-3 at pH 7.4 in the dark, followed by iodination of the peptide. The iodinated Ii-3-HSAB, which was separated from free 125I in a P-6 DG (Bio-Rad) gel filtration column, was incubated with affinity-purified class II MHC for 48 hr at room temperature. At the end of the incubation, the bound molecules were crosslinked by exposure to short wavelength ultraviolet light for 30 min and the complexes were subjected to SDS-PAGE and autoradiography. Fig.4.7 demonstrated clear binding of Ii-3 to both \( \alpha \) and \( \beta \) chains of the class II MHC molecules. Cold Ii-3 peptides could compete with such binding (Fig.4.7, lane 3) and several copurified proteins beyond the \( \alpha/\beta \) complex range were not bound by the peptide (Fig.4.6 and 4.7), suggesting that the Ii-3 peptide binding to class II MHC molecules was specific. The binding appeared to be saturable because addition of more [125I]-labeled Ii-3 did not show significant increase of
binding (Fig. 4.7, lane 2). The nature of a higher molecular weight band (~60 kD) was not clear, but it could be an α/β dimer of class II MHC molecules (see more in DISCUSSION).
Fig. 4.6 1-D SDS-PAGE analysis of unlabeled, affinity purified class II MHC molecules from Jesthom cells. The gel was stained by Coomassie blue.
Fig. 4.7 1-D SDS-PAGE and autoradiography analysis of affinity-purified molecules from J esthom cells (100 nM) crosslinked by 50 nM HSAB-coupled, iodinated li-3 peptide with (lane 3) or without (lane 1) 5 μM noniodinated li-3, or 150 nM (lane 2) HSAB-coupled, iodinated li-3 peptide alone.
B. CS-Ii Synthesis in the Absence of Class II-MHC Molecules.

P3HR-1 does not express class II-MHC. Jijoye, and its mutant, daughter cell line, P3HR-1, came from a Burkitt's lymphoma patient (Hinuma et al., 1967). The lack of class II MHC proteins in P3HR-1 cells was shown by its failure to immunoprecipitate class II MHC molecules with the anti-human class II MHC antibodies anti-23,30 and SG157 (Spiro et al., 1985). Therefore, P3HR-1 was selected to study whether CS-Ii could be synthesized in the absence of class II MHC molecules.

First, the lack of class II MHC molecules in P3HR-1 cells was further confirmed with another anti-human class II MHC mAb IVA-12 which has a broad specificity against various kinds of human HLA-D products. While the parent cell line Jijoye clearly demonstrated the synthesis of class II MHC molecules, immunoprecipitation of detergent-solubilized, \( ^{35} \text{S} \) methionine-labeled, P3HR-1 microsomal membrane with IVA-12 did not show any detectable expression of either \( \alpha \) or \( \beta \) chains of class II MHC (Fig.4.8). The expression of \( ^{35} \text{S} \) methionine-labeled Ii molecules in both cell lines appeared to be at same level. Results from 2-D gel analysis confirmed the identities of the bands shown by 1-D SDS-PAGE (Fig.4.9). The strong band at ~43 kD was actin and was used
Fig. 4.8  SDS-PAGE and autoradiography analysis of immunoprecipitated, [35S]methionine-labeled microsomal membrane proteins from Jijoye and P3HR-1 cell lines.
Fig. 4.9 2-D nonequilibrium pH gradient electrophoresis (NEPHGE) and autoradiography analysis of immunoprecipitated, [35S]methionine-labeled microsomal membrane proteins from Jijoye and P3HR-1 cell lines.
as the quantitative indicator for the relative synthesis of Ii and class II MHC in both cell lines.

**P3HR-1 could synthesize CS-Ii.** Following $[^{35}S]$sulfate-labeling and immunoprecipitation with anti-Ii mAb VIC-Y1, SDS-PAGE analysis demonstrated that a sulfate-bearing component was synthesized by P3HR-1 with considerable molecular weight heterogeneity (Fig. 4.10). Such a pattern was typical for CS-Ii as reported in other similar studies. After treatment of the precipitates with chondroitinase-ABC (CHase), the sulfate-bearing molecules from P3HR-1 cell line disappeared, confirming that above molecules were CS-Ii. This result provided direct evidence of the class II MHC-independent synthesis of CS-Ii.

As a positive control, the Jijoye cell line synthesized CS-Ii which was detected by both anti-class II MHC and anti-Ii monoclonal antibodies, showing that in Jijoye cells at least some CS-Ii was associated with class II MHC/Ii complex (Fig. 4.10). A separate, sulfate-labeled band around 35 kD was present in immunoprecipitates from Jijoye but not from P3HR-1, and it was not affected by CHase treatment. This pattern was typical of the sulfated α chain of class II MHC molecule as reported by Sant et al. (1988). Lack of this molecule in P3HR-1 supported the view that there was no class
II MHC synthesized in P3HR-1 and the higher molecular weight smear found in P3HR-1 with anti-Ii mAb was only CS-Ii.

Nevertheless, the CS-Ii found in P3HR-1 cells had several differences from that of Jijoye. More CS-Ii was synthesized when the cells did not express class II MHC molecules. Also, the relative molecular weight range of CS-Ii in P3HR-1 was somewhat higher than that of CS-Ii in Jijoye cells, shifting from 45-66 kD to 50-90 kD (Fig. 4.10).

From the strong smear on SDS-PAGE gel, it was possible to see several separable bands on the CS-Ii precipitated from P3HR-1 cells. Since all of the previously reported CS-Ii molecules were shown by 1-D gel as evenly spread smears, current results might suggest that the CS-Ii was formed mainly as a few core species in the absence of class II MHC. After processing of the autoradiographic film by computer scanning, such species were identified as 53, 56, 60 and 65 kD, respectively (Fig. 4.11).

The identity of CS-Ii from P3HR-1 cells was confirmed by another approach. The anion-exchange resin DEAE-Sephacel column was used to purify CS-Ii from P3HR-1 in a fashion similar to that reported by Giacoletto et al. (1986) (Fig. 4.12). The DEAE-Sephacel column not only purified [35S]sulfate-labeled CS-Ii, but also concentrated [35S]methionine-labeled CS-Ii which was not detectable by direct anti-Ii mAb precipitation, as only a small
Fig. 4.10 SDS-PAGE and autoradiography analysis of immunoprecipitated, $[^{35}\text{S}]$sulfate-labeled microsomal membrane proteins from Jijoye and P3HR-1 cell lines. Some samples (+) were treated with chondroitinase ABC (CHase) at a concentration of 2 U/ml. Others were mock-treated (-).
Fig. 4.11 The lane shown in Fig.4.10 as immunoprecipitated by VIC-Y1 and untreated with CHase was further processed by a Epson Equity III+ computer system with the scanning software Image Measure (Microscience, Inc., Federal Way, WA) to expose the core proteins. The picture shown here was taken from the computer monitor screen.
Fig. 4.12  SDS-PAGE and autoradiography analysis of [35S]methionine labeled (lanes 2, 3, 4, 6, 7, 8, 10, 11, 12) or [35S]sulfate labeled (lanes 1, 5, 9) microsomal membrane proteins from Jijoye and P3HR-1 cells. The radiolabeled cell lysates were applied to the anion-exchange resin DEAE-Sephacel (Pharmacia), which was well washed before the bound materials were eluted with a high salt solution. Lanes 1-4 are direct immunoprecipitations from cell lysates before added to the resin. Lanes 5-8 are immunoprecipitates of the pooled resin wash. Lanes 9-12 are immunoprecipitates of eluted bound materials. Anti-Ii mAb VIC-Y1 (lanes 1, 4, 5, 8, 9, 12), and anti-class II MHC mAb IVA-12 (lanes 2, 6, 10) and anti-class I MHC mAb W6/32 (lanes 3, 7, 11) were used.
percentage of Ii formed CS-Ii. The core protein of this DEAE-Sephacel eluate was shown to be Ii because only anti-Ii, but not anti-class I or anti-class II MHC antibodies, could immunoprecipitate materials contained in the column eluate.

**Cellular localization of Ii in Jijoye and P3HR-1.** Since the above results showed that CS-Ii could be synthesized in the absence of class II MHC but with some structural variation, the cellular localization pattern of Ii in P3HR-1 cells was further examined by indirect immunofluorescent microscopy analysis. For both Jijoye and P3HR-1 cells, most of the intracellular Ii molecules detected with anti-Ii mAb VIC-Y1 were localized at the Golgi complex region (Fig.4.13, 4.14). Anti-chondroitin sulfate mAb (7D4) stainings of both cells were not strongly positive (Fig.4.15, panel C and D). This result might be due partially to the limited amount of CS-Ii in each cell. Anti-class II MHC mAb (IVA-12) did not stain P3HR-1 cells (Fig.4.15, panel B). Therefore, no major difference in Ii distribution patterns between these two cell lines was observed by this technique.
Fig. 4.13  Immunofluorescent staining of Jijoye cells with VIC-Y1 (A) and wheat germ agglutinin (WGA) (B).
Fig. 4.14  Immunofluorescent staining of P3HR-1 cells with VIC-Y1 (A) and WGA (B).
Fig. 4.15 Immunofluorescent staining of Jijoye (A, C) and P3HR-1 (B, D) with IVA-12 (A, B) and 7D4 (C, D).
C. Ii Is Dissociated from Class II MHC by Proteolytic Cleavage

Proteolytic cleavage of Ii by cathepsin B. It was generally accepted that Ii was cotransported with class II MHC through several cellular compartments until later dissociated from class II MHC in an endosomal compartment. Such a dissociation step was critical because it would allow processed antigenic peptides to bind to the desetope of class II MHC molecules. It was suggested that the dissociation of Ii might be the result of protease cleavage, but there was no direct evidence to show which protease could play this role and whether the protease could selectively cleave Ii but not the closely associated class II MHC molecules.

While addressing this question, we have demonstrated that cathepsin B and cathepsin D could be the candidate proteases for this process (Reyes et al., 1990). With in vitro treatment of the lysate of B lymphoblastoid cell line Jesthom with cathepsin B, it was found that Ii could be cleaved and released from class II MHC molecules at pH 5.0 without apparent damage to the α and β chains of class II MHC (Fig. 4.16). Ii was cleaved in a dose dependent fashion with the complete removal of Ii at 5 U/ml cathepsin B. Leupeptin could block the activity of cathepsin B and restored the pattern of Ii molecules seen in the absence of cathepsin B.
(Fig. 4.17). Complete protection from cleavage was achieved with 10 μM leupeptin.

Cathepsin B cleavage of Ii generated a series of intermediate and low molecular weight fragments shown by SDS-PAGE (Fig. 4.16 and unpublished data). Some could match with the in vivo Ii cleavage products, such as p21 molecule, while some were only found in vitro cathepsin B treatment. It is not known yet how these fragments were generated.

Production of anti-Ii peptide sera. In order to study the cleavage of Ii in more detail, three polyclonal rabbit anti-Ii peptide sera were generated.

Two were produced by immunizing the rabbits with two Ii peptides within the hypothesized long helix region, Ii-2(146-169) and Ii-3(148-164) (Fig. 4.1), respectively. After several immunizations, anti-Ii-2 was able to precipitate a p18 molecule (Fig. 4.18) which was only present in Ii-positive cells (Fig. 4.19). Purification of this molecule for protein sequencing analysis was attempted, but I did not succeed to have enough for such a study.

Anti-Ii-3 could precipitate following denaturation a molecule with the molecular weight range similar to the p31-form of Ii (Fig. 4.20). In the absence of denaturing conditions, a p41 molecule was precipitated from some mutant Raji cells. This p41 molecule could overlap with the region
of highly processed p41-form Ii in 2-D gels (Fig. 4.21), but peptide mapping effort failed to reach a supportive conclusion. However, the dot blot (Fig. 4.22) and ELISA (Fig. 4.23) studies did suggest that anti-Ii-3 was a specific antibody against the original Ii-3 peptide.

The third anti-peptide serum was raised against Ii-4(78-92) which had a palindromic sequence with six positively charged residues (+) and four prolines (P) mixed with other residues (O): + O + O P + P P + P O O + O + (Table 3.3). This short sequence was located within the extracellular region of Ii immediately next to its transmembrane portion. Excepting for this region, almost all the other regions of the Ii sequence had been tested for raising anti-peptide serum by various labs. We felt that this anti-Ii-4 antibody would be useful to identify some of the Ii cleavage fragments. An ELISA study of this serum showed that it was specific for its peptide antigen (Fig. 4.24).
Fig. 4.16  Cathepsin B cleavage and release of li from class II MHC/li complex. Solubilized microsomal membranes from [35S]methionine-labeled Jesthom cell line were treated with cathepsin B (CB) at various concentrations and then immunoprecipitated with the anti-class II mAb IVA-12. (Courtesy of Dr. V.E. Reyes.)
Fig. 4.17 Inhibition by leupeptin of cathepsin B-induced cleavage and release of Ii from class II MHC molecules. Microsomal membrane preparations were each treated with: none (A); cathepsin B at 5U/ml alone (B); cathepsin B at 5U/ml plus leupeptin at 0.1 μM (C), at 1 μM (D), at 10 μM (E) and at 100 μM (F). The samples were then immunoprecipitated with IVA-12 mAb. (Courtesy of Dr. V.E. Reyes.)
Fig. 4.18 Immunoprecipitation of [35S]methionine-labeled Raji cell membrane proteins with prebleed, bleed 1, 2, 3, 4, and 5 from li-2 immunized rabbit (lanes 1, 2, 3, 4, 5, and 6, respectively). An anti-li antibody E1 was used as control (lane 7). (Courtesy of M-Z. Xu).
Fig. 4.19 Immunoprecipitation of [35S]methionine labeled Raji (lanes 1, 2), Jesthom (lanes 3, 4), Vavy (lane 5, 6) and CEM (lanes 7, 8) cell membrane proteins with anti-li-2 (lanes 2, 4, 6, 8) and anti-li antibody E1 (lanes 1, 3, 5, 7).
Fig. 4.20 Immunoprecipitation of $^{35}$S-methionine labeled Raji cell membrane proteins with anti-Ii-3 (lane 2, 4) and prebleed (lane 1, 3) in native (N) and denatured (D) conditions.
Fig. 4.21 2-D NEPHGE analysis of a p41 molecule immunoprecipitated from [35S]methionine labeled Raji cell membrane proteins by anti-ll-3.
Fig. 4.22 Dot blot analysis of anti-li-3 with serial dilution, 1:40, 1:160, 1:640 and 1:2560 (lane A, B, C and D, respectively).
Fig. 4.23  ELISA analysis of anti-li-3.
Fig. 4.24  ELISA analysis of anti-li-4.
CHAPTER V

DISCUSSION

There is one central question in the work of the previous chapters: why are only some peptides in antigenic proteins selected for recognition by T cells? The primary objective in my thesis research was to search for the prototypic structure of T cell-presented peptides. The theoretical and experimental studies with one model, PH-1.0, supported the hypothesis that recurrent hydrophobic residues in some peptides digested from a foreign antigen might stabilize helical coiling of those peptides by forming a longitudinal, hydrophobic strip against a hydrophobic surface. Such coiling could lead to protection from proteolysis and/or to scavenging and transfer of peptides to a MHC desetope. The finding of this biophysical principle leads to ideas about engineering of peptide vaccines and provides information about protein folding in general.

Origin of the SOHII. In an examination of the Hopp-Woods (Hopp & Woods, 1981) hydrophilicity plot of Ii, a six-cycle (Ii 146-169) oscillation in hydrophobicity was found at the frequency of an α-helix. Such an unusual pattern suggested that this segment might be important to maintain the general structure of Ii or even played the role of
binding to the antigen-binding site of class II MHC molecules (Elliott, 1987a, 1987b). If Ii_{146-169}, in fact, binds class II MHC molecules to cover the desetope, could one design an algorithm based on its general structure to further predict other T cell-presented peptides?

This idea was supported by the findings of Kaiser and his co-workers. They proposed that some biologically active peptides, e.g., hormones, assumed amphiphilic secondary structures in the presence of lipid membranes (Kaiser & Kezdy, 1984). When analogs were made with minimal specific sequence homologies but with similar secondary structures, they mimicked the membrane binding properties of the naturally occurring peptides. Analogs designed to optimize the predicted secondary structures could even induce stronger interactions than did the naturally occurring peptides. It appeared that the number of functional groups interacting with the hormone receptor was small and most of the amino acids in this type of peptide served a purely structural role, namely, the proper positioning of the ligand functions.

T cell antigens in many aspects were similar to peptide hormones. They were all short to intermediate-size peptides; they usually lacked disulfide bonds, they were linear in a sense that the functional domains were not formed by distal residues (Kaiser & Kezdy, 1984). Large foreign antigens were processed to expose the buried T cell epitopes, while quite a
few prohormones became activated only after proteolytic cleavage. Therefore, all these T cell-presented and hormone peptides faced a similar question, i.e., how to refold the shortened peptide sequence either to escape further degradation or to assume a conformation to play very specific functions. The conformation of these short peptides depended entirely on their environment. They could assume completely different secondary structures in water, in a detergent micelle, or in trifluoroethanol or other organic solvents (Kaiser & Kezdy, 1984). Since hormone receptors and MHC molecules were both membrane-integrated proteins, the preference of peptide hormones for amphiphilic secondary structure implied that there might be also some general structure patterns for T cell recognized antigens.

**Sensitivity and efficiency of SOHHA.** When the computer program SOHHA was used to select T cell-presented peptides and the selections were compared with known T cell determinants, it was found that the predicted sequences in most cases did not overlap completely with the sequences determined experimentally. This also happened when other predictive methods were used (Table 3.1). Two measures of proximity, overlapping and touching, were developed to evaluate the sensitivity and efficiency of the predictive methods.
It was difficult for a method to be both sensitive and efficient, but SOHHA was clearly more sensitive and efficient (0.47 and 0.35) than other methods in the more stringent level "overlapping" (Table 3.2). Thus, the structure predicted by SOHHA was presumably closer to the core sequence of T cell-presented peptides. The amphipathicity and motifs-4 methods could find a much higher fraction (0.71 and 0.79 respectively) of known T cell-presented peptides at a less stringent level "touching", indicating that these two methods actually predicted regions closely flanking the core sequence of T cell-presented peptides. However, these two methods achieved higher sensitivity at the expense of losing efficiency, i.e., more T cell determinants were predicted by making more predictions. The overall low efficiency of all three predictive methods listed in Table 3.2 might reflect our limited knowledge of known T cell determinants. It was possible that some sequences which were predicted with algorithms but not experimentally reported, would be found to be T cell-presented if additional mouse strains were tested over a range of antigen concentrations.

Role of recurrent hydrophobic residues. All three predictive methods depended, in varying degrees, on recurrent hydrophobicity. For the SOHHA, the measure of recurrent hydrophobicity was the mean hydrophobicity of amino acids,
falling in a longitudinal, hydrophobic strip of an α-helix. SOHHA could also be adapted to examine $3_{10}$ helix and β-pleated sheets (Reyes et al., 1988), but α-helix was used more frequently as a model system. The amphipathicity algorithm depended in part upon recurrent hydrophobicity but it weighed equally all amino acids of the sequence. Consequently, very hydrophilic amino acids were as influential in the score as highly hydrophobic residues and hence a sequence with only a high SOHII might not be selected. Likewise, we considered the motifs method to reflect alternating hydrophobicity, as each motif was centered on two or three hydrophobic residues and had hydrophilic boundaries. Frequently, two or more motifs were found within a range of 10 to 20 residues (Table 3.1) and if the sequence were coiled as an α-helix, the motifs would be placed at intervals that would display their hydrophobic residues on the same longitudinal surface.

Therefore, we speculated that the positioning of hydrophobic residues might be important in the selection of T cell-presented peptides. This view was supported by the hypothesized refolding process of newly digested antigenic peptides (Fig. 3.6), i.e., recurrent hydrophobic residues stabilized the growing secondary structure through their interactions with a hydrophobic surface. The biological membranes represented a characteristic, anisotropic environment due to the amphiphilic nature of the dividing
line between the aqueous solution and the lipid bilayer of the membranes. It was then possible that this environment could impose a secondary structure on the peptide. The free energy contribution of locating the amino acid residues in their optimal microenvironment was considerable and yielded additional stability. Secondary structures which would only have marginal or no stability in an aqueous solution could very well become stabilized once located in the membrane.

If the above hypothesis were true, the selection of T cell-presented peptides should be linked closely to the forming of secondary structures. There are at least two possibilities to explain the biological significance of this process. First, it is possible that peptides with certain secondary structures might be less susceptible to proteolytic degradation and therefore might escape complete destruction during antigen processing. A second explanation is that the peptides thus preserved as membrane-bound structures would have a better chance to bind to MHC molecules (Roof et al., 1990), either through higher local peptide concentrations which overcome the low binding affinity (Babbit et al., 1985; Buus et al., 1986b) or by specific conformation of the peptides. This idea is consistent with the observation that lipase could strip antigen from the surface of the antigen presenting cells and inhibited presentation of that antigen, without interfering with the general function of the cells as
measured by the ability to subsequently present other antigens (Falo et al., 1986, 1987).

Nevertheless, the study of peptide folding is a new area with very limited information since the rational design and construction of biologically active peptides becomes an attainable goal through the applications of the tools of modern chemistry, e.g., the technique of solid phase peptide synthesis. We decided to test ourselves how much the recurrent hydrophobic residues could contribute to the formation of a secondary structure of peptides, especially in a membrane-like structure.

Peptides assume completely different secondary structures in different environments. We first tested the secondary structures of a group of HPLC-purified peptides in 10 mM phosphate buffer, that buffer with 45% TFE and that buffer with lipid vesicles of DHPC. The peptides varied greatly in their sequence, proline and glycine content, values of mean hydrophilicity and the SOHHI (Table 3.3 & 3.4). Since this group of peptides included both T cell-presented peptides and non-T cell-presented peptides, their CD spectra represented very different structures. It also suggested that circular dichroism could be used in our studies to measure the helicity of peptides.
Short peptides (< 20 amino acids) were predicted by a classical view not to show observable helix formation in aqueous solution and truly there were only a few examples of such peptides, naturally occurring or of de novo design, which have shown stable α-helical structure in aqueous solutions. With some peptides, this was achieved by end-blocking to avoid the helix-destabilizing interactions with the helix dipole (Shoemaker et al., 1985; Marquesee & Baldwin, 1987). Since our interest was in antigen processing and presentation, none of our peptides was end-blocked. Therefore, it was not surprising to find that none of the peptides in our CD analysis formed detectable helical structure in 10 mM phosphate buffer alone (Table 3.5).

In 45% TFE, 5 peptides within the group formed α-helices. The dielectric constant of TFE is about one-third that of water, 26.67 vs. 78.54, respectively, at 25°C (Nelson & Kallenbach, 1986). Even though the exact biophysical nature of TFE's role to stabilize α-helical structures is not clear, it was suggested that (a) the stability of intrapeptide hydrogen bonds in TFE solution will be increased in comparison with peptides in aqueous solution; (b) the interactions between charged species would be stronger in TFE solution and such interactions might be responsible for stabilization of α-helix structures (Nelson & Kallenbach, 1986). Lark et al. (1989) also reported that two T cell
antigenic peptides from sperm whale myoglobin, SWMb 132-146 and SWMb 102-118, folded as α-helical structure in 50% TFE as shown by CD analysis while in aqueous buffer little ordered secondary structure was found. In our study, Ii-4, MA-2 and MA-3 could not form helical structures in 45% TFE, probably as a result of 2 to 4 prolines in the middle of their short sequences (Table 3.3). MT11-A could not form an α-helix in 45% TFE, but it was the only peptide doing so among 6 in this group without multiple prolines (Table 3.3 and 3.5). It had the lowest SOHBI among these six peptides, but since MT11-A was the only example in this group it was difficult to conclude whether its low helix-forming tendency in TFE was related to low SOHBI.

One major problem of using TFE to measure the helix-forming tendency of peptides was its strong helix-promoting effect which made many marginally stable peptides form helices. Also, there was no stable and compatible standard to judge the ability of a peptide to form a helical structure in TFE because the higher concentration of TFE usually led to a higher percentage of helicity for a peptide which could form helical structure.

It appears that such a shortcoming of TFE was overcome through the lipid vesicles experiments. In the presence of well-sonicated lipid vesicles, a helix-forming tendency was conserved for most peptides which had helical structure in
TFE, but their percentage helicities were lower in lipid vesicles (Table 3.5). The predominant form of secondary structure for MX in the presence of lipid vesicles was β-pleated sheet in contrast to α-helix in 45% TFE. Thus lipid vesicles were more stringent inducers of a peptide's secondary structure. When the percentage helicity of peptides in lipid vesicles, excluding those with multiple prolines, were plotted against the SOHHI, a general correlation was found (r = 0.77; p = 0.07), suggesting that the helix-forming tendency of peptides in lipid vesicles depended more on their SOHHI. However, the sample size was too small to draw a final conclusion.

**Prototypic helix peptide, PH-1.0.** PH-1.0, as a putative model, was built with two core components, an axial hydrophobic strip with a high SOHHI and a potential salt bridge based on recent findings about peptide folding in aqueous solutions (Shoemaker et al. 1987; Marqusee & Baldwin, 1987; Marqusee et al., 1989).

By CD measurements PH-1.0 did not show helical structure in 10 mM phosphate buffer (Fig. 3.7), but in lipid vesicles it formed the highest percentage helicity, 36% at room temperature and 40% at 4°C, among all the peptides we have tested (Table 3.5, 3.7, 3.10, 3.11). The mean hydrophilicity of PH-1.0 showed that it was not very hydrophobic in general
and certainly very much less hydrophobic than several other peptides tested. It appears that we generated a peptide that was water soluble at physiological pH (data not shown) and would partition into membrane in a controlled manner. The result that PH-1.0 had a superior helix-forming tendency in lipid vesicles highly supported our hypothesis that the recurrence of hydrophobic amino acids, in a polypeptide at positions falling in an axial, hydrophobic strip if the sequence were coiled as an α-helix, could lead to helical nucleation on a hydrophobic surface. CD analysis of MT11-A, which had very similar structural features as PH-1.0 and it had a low SOHHI, showed no helix formation and thus supported our hypothesis (Fig. 3.7-3.9).

When the helix-forming tendency of PH-1.0 in lipid vesicles was examined by CD at various experimental temperatures, it became clear that as the temperature was increased, a structural transition from α-helix to random-coil was taking place. When temperature was raised to 50°C, no clear helicity by CD measurement remained (Fig.3.12). The CD spectrum of PH-1.0 in lipid vesicles at 75°C was very close to the spectrum at 50°C (not shown). Since the phase transition temperature of the lipid (Tt) was about 30°C, this result probably reflected organizational changes of the lipid vesicles which thus lost their ability to induce helix formation of PH-1.0 at temperatures above Tt.
A clean isodichroic point at 202 nm was formed by the various CD spectra from different temperatures (Fig. 3.12), indicating that the transition was two-state, i.e., each amino acid was in either a helical state or a random coil state. This finding did not imply that the formation of the entire α-helix was two-state, or that the entire peptide was either completely a α-helix or a random coil (Nelson & Kallenbach, 1986). Based on this explanation and if we assume that every PH-1.0 peptide formed the same degree of helicity, the above result demonstrated that the transition between random-coil and helix was a stepwise process in which the recurrent hydrophobic residues played an important role in anchoring the successive loops of a growing helix to the lipid vesicles. Since CD analysis could not discriminate whether 50% of the peptide molecules formed 100% helicities or 100% of the peptides formed 50% helicities, the exact nature of PH-1.0 folding must be determined from other experiments, such as NMR study.

The experimental results also excluded the possibility that PH-1.0 formed helical structure through the aggregation of their strong hydrophobic residues. In 10 mM phosphate buffer alone, CD spectra for PH-1.0 did not show an observable helical structure (Fig. 3.7). When PH-1.0 was tested at a high peptide and salt concentration, there was still no obvious helical structure as shown by the CD
measurement (Fig. 3.10). This suggested that PH-1.0 would not aggregate in aqueous solution, at least not before a helical structure could be stabilized. Therefore, the strong helix-forming tendency of PH-1.0 could not be explained by self-aggregation.

There was additional evidence that the formation of helical structure of PH-1.0 in lipid vesicles was lipid-dependent. First, lipid vesicles only formed after sonication of the lipid solution, as judged by the formation of an optically translucent suspension, and our experience clearly suggested that PH-1.0 would not be induced to show a helical CD spectrum before lipid vesicle structure was formed by proper sonication. Once the sonication was successful, the secondary structure pattern and the percentage helicity of PH-1.0 in various lipid vesicles experiments were always very similar. Without the presence of lipid vesicles, sonicating PH-1.0 in aqueous solution did not induce helical structure. Second, an optimal lipid/peptide ratio was required for PH-1.0 to form a helical structure (CD spectra not shown). In most of our lipid vesicles experiments, the optimal ratio of lipid to peptides was ~50/1 on a molar basis. PH-1.0 would not form α-helix with less lipid, but more lipid than the optimal range did not further increase the percentage helicity. This again demonstrated that the helix-like CD spectra in lipid vesicles experiments truly
represented the structure of the peptide rather than the interference of lipid vesicles.

An ESR study of the interaction between PH-1.0 and lipid vesicles further showed that the formation of helical structure was related to the direct binding of peptide into lipid vesicles (unpublished observation). It will be possible to achieve more detailed information about the orientation of PH-1.0 in lipid vesicles if additional peptide analogs are made with the tyrosine residue placed in various positions.

As expected, PH-1.0 formed an α-helix in TFE solution (Fig. 3.11) with a high percentage helicity, ~60% at room temperature and 76% at 4°C (Table 3.5, and 3.7). The higher the concentration of TFE, the higher the percentage helicity was induced (Table 3.6). Since we only tried 30-45% TFE in these experiments, it might be possible to induce higher percentage helicity with more TFE.

Number and placement of hydrophobic residues in a longitudinal strip governs helix formation of peptides in the presence of lipid vesicles. As discussed previously, two core components, the longitudinal, hydrophobic strip and the potential salt bridge (including the orientation of its charged residues), were considered to stabilize the helical structure of PH-1.0. We designed a large number of analogs
for PH-1.0 to study the contribution of each of these two components (Fig. 3.13-3.15).

The simplest way to analyze the importance of recurrent hydrophobic residues in PH-1.0 was to replace them with much less hydrophobic residues, such as threonine. The substitution could be made with one residue only, or several at the same time with various combinations at different positions along the longitudinal, hydrophobic strip (Fig. 3.13). Another way to examine the importance of the recurrent pattern of hydrophobic residues was to alternate the leucine residues in PH-1.0 to other locations than the hypothetical hydrophobic strip to see if the helix-forming tendency of this peptide was decreased (Fig. 3.15.A).

In order to study the contribution of charged residues in PH-1.0, the potential salt bridge Glu⁴-Lys⁷ could be broken by (a) replacing one of these two residues with non-charged residue (PH-2.1-2.3) or with the residue of opposite charge (PH-2.5-2.6), and (b) placing these two residues at such a distance that they would not be close to each other when the peptide coils as an α-helix (PH-2.4) (Fig. 3.14). The effect of charged residues on the macrodipole of an α-helix could be studied with similar logic (Fig. 3.14).

Eight analogs of PH-1.0 were synthesized with varied number and position of leucine residues in the longitudinal hydrophobic strip (PH-1.1 to 1.7, 1.10). While none of these
eight analogs, like PH-1.0, formed α-helix in 10 mM phosphate buffer alone (Fig. 3.17), some of them formed distinctive helical structure in the presence of lipid vesicles at 4°C (Fig. 3.19). Careful examination of these CD spectra led to the following findings: (a) substituting even one of the leucine residues in the longitudinal strip with threonine, independent of the location, led to the reduction of peptides' helix-forming tendency in lipid vesicles; (b) such an effect was more damaging when the substitutions were made in the middle of this strip (PH-1.2 and 1.3); (c) when several substitutions were made simultaneously, maintaining at least two leucine residues in the middle of the strip was necessary to form a helical structure (Ph-1.7); (d) the leucine residues at two ends of the strip contributed differently in the induction of helical structure in lipid vesicles, i.e., PH-1.4, with N-terminal leucine, had a higher percentage helicity than the C-end leucine preserving PH-1.1 (Fig. 3.19, Table 3.10), suggesting an orientation preference of peptides in interacting with lipid vesicles.

We conclude that preserving two strong hydrophobic residues in the middle of a longitudinal hydrophobic strip for a 13-residue peptide like PH-1.0 is critical to induction of an α-helix in lipid vesicles. Such a sequence-dependent pattern was unique to lipid vesicles because all of these eight analogs demonstrated very typical, helix-like CD
spectra with close percentage helicities when they were examined in 45% TFE solution (Fig. 3.17, Table 3.8).

Again, the helix-forming tendency of analog peptides was temperature dependent. PH-1.1 maintained 26% helicity even at 75°C in 30% TFE, (Fig. 3.18, Table 3.9), while in lipid vesicles, CD spectra of these analogs did not show helical structure when the temperature was raised to 25°C (Fig. 3.19). These and previous results about PH-1.0 showing a thermal transition were consistent with other classical studies of peptide folding, e.g., C-peptide of ribonuclease (Bierzynski et al., 1982; Shoemaker et al., 1987), indicating that the helix formation of our prototypic helix peptide was enthalpy-driven (Marqusee & Baldwin, 1987). It was suggested that one candidate for the enthalpic contribution to helix formation was the hydrophobic interaction, which becomes increasingly enthalpy-driven at higher temperatures (Baldwin, 1986; Marqusee & Baldwin, 1987). In fact, the successful examples of peptides folding to α-helices in many studies were found at or close to 0°C, e.g., C-peptide at 3°C (Shoemaker et al., 1985) and at 5°C (Shoemaker et al., 1987), Ala-based peptide of de novo design at 1°C (Marqusee & Baldwin, 1987; Marqusee et al., 1989) and at 0°C (Padmanabhan et al., 1990). Recently Talanian et al. (1990) reported that a sequence-specific DNA binding peptide, GCN4-brl, which adopted a helical structure when bound to DNA, bound DNA
better at 4°C than at 24°C as tested by deoxyribonuclease (DNase) I footprinting analysis. Therefore, we hypothesized that the percentage helix formation at the low temperature shown by in vitro biophysical studies may represent the true helix-forming tendency of that peptide in vivo, but there might be additional unknown factors to stabilize such structure at physiological temperatures.

The ninth analog of PH-1.0 was synthesized with a leucine at position 3 (Fig. 3.16). Because Leu³ in this analog, PH-4.2, changed the recurrent hydrophobic pattern in the N-terminal part of the peptide, we tried to test whether hydrophobic residues in strips other than the hydrophobic one in a potential α-helix could compete to break the helical structure. The CD analysis showed that PH-4.2, different from other analogs, formed helical structure in lipid vesicles at both 4°C and at room temperature (Fig. 3.20). However, the helix-forming tendency of PH-4.2 was not as strong as PH-1.0, as judged by their percentage helicities (Table 3.11). Thus, addition of a hydrophobic residue at position 3 while preserving the strong longitudinal strip and other structural features could reduce, but not completely inhibit, the coiling of this peptide into an α-helix. From the current CD result, we could not tell whether PH-4.2 actually formed other secondary structures, such as β-pleated
sheet to compete with the strong hydrophobic strip in the presence of lipid vesicles.

Functional significance of the prototypic helix peptide.

The immunological relevance of PH-1.0 in antigen presentation was shown in one functional study in which PH-1.0 could inhibit the presentation of a DR 1-restricted peptide M1 17-31 of M1 matrix protein of influenza A virus. PH-1.0 had very little primary sequence homology with M1 17-31 (Fig. 3.21) but its ability to inhibit the presentation of M1 17-31 was as good as another DR 1-restricted peptide, HA 307-318 (Fig. 3.22 and 3.23). Since M1 17-31 could form a longitudinal, hydrophobic strip if the peptide coiled as an α-helix (Fig. 3.21), the results supported our hypothesis that the foreign antigenic peptides could be selected through their potential hydrophobic strip of a helical structure and it was highly possible that the prototypic helix peptide, PH-1.0, competed with M1 17-31 as a result of its high SOHHI value.

The above results, both biophysical and functional, raised the question of whether a helical structure was required for T cell-presented peptide. Statistical (Spouge et al., 1987; Margalit et al., 1987; Stille et al., 1987; Reyes et al., 1988) and some functional (Schwartz et al., 1985; Berkower et al., 1986; Cease et al., 1986; Carbone et
al., 1987; Allen et al., 1987c) results indicated that an α-helical structure might be required for many T cell antigenic peptides. There were also functional studies arguing that such a structure might not be an obligatory requirement for some other T cell determinants (Heber-Katz et al., 1985; Sette et al., 1987; Ogasawara et al., 1989; Bhayani & Paterson, 1989). Most of the above conclusions were reached by structural modeling of peptide sequences with very limited experimental data from direct structural measurements, e.g., CD and 2D NMR. Peptide structures were proposed in some studies based on the assignment of certain amino acids within the peptide as MHC-contacting or TCR-contacting residues by functional analysis of a series of analogs containing single amino acid substitutions (Sette et al., 1987; Ogasawara et al., 1989), but it was impossible to be sure that any particular amino acid substitution only influenced direct contact with MHC or TCR and did not affect the general structure of the peptides. It was possible that some secondary structures, e.g., an α-helix, could maintain a short peptide in a relatively stable conformation only when placed in the right environment. Our results with PH-1.0 and its analogs were consistent with this view.

The hypothesis that there were relatively common structural features for immunogenic peptides was supported by the concept of degenerate binding of peptides to class II MHC
molecules (Roche & Cresswell, 1990) since there was neither a significant affinity difference nor a kinetic preference for the association of an influenza hemagglutinin peptide, HAp, with either DR1, DR5, or DR8 molecules. Grey and co-workers (Buus et al., 1987; Sette et al., 1989) reported that when data from a large panel of peptides were analyzed, a peptide that bound to an individual I-A or I-E allele had a significant probability of binding to other alleles of the same locus. Sinigaglia et al. (1988) found that a circumsporozoite protein-derived peptide was capable of being recognized by T cells in association with many different human and mouse class II MHC molecules. An influenza virus matrix protein-derived peptide could bind to both DR1- and DRw13-positive B cells (Ceppellini et al., 1989). Thus, even though it was generally assumed that the specific association of a given immunogenic peptide with a particular class II MHC molecule was directly responsible for the observed MHC-restriction of T cells, these recent results did suggest that the specific association of the TCR with the peptide-class II MHC complex might be more important in determining the final T cell response. Therefore, a clear pattern of recurrent hydrophobic residues may only promote the association of immunogenic peptide with MHC molecules but the peptide/MHC complex expose different sets of residues to different T cell clones (Bhayani & Paterson, 1989; Kurata & Berzofsky, 1990).
Our results clearly showed that only a few critical, recurrent hydrophobic residues might be enough to induce a helical structure (e.g., PH-1.7), thus various peptides could form a similar secondary structure and at the same time still maintain their fine specificity to induce very specific T cell response. In addition, a change in the positioning of hydrophobic residues (e.g., PH-4.2) might generate more fine specificities.

It is still not clear how an immunogenic peptide can be fitted into the peptide-binding cleft on the class I MHC molecules (Bjorkman et al., 1987) and the similar yet hypothetical structure of the class II MHC molecules (Brown et al., 1988). Comparison of X-ray crystallography structures of HLA-Aw68 and HLA-A2 found that side chains of peptide antigens could interact with specificity pockets in the peptide-binding cleft and one prominent pocket, a hydrophobic cavity with Met\textsuperscript{45} at its base (the "45 pocket"), remained essentially conserved between these two alleles (Garrett et al., 1989). Since the cleft was too small to hold the whole length of many known T cell immunogenic peptides, it is possible that only part of those peptides, or a core sequence, could actually access the binding pockets (Sette et al., 1987). Hence, residues outside the minimal immunodominant site might play other roles, such as to
maintain certain important conformations for T cell-presented peptides.

At least four explanations for the need of certain conformations could be suggested. First, a stable conformation might position the critical residues of a peptide in a proper orientation in order to induce the very specific T cell response. Many studies have suggested that different T cell clones could recognize a single peptide bound with the same MHC molecules differently (Bhayani & Paterson, 1989; Kurata & Berzofsky, 1990; Boyer et al., 1990).

Second, it was possible that structural features outside the core antigenic determinant could influence the magnitude of the T cell response, either increasing the potency of the peptide (Schwartz et al., 1985) or exerting negative effects on recognition by antigen-specific T cell clones (Vacchio et al., 1989).

A third possibility, independent of binding to the MHC molecule, but not mutually exclusive, was the ability of those peptides with α-helical conformation, to intercalate into membranes (Collawn et al., 1989, Collawn & Paterson, 1990). Mecheri et al. (1990) have shown that immunogenic peptides require an undisturbed phospholipid cell membrane environment and must be amphipathic to immobilize class II MHC molecules on B cells.
A fourth explanation for the importance of some structural features, especially for the helical structure, was that such structure might be less susceptible to proteolytic degradation and therefore might escape destruction during processing. This hypothesis is currently under active investigation in our lab.

**Ii(148-164)** forms an α-helix in lipid vesicles and binds to both α and β chains of class II MHC molecules. More than three years ago, Elliott et al. (1987a, 1987b) hypothesized that (a) a 24-residue segment in Ii sequence (Ii\textsubscript{146-169}) might form an amphipathic, α-helical structure and (b) it would be the sequence to bind to the class II MHC molecules in order to cover the antigen binding site (desetope) until dissociation in endosomes where foreign peptide would be bound to class II MHC molecules. This blocking function of Ii has been supported by more and more studies on intracellular traffic and processing of protein antigens, especially by Long's hypothesis (1989) that the invariant chain might be involved in the distinction between class I and class II MHC molecules in presentation of endogenous antigens. Nevertheless, the hypothesis of Elliott et al. was directly confirmed by the biophysical and biochemical studies of Ii-3 presented in this work.
CD measurement of the secondary structure of Ii-3, a 17-residue (148-164) peptide from the proposed α-helical region, clearly demonstrated that it formed 51% helicity in 45% TFE solution and 18% helicity in lipid vesicles (Fig. 4.4, Table 3.5). From the studies of PH-1.0 and its analogs, it appeared that the recurrent hydrophobic residues in Ii-3 might promote the nucleation of the helix because the hydrophobic residues in this short peptide only fell within a longitudinal strip when the peptide coiled as an α-helix (Fig. 4.1).

The subsequent crosslinking experiment of Ii-3 provided direct evidence that Ii-3 was capable of binding both α and β chains of immunopurified class II MHC molecules (Fig. 4.7). This was consistent with a report that T cell-presented peptides could bind to both α and β chains of class II MHC (Buus et al., 1987) and was supported by the hypothesized structure of peptide-binding cleft on the class II MHC molecules (Brown et al, 1988) which was formed by both α and β chains.

Teyton et al. (1990) recently showed by transfection experiments that the invariant chain could independently bind both α and β chains of the class II MHC molecules, but they did not know whether the invariant chain had two independent sites for binding of the α and β chains, or whether a single site on Ii recognized similar structural features of the two
subunits of the class II MHC molecules. Our result that the same Ii-3 peptide bound both α and β chains of the class II MHC molecules supported the second possibility.

There was an additional higher molecular weight band (~60 kD) crosslinked by Ii-3 and shown by SDS-PAGE analysis (Fig. 4.7). It was not clear about the nature of this band, but similar findings in other studies suggested that it could be the α/β dimers (Buus et al., 1986b; 1987; Roche & Cresswell, 1990b), because HLA-DR α/β dimers were previously shown to be remarkably stable to dissociation in SDS (Springer et al., 1977; Cresswell, 1977). The complete dissociation of α/β dimers might need boiling the sample at 100°C for 5 min (Roche & Cresswell, 1990b) or even 15 min (Wraith et al., 1989), while our sample was only boiled 1 min before electrophoresis. It was also postulated that this unknown molecule found during peptide-class II MHC binding might be a high molecular weight Ii species (Roche & Cresswell, 1990a), but its nature has not been confirmed.

The fact that Ii-3 formed an α-helix in lipid vesicles and bound class II MHC molecules was consistent with the view that T cell-presented peptides have the potential to form helical structures and further supported the hypothesis that Ii's function was to protect the desetope of class II MHC molecules (Elliott et al. 1987a). This hypothesis was also supported by recent studies which showed that invariant chain
association with HLA-DR molecules inhibited immunogenic peptide binding (Roche & Cresswell, 1990a) and that the bindings of peptide and invariant chain to class II molecules were mutually exclusive (Teyton et al., 1990).

However, our data did not imply that a helical structure of Ii-3 must directly contact the desetope but rather it might be needed for invariant chain to bind tightly to class II MHC molecules and to prevent the binding of immunogenic peptides. It was shown that the $K_D$ for the interaction between the soluble Ii and affinity-purified HLA-DR molecules was $1.5 \times 10^{-8}$ to $3.0 \times 10^{-9}$ M (Teyton et al., 1990), indicating a much higher affinity than that of most immunogenic peptides, $2 \times 10^{-6}$ M (Babbitt et al., 1985; Buus et al., 1986b). But if the class II MHC molecules were highly purified without the association of Ii, the binding affinity of immunogenic peptides was increased to $1.3-2.8 \times 10^{-8}$ M (Roche & Cresswell, 1990b). Therefore, if Ii-3 really represented the site on Ii to bind to class II MHC molecules, a helical structure with a narrow, strong, hydrophobic strip might contribute significantly to the above high binding affinity of Ii. It would also make sense for immunogenic peptides to assume a similar secondary structure in order to support their core determinants to interact with the desetope with a higher affinity, thus demonstrating a higher potency in the induction of a T cell response. A competitive binding
analysis between Ii-3 and immunogenic peptides would be able to show whether they bind same site on class II MHC molecules. The binding site could be further identified by mapping the crosslinked sequence on MHC molecules.

**CS-Ii synthesis in the absence of class II MHC molecules.** It was generally believed that Ii was associated with class II MHC molecules throughout post-translational processing (Jones et al., 1978) until their dissociation in endosomal compartments. Some of the Ii molecules could be converted to a proteoglycan form (Sant et al., 1983, 1984, 1985a, 1985b; Giacoletto et al., 1986). It was estimated that only a fraction (2-5%) of the total cellular class II MHC molecules contained CS-Ii (Sant et al., 1985b). It was noted that CS-Ii might function in antigen processing and presentation events (Sivak et al., 1987; Rosamond et al., 1987), but the detailed mechanism was not known. It was not known what was the role of class II MHC molecules during the synthesis of CS-Ii.

Our study of the synthesis of CS-Ii in a class II MHC negative cell line P3HR-1 clearly demonstrated that CS-Ii could be synthesized in the absence of class II MHC molecules (Fig. 4.10). This finding showed that intracellular trafficking of the core proteins of CS-Ii did not need the presence of class II MHC molecules and it was consistent with
the report that CS-Ii might only be associated with class II MHC molecules transiently (Sant et al., 1985b).

The synthesis of CS-Ii was increased in P3HR-1 in comparison with Jijoye, its class II MHC-expressing, parent cell line (Fig. 4.10), while at the same time it appeared that Ii was terminally processed to more acidic, sialic acid-derivatized forms only in Jijoye cells but not in P3HR-1 cells (Fig. 4.9) (Spiro et al., 1985). This finding suggested that the regular processing of Ii to Ip and the synthesis of CS-Ii might represent two mutually exclusive events regulated by class II MHC molecules. In the presence of class II MHC expression, most Ii was processed to Ip and the synthesis of CS-Ii was only minimal, while in the absence of class II MHC molecules, more Ii was converted to CS-Ii rather than moved through its regular processing pathway. Such pathway selection for processing of Ii was consistent with the report that addition of glycosaminoglycan (GAG) to the core protein of CS-Ii happened in a cis-Golgi compartment (Spiro et al., 1989a). The results of immunofluorescent microscopy analysis suggested that the majority of Ii molecules in Jijoye and P3HR-1 cells were distributed within Golgi complexes and there was no clear difference between these two cell lines in their distribution patterns.

The average molecular weight of CS-Ii was greater in P3HR-1 cells in the absence of class II MHC molecules than in
Jijoye cells (Fig. 4.10). It was not clear why CS-II synthesized in P3HR-1 had a larger molecular weight. Simonis et al. (1989) also reported a CS-II molecular weight change in the absence of class II MHC molecules, but that CS-II appeared to be smaller, with a molecular range of 39-61 kD in comparison to 43-80 kD found in the presence of class II MHC molecules. While their result was based on a transfection study with mouse cells, which could account for some of the differences between the two studies, it was clear that the presence or absence of class II MHC molecules certainly could affect the GAG chain length of CS-II molecules (Simonis et al., 1989).

The multiple cleavage pathways for Ii molecules. There were at least two major Ii proteolysis pathways. One appeared to be responsible for the proteolysis of Ii to p25 which represented the C-terminal portion of Ii and was generated in an ER or cis-Golgi compartment (Thomas et al., 1988). Another pathway of Ii proteolysis has been described (Blum & Cresswell, 1988; Nguyen et al., 1988). Two proteolytic intermediates, p21 and p10, from a sialic acid-derivatized, complex sugar form of Ii were induced by the thiol-protease inhibitor leupeptin. However, those fragments were still associated with class II MHC molecules. It was
not clear how Ii was actually released from class II MHC molecules in an endosomal compartment.

The results presented in this work showed that cathepsin B could completely cleave and release Ii from α and β chains of class II MHC molecules without apparent damage to those chains (Fig. 4.16). Such a cleavage could be blocked by leupeptin (Fig. 4.17). Cathepsin B digested Ii in a dose dependent fashion yielding multiple fragments, including a p21 molecule (Fig. 4.16). Since cathepsin B was found in endocytic compartments where Ii appeared to be dissociated from class II MHC molecules (Guagliardi et al., 1990) and T cell determinants produced from myoglobin by cathepsin B digestion were presented to T cells without further processing (Takahashi et al., 1989), it was highly possible that for some antigens, a single protease, cathepsin B, might be both necessary and sufficient for antigen processing and Ii release. In this way, cathepsin B might work efficiently to catalyze the binding of immunogenic peptides to the desetope of class II MHC molecules in order to overcome the slow association rate for peptide charging of isolated class II MHC molecules (Buus et al., 1986b).

Since cathepsin B at low doses generated p21 molecule in vitro while leupeptin treatment of whole cells could only block the further digestion of p21, it appears that p21 found with leupeptin treatment was the result of incomplete
inhibition of cathepsin B activity because a much higher concentration of leupeptin to whole cells might be required to achieve a similar effect as in our *in vitro* experiments. Therefore, Ii could be released from class II MHC molecules by one protease rather than by a previously proposed two-step proteolysis mechanism (Blum & Cresswell, 1988).

However, it is important to make sure that the p21 generated in our cathepsin B experiment was same as the p21 molecule generated when the whole cells were treated with leupeptin. Towards this goal and to characterize the various digested fragments during the proteolysis, several polyclonal anti-Ii sera were produced by immunizing rabbits with various synthesized Ii peptides coupled with KLH as carrier. Anti-Ii-2 precipitated a p18 molecule which might represent a minor species of leupeptin-induced Ii fragments (Blum & Cresswell, 1988). Anti-Ii-3 and anti-Ii-4 was each specific for their respective antigenic peptides in ELISA. They should be useful in identifying respective Ii sequences in proteolytic fragments and for additional experiments.

Overall, the structural and functional studies on PH-1.0 and its analogs presented in this work showed that the recurrent hydrophobic residues might play an important role in the selection of T cell-presented peptides by formation of
helical structures, presumably in the endosomal vesicles. PH-1.0 and its analogs should be tested further for inhibition of additional antigens in more antigen presentation systems. Thus, the relationship between the SOHHI of a peptide and its function of blocking antigen presentation can be probed. New analogs, with other types of hydrophobic residues falling in the longitudinal strip, will provide more information about the contribution of those residues in the formation of an \( \alpha \)-helix against a hydrophobic surface. The study of peptides with alternately placed hydrophobic residues, like those shown by Fig. 3.15, may demonstrate how different types of secondary structures can be induced based on the positioning of hydrophobic residues and their interactions with a hydrophobic surface. Future work in this area will certainly build the knowledge about the structural requirements for T cell-presented antigens and may facilitate the design of peptide vaccines.

The finding that Ii\(_{148-164}\) formed an \( \alpha \)-helix in lipid vesicles and could bind class II MHC molecules offered another useful tool to study, both structurally and functionally, the interactions between T cell-presented antigens and class II MHC molecules. This peptide should be tested in functional studies to see whether it can compete with foreign, antigenic peptides. Biochemical characterization of crosslinked Ii-3/class II MHC complex may reveal
its actual binding site on class II MHC molecules. Further modification of this peptide with knowledge achieved from PH-1.0 studies will be useful in further examinations of structural requirements for T cell-presented peptides.

The studies of CS-Ii and the proteolytic cleavage pathways of Ii provided additional information regarding the complicated process of post-translational modification of Ii. It will help to understand finally the functions of this molecule and the biochemical mechanisms controlling these functions. Complete removal of the invariant chain from the Ii/class II MHC complex also produced an experimental model to directly test the mechanism of binding of various T cell-presented peptides to Ii-depleted class II MHC molecules. All of the above studies should allow us to understand better the mechanisms that control antigen processing and presentation of immunogenic peptides by major histocompatibility complex molecules.
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